

ORIGINAL ARTICLE

Antibody-directed coupling of endoglin and MMP-14 is a key mechanism for endoglin shedding and deregulation of TGF- β signalingS Kumar¹, CC Pan¹, JC Bloodworth¹, AB Nixon², C Theuer³, DG Hoyt¹ and NY Lee^{1,4}

Endoglin is a transforming growth factor β (TGF- β) coreceptor that serves as a prognostic, diagnostic and therapeutic vascular target in human cancer. A number of endoglin ectodomain-targeting antibodies (Abs) can effectively suppress both normal and tumor-associated angiogenesis, but their molecular actions remain poorly characterized. Here we define a key mechanism for TRACON105 (TRC105), a humanized monoclonal Ab in clinical trials for treatment of advanced or metastatic tumors. TRC105, along with several other endoglin Abs tested, enhance endoglin shedding through direct coupling of endoglin and the membrane-type 1 matrix metalloproteinase (MMP)-14 at the cell surface to release the antiangiogenic factor, soluble endoglin (sEng). In addition to this coupling process, endoglin shedding is further amplified by increased MMP-14 expression that requires TRC105 concentration-dependent c-Jun N-terminal kinase (JNK) activation. There were also notable counterbalancing effects on canonical Smad signaling in which TRC105 abrogated both the steady-state and TGF- β -induced Smad1/5/8 activation while augmenting Smad2/3 activation. Interestingly, TRC105-induced sEng and aberrant Smad signaling resulted in an excessive migratory response through enhanced stress fiber formation and disruption of endothelial cell–cell junctions. Collectively, our study defines endoglin shedding and deregulated TGF- β signaling during migration as major mechanisms by which TRC105 inhibits angiogenesis.

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INTRODUCTION

Angiogenesis is a process in which new blood vessels are formed from pre-existing vasculature. As tumors require angiogenesis to grow and metastasize to distant organs, reducing tumor vascularization is a promising strategy in limiting cancer progression.¹ Endoglin is a transforming growth factor- β (TGF- β) coreceptor that is emerging as a unique vascular target in anticancer therapy.^{2–5} Numerous studies have shown that endoglin is required for both normal and tumor-induced angiogenesis, and is considered as the gold standard biomarker of tumor vasculatures for tumor progression, survival rate and metastases.⁶ Despite a growing number of endoglin monoclonal antibodies (Abs) under evaluation for clinical applications, however, the underlying molecular mechanisms of antibody (Ab) action remain poorly defined.

As part of an integral TGF- β signaling complex in endothelial cells, the essential role of endoglin in angiogenesis during embryonic development and tumor growth is well established.⁷ Endoglin knockout in mice is embryonic lethal due to defective angiogenesis, and multiple studies have shown that either depletion or targeted inhibition of endoglin expression impairs tumor vascularization.^{5,8,9} These effects are mediated in large part by two canonical TGF- β signaling pathways.

In endothelial cells, TGF- β can either promote or inhibit angiogenesis depending on its association with two types of signaling receptors. The endothelial-specific receptor, ALK1, enhances a proangiogenic transcriptional response by phosphorylating and

activating Smad1/5/8 transcriptional factors.¹⁰ Alternatively, TGF- β can signal through ALK5, a ubiquitously expressed signaling receptor that suppresses angiogenesis by activating Smad2/3.¹⁰ In addition, endoglin and ALK1 can each bind a structurally related TGF- β superfamily ligand, bone morphogenetic protein 9 (BMP-9), which signals through the ALK1/Smad1/5/8 pathway.¹¹ Although the precise mechanisms by which TGF- β and BMP-9 regulate angiogenesis is still an active area of investigation, endoglin is considered a critical component in modulating the balance between ALK1 and ALK5 signaling to exert either pro- or antiangiogenic signals.

The complex role of endoglin in angiogenesis is also evident at the level of extracellular domain shedding.¹² A recent study has demonstrated that endoglin is cleaved near the plasma membrane by membrane-anchored matrix metalloproteinase (MMP)-14 to release a soluble form of endoglin (sEng) into the circulation.¹³ And although membrane-bound endoglin promotes angiogenesis, sEng antagonizes this process through multiple mechanisms including cell surface receptor downregulation and ligand sequestration. More recently, a surface plasmon resonance study demonstrated direct binding of sEng with BMP-9 but not TGF- β , suggesting that sEng inhibits angiogenesis in part by dampening the BMP-9/Smad1/5/8 signaling axis.¹⁴ Overall, endoglin shedding has a critical role in regulating TGF- β signaling during angiogenesis.

Numerous preclinical studies have so far demonstrated the efficacy of endoglin-targeted therapies. Of particular interest is

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TRACON105 (TRC105), the first humanized monoclonal Ab currently in phase I/II clinical trials for treatment of advanced or metastatic solid tumors.¹⁵ To date, it is unclear whether TRC105 and its variants inhibit angiogenesis by neutralizing endoglin signaling, by mediating Ab-dependent cell-mediated cytotoxicity or by inducing endothelial cell dysfunction through a combination of mechanisms. In the present study, we examined the mechanistic basis for how TRC105 and other endoglin-targeting Abs inhibit angiogenesis.

RESULTS

We chose to study the effects of TRC105 on two types of human endothelial cells, a human microvascular endothelial cell line (HMEC-1) and primary human umbilical vein endothelial cells (HUVEC), to define the mechanisms by which endoglin-targeting Abs inhibit angiogenesis. We first examined the effects of TRC105 on the steady-state TGF- β signaling to the canonical ALK1/Smad1/5/8 and ALK5/Smad2/3 pathways. A 24 h treatment with TRC105 in growth media caused a significant enhancement in Smad2/3 activation, whereas Smad1/5/8 activation was markedly reduced in a concentration-dependent manner (Figures 1a and b). The Smads appeared as either single or doublet bands when immunoblotted with their respective phospho-specific and total Smad Abs, depending on the gel electrophoresis conditions as previously reported.^{16,17} We next tested for ligand responsiveness by subjecting the cells to serum deprivation, followed by a brief pretreatment with TRC105 (10 min) before either TGF- β or BMP-9 stimulation. Interestingly, TRC105 treatment abrogated TGF- β -induced Smad1/5/8 activation, whereas its effect on Smad2/3 was enhanced (Figure 1c). Given that TGF- β -induced Smad1/5/8 activation first requires TGF- β binding to the endothelial-specific endoglin/ALK1 complex, these results suggest that TRC105 prevents the TGF- β /endoglin interaction, while presumably providing ALK5 a greater access to the ligand for Smad2/3 activation. In the case of BMP-9, there was a robust Smad1/5/8 activation irrespective of TRC105 treatment (Figure 1d). Together, these results suggest that by targeting endoglin, TRC105

selectively inhibits the regulation of TGF- β but not BMP-9 signaling, and that overall, TRC105 attenuates the activation of Smad1/5/8 in favor of Smad2/3.

