Src-mediated Post-translational Regulation of Endoglin Stability and Function Is Critical for Angiogenesis*

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Christopher C. Pan‡, Sanjay Kumar‡, Nirav Shah§, Dale G. Hoyt¶, Lukas J. A. C. Hawinkels†, Karthikeyan Mythreye‡, and Nam Y. Lee‡

From the ‡Division of Pharmacology, College of Pharmacy, and ¶Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio 43210, the §Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208, and the †Department of Molecular Cell Biology, Leiden University Medical Center, Leiden University, 2333 Leiden, the Netherlands.

Background: Endoglin overexpression promotes angiogenesis but the mechanism of endoglin down-regulation is largely unknown.

Results: Endoglin YIY motif is phosphorylated by Src and induces receptor down-regulation.

Conclusion: The YIY motif is an endocytic signal for endoglin turnover.

Significance: Given that endoglin is a vascular target, defining how endoglin expression is post-translationally regulated is crucial for anti-angiogenic therapies.

Endoglin is a transforming growth factor β (TGF-β) co-receptor essential for angiogenesis and tumor vascularization. Endoglin modulates the crucial balance between pro- and anti-angiogenic signaling by activin receptor-like kinase (ALK) 1, 5, and TGF-β type II (TβRII) receptors. Despite its established role in physiology and disease, the mechanism of endoglin down-regulation remains unknown. Here we report that the conserved juxtamembrane cytoplasmic tyrosine motif (612YIY614) is a critical determinant of angiogenesis. Src directly phosphorylates this motif to induce endoglin internalization and degradation via the lysosome. We identified epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) as Src-activators that induce endoglin turnover following 612YIY614 phosphorylation. Interestingly, Src phosphorylation of endoglin-612YIY614 was also an important process for receptor down-regulation by TRACON105 (TRC105), an endoglin-targeting antibody currently in clinical trials. The regulation of 612YIY614 phosphorylation was critical for angiogenesis, as both the phosphomimetic and unphosphorylatable mutants impaired endothelial functions including proliferation, migration, and capillary tube formation. Collectively, these findings establish Src and pro-angiogenic mitogens as critical mediators of endoglin stability and function.

Transforming growth factor β (TGF-β) is a multifunctional cytokine that exerts a wide range of biological and cellular effects (1–7). In the vascular system, TGF-β has dichotomous roles in endothelial functions such as proliferation and migration during angiogenesis (8). The canonical TGF-β signaling pathway, present in all cell types, causes endothelial cell (EC) growth-arrest or apoptosis through the TGF-β type II (TβRII) and activin receptor-like kinase (ALK) 5 receptor complex that activates downstream Smad 2/3 transcriptional effectors (4). Opposing this process is an endothelial-specific transcriptional response elicited by TGF-β and a structurally related member, bone morphogenetic protein (BMP)-9, which signals through ALK1 to activate Smad1/5/8 (8). As a co-receptor, endoglin modulates the crucial balance between ALK1 and ALK5 signaling during angiogenic progression (9–13).

Endoglin expression is markedly elevated in actively proliferating ECs during angiogenesis and vascular remodeling (10). Many growth factors or conditions known to promote tumor progression also stimulate endoglin gene expression, including TGF-β, hypoxia, and inflammation (12). Indeed, its robust expression serves as a highly sensitive marker of tumor vascularization, and strongly correlates with tumor growth, metastases, and poor overall prognosis (11, 14, 15). Endoglin is also recognized as a highly effective vascular target, as demonstrated by TRC105, the first humanized endoglin antibody used in clinical trials for treatment of advanced or metastatic tumors (16–18). But despite recent advances, many facets of endoglin biology are still poorly understood at the molecular and cellular level. In particular, while extensive efforts have aimed at suppressing endoglin function to block tumor vascularization, the normal post-translational mechanism(s) mediating endoglin down-regulation remains virtually unexplored.

Previous endoglin structure-function studies reveal a large extracellular ligand-binding domain that binds to BMP-9/10, while TGF-β and other structurally-related family members including activins, can bind to this domain in a heteromeric complex with ALK1, TβRII, BMP, or activin receptors (2, 12). The extracellular domain is linked to a single transmembrane segment, followed by a short cytoplasmic domain that serve as key docking sites for a number of signaling-trafficking adaptor partners including zyxin, Tctex2β, and β-arrestin2 (12, 19–22). Many serine/threonine phosphorylation sites within this domain are