To determine the cellular effects of TRC105, we first tested its impact on endothelial cell growth using the MTT assay. We and others^{18,19} have previously reported that endoglin suppresses cell growth in part by downregulation of extracellular signal-regulated kinase (ERK) activation and c-Myc expression. Interestingly, endoglin targeting by TRC105 had minimal effect on cell growth relative to the control immunoglobulin G (IgG) over the course of 48 h treatment (Figure 2a, left graph) or by cell doubling time (data not shown), whereas endoglin depletion predictably yielded increased cell growth relative to the control (Figure 2a, right graph). We further compared the mitogenic response between endoglin targeting by TRC105 and endoglin depletion (Supplementary Figure 1A). Here, endoglin depletion by short hairpin RNA (shRNA) stable knockdown caused a notable increase in ERK activation that activates c-Myc expression compared with the non-targeting control Ab, whereas endoglin targeting by TRC105 or control Ab had minimal effects (Supplementary Figure 1A). In addition to proliferation, cells were assessed for TRC105 dose-dependent cytochrome c release as an indicator of mitochondrial dissolution and apoptosis. Consistent with the cell proliferation data, TRC105 did not induce a significant cytosolic cytochrome c release relative to untreated cells (~3–5%) (Figure 2b, graph). In comparison, TGF- β , as a known inducer of apoptosis, yielded 25–30% cytochrome c release (Figure 2b; graph). Furthermore, there was no detectable difference in caspase cleavage relative to control IgG (Figure 2c), indicating that TRC105 does not have a direct role in growth inhibition or apoptosis.

Although previous studies have established sEng as an antiangiogenic factor *in vitro* and *in vivo*,^{20–23} whether endoglin Abs regulate sEng production has not been examined. To test whether TRC105 has a functional role in regulating sEng production, we measured for endogenous sEng in the conditioned media of HUVECs and HMEC-1s upon TRC105 treatment for 24 h. Intriguingly, there was a distinct TRC105

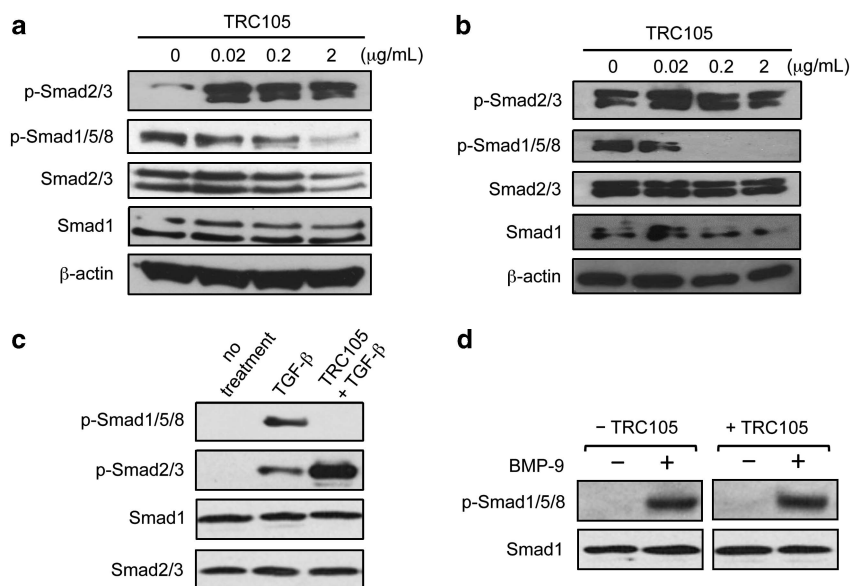


Figure 1. TRC105 promotes steady-state and ligand-induced Smad2/3 activation while inhibiting Smad1/5/8 signaling. Western blot analyses show endogenous expression of phosphorylated (p-) Smad2/3 (activated), total Smad2/3, p-Smad1/5/8 and total Smad1 in cell lysate following a 24 h treatment with TRC105 (0–2 μ g/ml) in HMEC-1 (a) and HUVEC (b). β -actin levels indicate equal loading of lysates in both cell types. (c) Western blot shows Smad1/5/8 and Smad2/3 activation in HMEC-1 under no treatment, TGF- β (100 pM) and TRC105 (0.2 μ g/ml) plus TGF- β (100 pM) for 30 min. Lower panels show total Smad1 and Smad2/3 from the same cell lysate. (d) Western blot shows Smad1/5/8 activation in response to BMP-9 treatment (30 min) in HUVEC in the presence or absence of TRC105 (0.2 μ g/ml) for 30 min. Data are representative of at least three independent experiments.

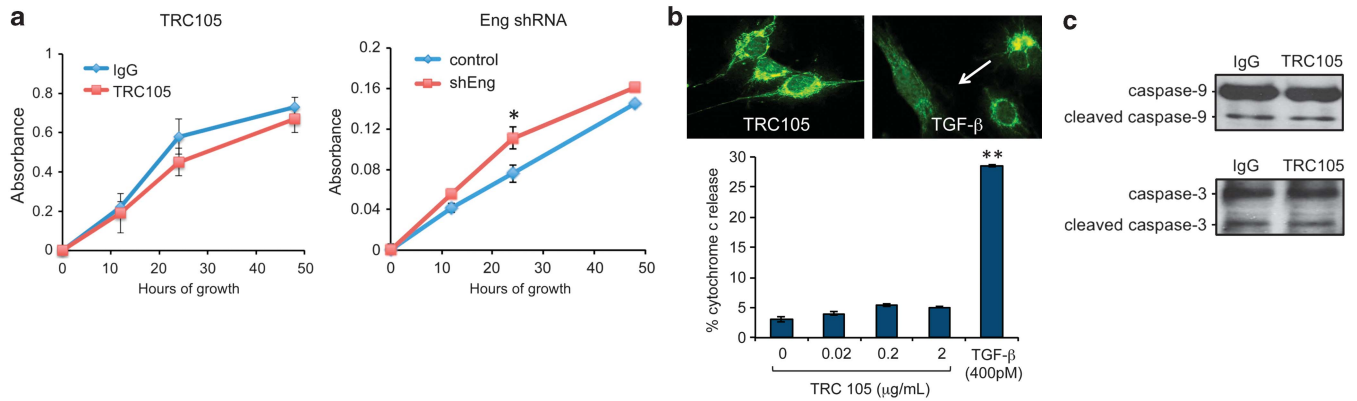


Figure 2. TRC105 does not induce endothelial growth arrest or apoptosis. **(a)** MTT assay showing the HUVEC growth pattern following treatment with either control IgG or TRC105 (2 µg/ml) for 12, 24, 48 h (left graph). A parallel MTT assay showing the effects of control and stable endoglin depletion through shRNA (shEng) in HMEC-1 (right graph). * $P = 0.001$ comparing control versus shEng at 24 h. **(b)** HMEC-1 cells were treated with different concentrations of TRC105 (0–2 µg/ml) and TGF-β1 (400 pM) for 24 h, and assessed for cytochrome c release via immunofluorescence. Shown are representative images of cells treated with TRC105 and TGF-β1. Arrow identifies a cell from which cytochrome c was released. Data are mean \pm s.d. of at least 30 cells counted for each condition (** $P < 0.01$ compared with control 0 µg/ml). **(c)** The western blot shows caspase-9 and -3-cleaved products upon treatment of HMEC with either IgG control or TRC105 (2 µg/ml) for 16 h.

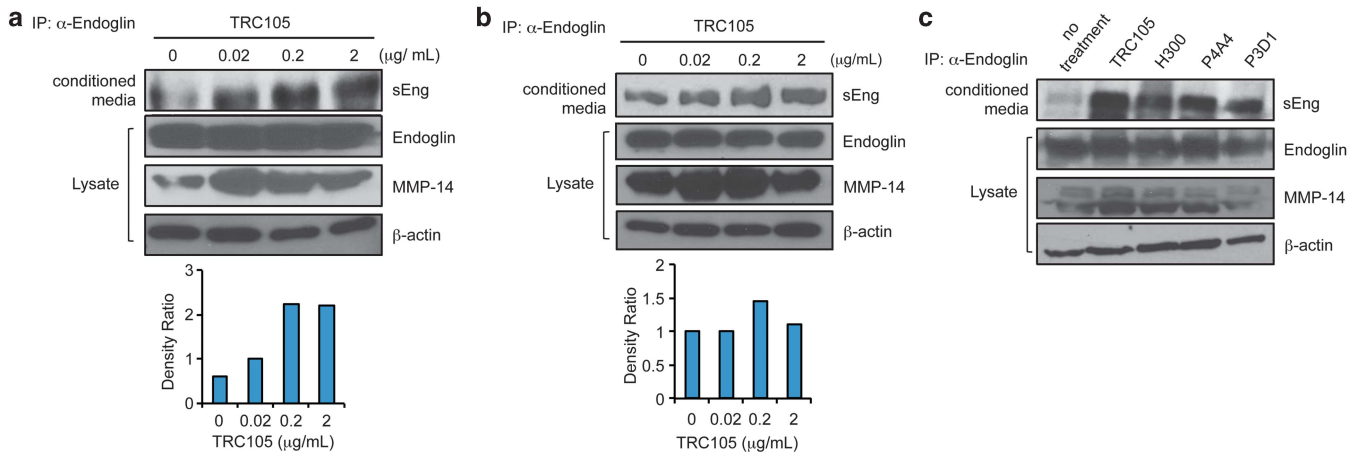


Figure 3. Endoglin-targeting Abs enhance sEng production. **(a, b)** Western blot of sEng immunoprecipitated from conditioned media (Abs P3D1 or H-300) after 24 h treatment with TRC105 (0–2 µg/ml) in HMEC-1 **(a)** and HUVEC **(b)** (top panels). Middle and lower panels show endogenous expression of membrane-anchored full-length endoglin, MMP-14 and β-actin in cell lysates. Graphs represent the density ratio of sEng to β-actin. **(c)** Endogenous sEng immunoprecipitated from the conditioned media upon 24 h treatment with indicated Abs directed against different epitopes on endoglin extracellular domain (200 ng/ml). Lower panels show expression of full-length endoglin, MMP-14 and β-actin in cell lysates. All of the western blots are representative of at least four independent experiments.

concentration-dependent increase in sEng when released into the media from both cell types, whereas expression of total endogenous cellular endoglin remained constant (Figures 3a and b upper and middle panels). As TRC105 caused endoglin downregulation by promoting shedding rather than degradation, we explored the possibility that TRC105 regulates the MMP-14 activity. Notably, MMP-14 expression was moderately increased as a result of TRC105 treatment (Figures 3a and b third panels). To test whether this was an epitope-related effect, three additional Abs targeting different epitopes were measured for their endoglin-shedding effects. Similar to TRC105, all three Abs tested significantly enhanced the level of sEng compared with no treatment (Figure 3c), indicating a general role for endoglin-targeting Abs in sEng production. Moreover, three of the four Abs tested, including TRC105, also promoted MMP-14 expression relative to the control (Figure 3c, third panel).

To define the mechanism of Ab-induced endoglin shedding, we first examined their direct effects on MMP-14 proteolytic activity. We hypothesized that endoglin-targeting Abs promote the

coupling of endoglin and MMP-14 into a complex at the cell surface. To test this, cells expressing myc-tagged endoglin and human influenza hemagglutinin (HA)-tagged MMP-14 were subjected to a brief pretreatment with TRC105 at 4 °C to allow TRC105 to react with membrane-localized endoglin while preventing their endocytosis. Although the immunoprecipitation with either anti-HA or MMP-14-specific Ab yielded a barely detectable transient interaction with full-length endoglin in the absence of pretreatment, TRC105 pretreatment dramatically enhanced their interaction, suggesting an Ab-induced coupling of endoglin/MMP-14 at the cell surface (Figures 4a and b). To exclude the possibility of TRC105 non-specifically targeting MMP-14, a parallel experiment was performed in which cells expressing MMP-14 alone, or MMP-14 and endoglin, were treated with TRC105 and then immunoprecipitated with TRC105 (Supplementary Figure 1B). As predicted, TRC105 failed to react with MMP-14 irrespective of pretreatment (lanes 1 and 2), and only co-immunoprecipitated MMP-14 when endoglin was co-expressed (lane 3, Supplementary Figure 1B). To specifically examine whether

the Ab-induced coupling of the endoglin/MMP-14 complex occurred at the cell surface, cells expressing MMP-14 and endoglin were pretreated with or without TRC105 at 4 °C before cell surface biotinylation, followed by immunoprecipitation of MMP-14. Consistent with the co-immunoprecipitation results, TRC105 pretreatment enabled the detection of biotinylated endoglin/MMP-14 complex, confirming TRC105 action at the cell surface (Figure 4c). Finally, to demonstrate that endoglin shedding specifically requires MMP-14 proteolytic activity, we used an MMP-14 blocking Ab (α -MMP-14) that neutralizes its catalytic function.^{24–26} Although TRC105 markedly enhanced endoglin shedding relative to the basal state, we repeatedly observed only slight increases in sEng upon MMP-14 inhibition (Figure 4d), presumably through indirect proteolytic actions of other MMPs. However, the level of sEng upon co-treatment of TRC105 and α -MMP-14 was lower than TRC105 alone, indicating that the TRC105-induced shedding requires MMP-14 activity (Figure 4d). Taken together, these biochemical studies indicate that TRC105 induces endoglin shedding by promoting its interaction with MMP-14 for proteolytic cleavage.

In addition to the biochemical approaches, we employed an immunofluorescence co-patching method to visualize TRC105-induced cell surface clustering of the endoglin/MMP-14 complex. Originally developed by Henis and co-workers^{27–29}, this method provides a semi-quantitative analysis of two known interacting proteins to form heteromeric co-patched clusters at the cell

surface. In control experiments, we measured the degree of random co-patching between MMP-14 with ALK3, another TGF- β superfamily membrane receptor. Upon expressing Myc-tagged ALK3 with HA-tagged MMP-14, each protein was allowed to aggregate into patched clusters by probing with its respective primary and then fluorophore-conjugated secondary Abs at 4 °C before fixation. This Ab-induced aggregation yielded mostly distinct green and red patches, indicating that TRC105 did not promote the dimerization of ALK3 and MMP-14 (Figures 5d and e; random overlay image and graph). In contrast, endoglin and MMP-14 co-expression followed by immunoprobining with TRC105 and either anti-HA or MMP-14-specific Ab resulted in significant co-patching and co-localization (Figures 5a–c, inset in image c and overlay in d). The level of TRC105-induced endoglin/MMP-14 co-patching was significantly greater than the random control (60 versus 10%) regardless of whether HA or MMP-14-specific Ab was used (Figure 5e graph). To further test whether this coupling is TRC105-specific or a general endoglin-Ab-directed effect, we used P3D1 Ab that also promoted sEng production (Figure 3c). P3D1 and MMP-14-specific Abs yielded ~40% co-patching (Figure 5e graph), strongly supporting a general role for endoglin-targeting Abs in endoglin/MMP-14 coupling at the cell surface. Taken together, these biochemical and immunofluorescence data demonstrate that endoglin-targeting Abs mediate endoglin/MMP-14 coupling at the cell surface to promote endoglin shedding.

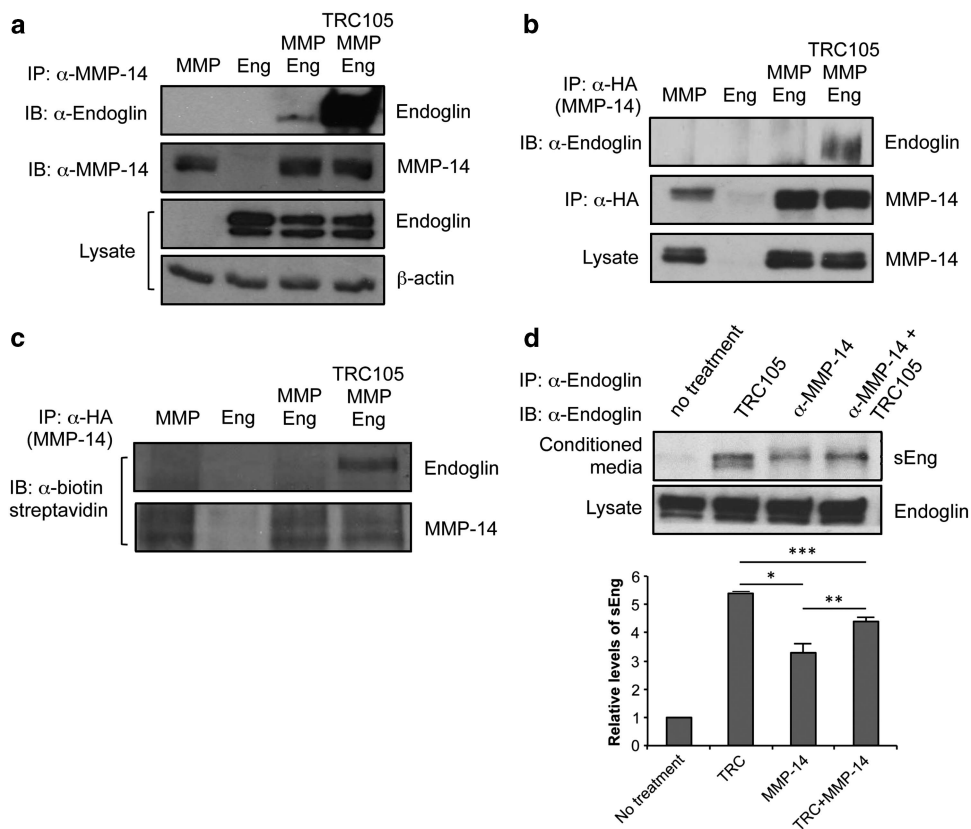


Figure 4. TRC105 enhances endoglin/MMP-14 association at the cell surface. (a) Western blot analysis shows co-immunoprecipitation of endoglin and MMP-14. COS-7 cells expressing HA-tagged MMP-14 (MMP), endoglin (Eng), MMP and Eng in the presence or absence of TRC105 (200 ng/ml) pretreatment for 10 min. Lysates were immunoprecipitated with MMP-14-specific Ab and immunoblotted for co-immunoprecipitated endoglin, MMP-14, total endoglin, and β -actin. (b) Western blot analysis shows co-immunoprecipitation of Eng and MMP-14 following immunoprecipitation with HA-Ab (MMP-14). (c) Cell surface biotinylation assay was performed in COS-7s expressing HA-tagged MMP-14 (MMP), Eng, MMP and Eng in the presence or absence of TRC105 pretreatment (200 ng/ml) at 4 °C. Cell lysates were prepared following biotinylation, and MMP-14 was immunoprecipitated with HA-Ab. The western blot shows biotinylated endoglin and MMP-14 detected with streptavidin-horseradish peroxidase (HRP). Data are representative of at least three independent experiments. (d) HUVECs were treated with TRC105 alone (200 ng/ml), MMP-14 blocking Ab alone (2 μ g/ml), or co-treated for 24 h. Graph represents a densitometry analysis of sEng present in each condition as a ratio compared with no treatment. Each error bar represents the s.d. derived from three independent experiments normalized to no treatment. * P < 0.001, ** P = 0.0014, *** P < 0.001.

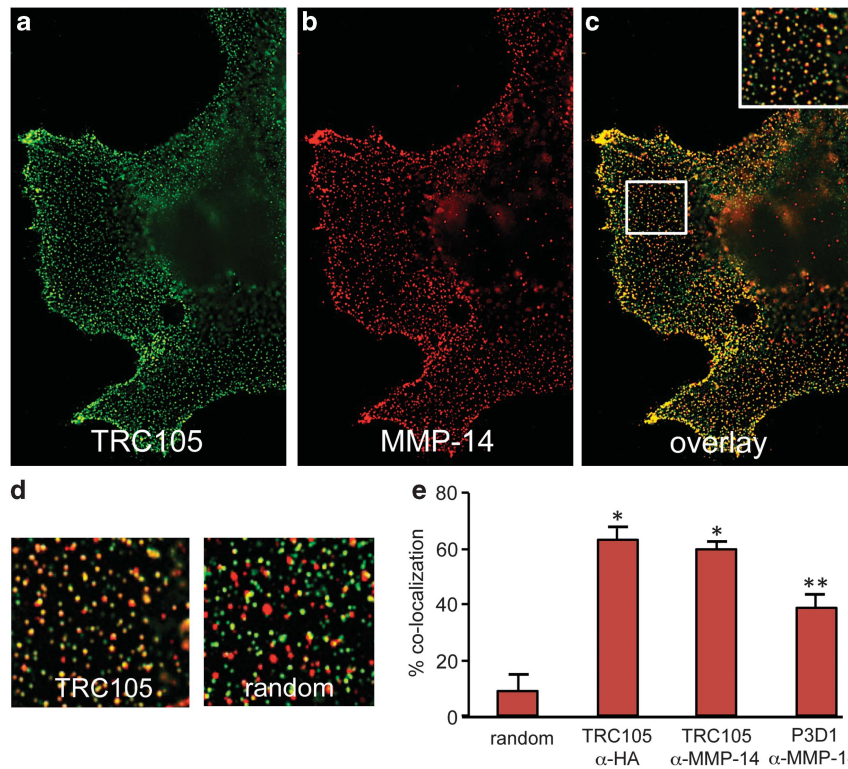


Figure 5. TRC105 couples endoglin/MMP-14 into co-patched clusters at the cell surface. (a) Green immunofluorescence indicates cell surface TRC105-mediated clusters of endoglin. (b) Red immunofluorescence indicates cell surface HA-Ab-mediated clusters of MMP-14. (c) Shown is the overlay image of endoglin/MMP-14 clusters. (d) Enlarged overlay images of endoglin/MMP-14 co-patched clusters in COS-7 cells treated with TRC105, or overlay images of MMP-14 and Myc-tagged ALK3 in cells probed with MMP-14 and anti-Myc-specific Abs, respectively. (e) Graph represents the quantification co-patched clusters of endoglin/MMP-14 or ALK3/MMP-14 (random). Cells were treated with TRC105 and HA-Ab (MMP-14), TRC105 and MMP-14-specific Ab, or P3D1 (endoglin) and MMP-14-specific Ab. For random quantification, Myc and MMP-14-specific Abs were used to induce patching. % Co-localization was quantified based on Pearson correlation coefficient measurements of at least 20 cells per condition. * $P < 0.01$, ** $P < 0.04$ compared with control (random).

Given the observation that TRC105 also promotes MMP-14 expression (Figure 3) that likely further potentiates endoglin shedding, we measured the effect of TRC105 on gene expression and found that TRC105 typically amplified *MMP-14* gene expression 1.5–2-fold relative to the control (Figure 6a). As TGF- β has been shown to transcriptionally regulate several members of the MMP family in other cell types by Smad2/3 induction of Snail transcription factor,^{30,31} we tested this pathway as a possible mechanism for TRC105-induced *MMP-14* gene expression. Contrary to expectations, blocking Smad2/3 activation with the ALK5 inhibitor (SB431542) markedly enhanced MMP-14 transcription relative to the control or TRC105 treatment (Figure 6b graph). Co-treatment with the ALK5 inhibitor and TRC105 failed to suppress MMP-14 transcription, suggesting that the TRC105-induced MMP-14 expression is Smad2/3-independent. We next screened several small-molecule inhibitors to identify other potential signaling effectors mediating this process. Induction of MMP-14 mRNA by TRC105 was most sensitive to JNK inhibition (Figure 6c). Consistent with this finding, there was a distinct concentration-dependent increase in JNK activation by TRC105 (Figure 6d), supporting the novel role of TRC105 in JNK-mediated MMP-14 transcriptional regulation.

Although sEng is a well-established antiangiogenic factor *in vivo*, the underlying molecular and cellular mechanisms have not been fully elucidated. Having ruled out growth inhibition and apoptosis as major cellular mechanisms, we tested whether TRC105 and sEng production disrupt cell motility. HUVEC and HMEC-1 were treated with either low or high concentrations of TRC105 and allowed to migrate in a transwell system.

Interestingly, TRC105 increased the migratory response in both the HMEC-1 and HUVEC (Figures 7a and b), a result consistent with the anti-migratory role for endoglin in previous studies using the endoglin knockout and knockdown systems.^{32–34} Next, given that MMP-14 activity is required for efficient motility in many cell types, we examined whether the TRC105 enhances migration by upregulating MMP-14 activity. To do so, we co-treated cells with TRC105 in the presence or absence of the MMP-14 blocking Ab. Consistent with previous reports of MMP-14-neutralizing function, α -MMP-14 treatment alone effectively suppressed cell migration relative to the control (Figure 7c). Furthermore, TRC105 treatment failed to override the inhibitory effects of α -MMP-14 in cells treated with both Abs (Figure 7c), suggesting that MMP-14 activity mediates TRC105-induced migration. Finally, to specifically test the role of sEng in cell motility, we compared the migratory response of TRC105-treated cells with those expressing a secreted form of endoglin into the conditioned medium (Eng-E: extracellular domain). As predicted, the expression of Eng-ECD resulted in a significant enhancement of migration similar to that of TRC105 treatment, the effects of which were not significantly reduced even when incubated with α -MMP-14 Ab (Figure 7d).

To determine the mechanisms by which increased endothelial migration might contribute to reduced angiogenesis, we examined several cellular properties including the actin cytoskeleton organization, and more the recently described phenomenon of endothelial-to-mesenchymal transition.^{35,36} Given that α -smooth muscle actin (α -SMA) is a marker of endothelial-to-mesenchymal transition, we tested for α -SMA expression in HUVEC and HMEC-1. Although α -SMA expression could not be detected by

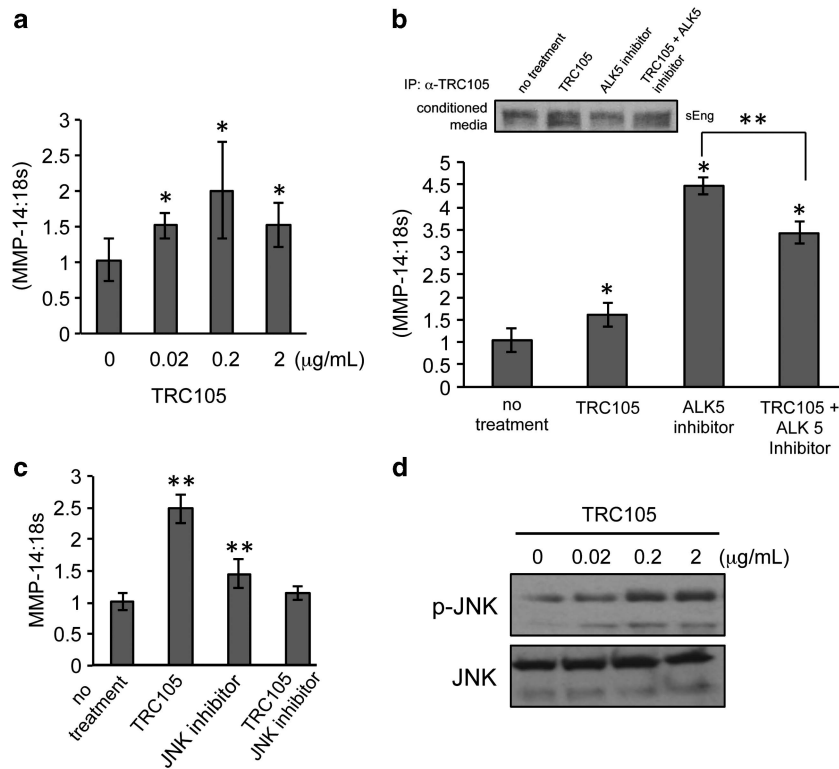


Figure 6. TRC105 promotes *MMP-14* gene expression in HUVEC. **(a)** Cells treated with TRC105 (200 ng/ml) for 24 h were quantified by SYBR green based quantitative PCR and analyzed by delta-delta-CT (ddCT) methods using 18S rRNA as internal control. Fold changes were calculated by setting the mean fractions of untreated cells as one. Bars indicate mean \pm s.d. in cells from TRC105-treated and -untreated cells. **(b)** Graph shows the effect of the ALK5 inhibitor (SB431542, 5 μ M) and/or TRC105 (200 ng/ml) for 24 h on *MMP-14* gene expression in HUVEC. Inset of western blot shows endogenous expression of sEng immunoprecipitated from the conditioned media of the same cells that were used to isolate RNA for *MMP-14* gene expression study. **(c)** Graph shows *MMP-14* gene expression upon treatment for 24 h with TRC105, JNK inhibitor (SP600125, 5 μ M) and TRC105 with JNK inhibitor. **(d)** Western blot analysis shows TRC105 concentration-dependent phosphorylation (activation) of JNK (upper panel) with t (lower panel) as loading control. * $P < 0.05$, ** $P < 0.001$.

immunofluorescence staining or immunoblotting (data not shown), we observed a striking increase in actin stress fibers in HMEC-1 and HUVEC when treated with TRC105 (Figure 8A). Moreover, while the additional contractile forces generated by the stress fibers likely contribute to enhanced cell motility, we also measured how TRC105 influences endothelial cell-cell contacts during the maturation phase of angiogenesis. Near confluent monolayers of HUVEC were treated with or without TRC105 for 12–24 h, then stained with endothelial-specific adherens junctions markers (Figure 8B). Whereas untreated cells formed a complete monolayer accompanied by prominent vascular endothelial (VE)-cadherin and platelet endothelial cell adhesion (PECAM) staining along cell-cell junctions. TRC105 treatment prevented the formation of efficient cell-cell contacts as indicated by the reduced VE-cadherin and PECAM localization along cell membranes (Figure 8B, I–III versus IV–VI). Taken together, our data here strongly support the role of TRC105 in perturbing normal endoglin regulation of endothelial migration and the formation of endothelial cell junctions.

DISCUSSION

Endoglin shedding is an important process in the regulation of angiogenesis and endothelial homeostasis. Not only does shedding reduce the overall cell surface level of endoglin, the resulting product acts as an antiangiogenic factor by sequestering circulating BMP-9.¹⁴ The recent discovery of MMP-14 as the major protease responsible for endoglin shedding has raised an important question as to whether this proteolytic processing is

actively regulated or whether it involves a general housekeeping mechanism. Our present study provides novel evidence that endoglin Abs have an important role in endoglin shedding—a key finding that is now supported by clinical evidence. Indeed, results from the first-in-human, phase I trial now reveal that, among the 37 plasma-based protein biomarkers tested, there is a dramatic dose-dependent increase in sEng levels in patients treated with TRC105 (personal communication with Drs Y Liu and A Nixon, Duke University, manuscript submitted).

Notably, our data indicate that TRC105 induces endoglin shedding through two overlapping mechanisms. First, we used multiple biochemical and immunofluorescence approaches to demonstrate that endoglin Abs enhance shedding by directly coupling endoglin and MMP-14 into complex at the cell surface (Figure 8). This process likely involves the stabilization of a preformed endoglin/MMP-14 complex, as our control experiments demonstrate that TRC105 specifically targets endoglin and does not directly react with MMP-14. Second, our small-molecule kinase inhibitor screening identified JNK signaling as the key mediator of TRC105-induced *MMP-14* gene expression instead of Smad2/3, which has been previously shown to induce *MMP-14* gene expression through Snail transcription factor. This finding was rather unexpected, as TRC105 promoted Smad2/3 activation at steady state and in rapid response to TGF- β (Figure 1). Instead, the Smad2/3 upregulation may contribute toward pro-migratory phenotype through transcriptional regulation of known mediators of cell motility, including PAI-1 (schematic, Supplementary Figure 2). Given that ALK5 is capable of eliciting mitogenic and pro-migratory signals through TGF- β -activated kinase (TAK1), our

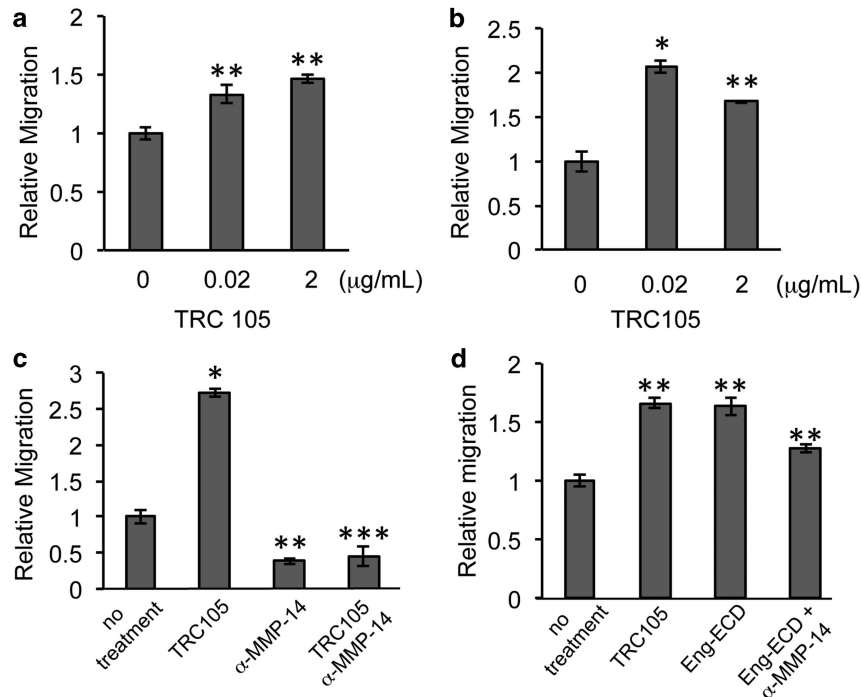


Figure 7. TRC105 and sEng induce cell migration. (a) HUVEC and (b) HMEC-1 were plated on transwells coated with 0.02% gelatin and treated with TRC105 (0 to 2 $\mu\text{g/ml}$) in growth media for 16 h. Cells that migrated to the bottom side of the membrane were fixed, stained for their nuclei and imaged for counting by using ImageJ software. (c) The graph represents the effect of TRC105 treatment (200 ng/ml with or without MMP-14-neutralizing Ab (1:500 v/v)) treatment on migration of HMEC-1. (d) The graph shows migration of HMEC-1 expressing the secreted endoglin extracellular domain (Eng-ECD), treated with or without TRC105 or MMP-14 Ab. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.08$. Relative cell migration is represented as a percentage compared with untreated cells, from triplicates for each of the three independent experiments. The error bars indicate the s.e.m. of the percentage of the cells that migrated.

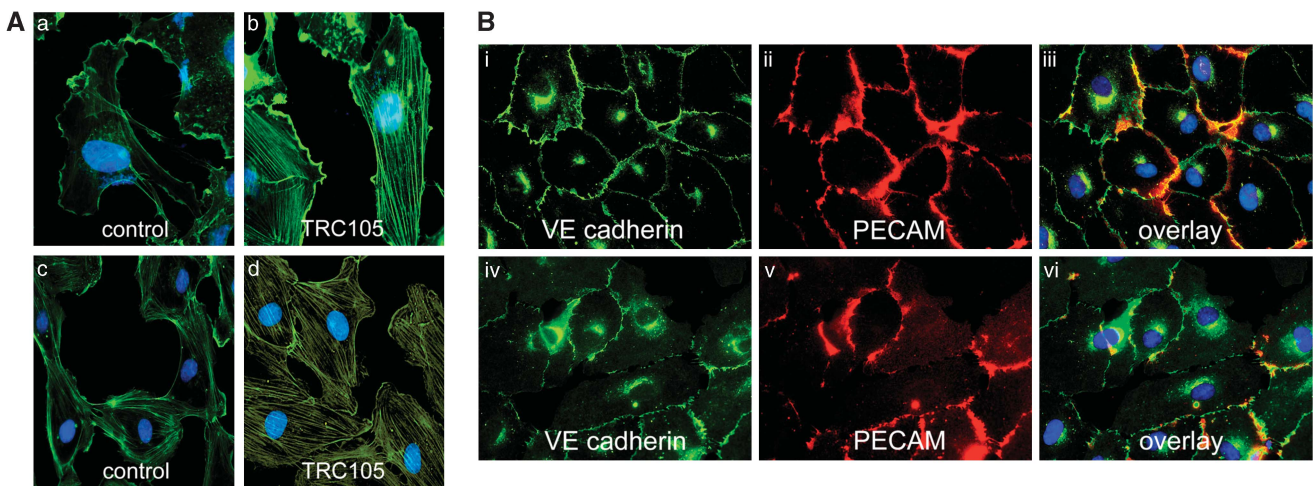


Figure 8. TRC105 induces stress fiber formation and dissolution of endothelial cell-cell junctions. (A) Alexa-phalloidin staining shows the detection of actin stress fiber organization in HMEC-1 (a, b) and HUVEC (c, d) in control versus treatment with TRC105 (0.2 $\mu\text{g/ml}$) for 16 to 24 h. (B) HUVEC were grown to near confluent monolayer (~80–90%) before either no treatment (I–III) or TRC105 treatment (IV–VI) (0.2 $\mu\text{g/ml}$) for 16–24 h. Cells were fixed, and then co-stained for VE-cadherin (green), PECAM (red) and DAPI (blue).

data is also consistent with the role of TRC105 in stimulating cell motility through ALK5/JNK-induced stress fiber formation. Our data here also reveal important clues as to how endoglin Abs may alter receptor oligomerization at the cell surface, not only with ALK1 and ALK5, but also another subset of TGF- β superfamily receptors such as ALK3 and ALK6, which are known to interact with endoglin in various contexts.^{22,23,37,38} How TRC105 and

related Abs alter these heteromeric receptor complexes need to be studied to more fully understand the molecular basis for their antiangiogenic effects.

Although it is clear that endoglin Abs cause endoglin shedding, the fate of the remaining membrane-anchored intracellular domain is unclear. Although this relatively short cytoplasmic domain lacks a catalytic function, it contains important structural

elements that serve as docking sites for multiple adaptor proteins. To date, at least five binding partners have been identified: zyxin, zyxin-related protein 1, β -arrestin2, Tctex2 β and GIPC.^{32–34,39,40} Given that these proteins differentially regulate endoglin signaling, trafficking or exert distinct cellular effects during angiogenesis, it will be critical to define precisely how endoglin Abs affect these important interactions.

Apart from the mechanism of endoglin shedding, understanding the overall effects of TRC105 on endoglin signaling and biology appears to be much more complex than anticipated. For instance, endoglin depletion in HMEC-1 increased the mitogenic response (that is, ERK activation, c-Myc expression and cell proliferation), whereas TRC105 yielded no changes, suggesting that, overall, TRC105 alters rather than neutralizes endoglin function. This notion is consistent with our data showing that endoglin expression is not significantly altered by TRC105 even though a subpopulation of endoglin undergoes shedding. Another related but surprising finding in our studies was that TRC105 and its sEng byproduct minimally induced growth arrest or apoptosis, two widely recognized cellular outcomes of TGF- β signaling to the ALK5/Smad2/3 pathway (Figure 2). These results are in contrast with previous preclinical studies in which TRC105-induced apoptosis in HUVEC.⁴ Although our results showed no such TRC105-related effects, a notable difference between the two studies is the markedly higher concentration of TRC105 used to induce apoptosis (50–100 μ g/ml) compared with our experimental conditions (0.02–2 μ g/ml). Preclinical pharmacokinetic studies suggest that TRC105 binds to human endoglin with a K_d of 4.6 ng/ml (31 pM), and achieves saturation at 200 ng/ml in proliferating HUVEC. Although we also did observe growth inhibition and apoptosis at significantly higher doses (500–1000 μ g/ml), these effects were relatively small (~10%) and statistically similar to that of IgG control (data not shown). Still, there are other discrepancies that cannot be accounted for, such as the recent study demonstrating that a relatively high dose of TRC105 (6 μ g/ml or higher) effectively blocks BMP-9-induced Smad1/5/8 activation in HUVEC.⁴¹ In direct contrast, our data indicate little or no effects on BMP-9/Smad1/5/8 activation either at low (0.2 μ g/ml; Figure 1d) or high concentrations (10 μ g/ml; data not shown).

In our present work, a much more defined endothelial dysfunction was evidenced in uncontrolled migration of two different endothelial cell types. To date, the role of endoglin in endothelial migration remains controversial, with earlier studies demonstrating that endoglin and ALK1 together promote migration, whereas others^{10,23,33,42} have reported the opposite finding. Although certainly the varying experimental conditions may help explain these disparate findings, it is noteworthy that the role of sEng in these studies had not been examined as a critical variable. Our data suggest that the degree of sEng production may have a key role in fine-tuning cell motility, and is consistent with previous endoglin knockout and knockdown studies, demonstrating that endoglin inhibits migration and proliferation in certain contexts in order to facilitate neovessel maturation.^{18,19,32,33,43}

In summary, we have examined the multifunctional role of TRC105 to provide immediate and clinically relevant mechanistic insights for rapid improvement of existing antiangiogenic therapies. Importantly, our results demonstrate a previously unanticipated role of TRC105 in upregulating sEng production that contributes to deregulated endothelial migration and significantly alters TGF- β signaling to impair angiogenesis.

MATERIALS AND METHODS

Cell culture, transfection and Abs

HUVEC were purchased from Lonza and maintained in endothelial growth medium (EGM)-2 growth medium (Lonza, Walkersville, MD, USA). HUVEC from passages 2 to 9 were used for experiments. HMEC-1 were maintained

in MCDB-131 medium (Invitrogen, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 1 μ g/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (Sigma, St Louis, MO, USA) and 2 mM L-glutamine. COS-7 cells were maintained in DMEM with 10% fetal bovine serum. Lipofectamine 2000 was used to transfect COS-7 cells as described according to manufacturer's protocol (Invitrogen). HMEC-1 were either transfected with Lipofectamine or Amaxa 4D nucleofection system according to manufacturer's protocol. Primary Abs used in this study were: TRC105 (TRACON Pharmaceuticals, San Diego, CA, USA), endoglin (H-300, Santa Cruz Biotechnology, Dallas, TX, USA), endoglin P4A4, P3D1, and PECAM (University of Iowa Hybridoma, Iowa city, IA, USA), HA-Ab (Roche, Applied Sciences, Indianapolis, IN, USA), MMP-14 (Abcam, Cambridge, MA, USA) and β -actin (Sigma-Aldrich, St Louis, MO, USA). The following Abs were all purchased from Cell Signaling (Danvers, MA, USA): total Smad2/3 (no. 8685), total Smad 1 (no. 6944) and phospho-Smad2/3 (no. 9510), phospho-Smad 1/5/8 (no. 9511), VE-Cadherin (no. 2500), total SAPK/JNK (no. 9258), phospho-SAPK/JNK (no. 9665), caspase-3 (no. 9665) and caspase-9 (no. 9508).

Immunoprecipitation

Cells were washed with PBS, then lysed on ice with lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 10% (w/v) glycerol, 1% Nonidet NP-40) and supplemented with protease inhibitors (Sigma protease inhibitor cocktail) and phosphatase inhibitors (Sigma phosphatase inhibitor cocktail). The lysates were precleared by centrifugation and incubated with appropriate Abs and protein agarose G for 4–6 h at 4 °C. The immunoprecipitates were collected by centrifugation; pellets were washed with lysis buffer, and stored in 2 \times sample buffer before western blot analyses. For immunoprecipitation of sEng from the conditioned media, typically media from 10-cm plates of HUVEC and HMEC-1 were collected and concentrated by Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA, USA) before immunoprecipitation with appropriate Abs.

Reverse transcriptase, real-time PCR and shRNAs

Total RNA was extracted from the cells with Trizol reagent (Invitrogen), and 2 μ g RNA was then converted to cDNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY, USA). Gene expression of MMP-14 was quantified by real-time reverse transcriptase PCR (Applied Biosystems, Step One) using the SYBR green assay reagent and gene-specific primer (Forward 5'-CCTGCCTGCGTCCATCA-3'; and Reverse 5'-TCCAGGGACGCTCATCA-3'). Relative amplification was quantified by normalizing the gene-specific amplification to that of 18s rRNA Forward (5'-GCTCTAGAATTACCACAGTTATC-3') and Reverse (5'-AAATCAGTTATGGTTCCTTTGGT-3') in each sample. The specific products were confirmed by SYBR green single-melting curve and a single correct-size product when running in 2% agarose gel. Changes in mRNA abundance were calculated using $2^{-\Delta\Delta C_T}$ method.^{44,45} Quantitative PCR were run in triplicate. Statistical significance is presented as mean \pm s.d. The knockdown of human endoglin in HMEC-1 was achieved by transfecting cells with a shRNA vector purchased from Sigma (Mission shRNA) (5'-CCGGCCACTTCTACACAGTACCCATCTCGAGATGGGACTGTGTAG AAGTGGTTTTG-3') and stably selected using puromycin.

Immunofluorescence

HMEC-1s and HUVECs grown on coverslips were serum starved for 3–4 h, washed with PBS and then fixed with 4% paraformaldehyde. Cells were permeabilized in 0.1% Triton X-100 in PBS for 4 min, then blocked with 5% bovine serum albumin in PBS containing 0.05% Triton X-100 for 20 min. All primary Abs were incubated at room temperature for 1 h unless noted otherwise. Cytochrome *c* was detected using cytochrome *c* Ab (Invitrogen). VE-cadherin (Cell Signaling) and PECAM Abs (University of Iowa Hybridoma) were used to detect endothelial cell–cell junctions. Actin was detected using Alexa Fluor-conjugated phalloidin for 30 min. Wherever appropriate, cells were co-stained with DAPI (Sigma) immediately before immunofluorescence microscopy analyses. For immunofluorescence co-patching, cells expressing endoglin and MMP-14 were washed with cold PBS, blocked with 5% bovine serum albumin in PBS before 1 h incubation with appropriate Abs (TRC105 and Myc or HA) at 4 °C to allow cell surface labeling while preventing internalization. Cells were then washed three times with cold PBS, then incubated with secondary fluorophore-conjugated Abs (for example, anti-human for TRC105 and anti-mouse for HA or Myc-Ab) for 30 min at 4 °C. To analyze the co-patching, Pearson correlation coefficient software (<http://www.wessa.net/>)

rwasp_correlation.wasp/) was used with ImageJ (<http://rsbweb.nih.gov/ij/index.html>) to calculate the degree of co-localization of two fluorophores.

Transwell migration assays

Cells were seeded in the upper chamber of a transwell filter in growth media, coated both at the top and bottom with gelatin to assess cell migration. Cells were allowed to migrate for 16 h at 37 °C through the gelatin-coated toward the lower chamber containing growth media with TRC105 (0.02–2 µg/ml). Migrated cells on the bottom surface of the filter were fixed, stained and then digitally imaged before counting.

Cell surface biotinylation assay

Cells were washed briefly with cold PBS before biotinylation with membrane-impermeable biotinylation reagent (Sulfo-NHS-LC-Biotin, Thermo Scientific, Pierce, Rockford, IL, USA) according to manufacturer protocol. The biotinylation reaction was neutralized and cells were washed three times with cold PBS, then lysed and prepared for immunoprecipitation with MMP-14 (HA-Ab). The immunoprecipitated MMP-14 along with interacting proteins were resolved on SDS-PAGE and immunoblotted with streptavidin-horseradish peroxidase.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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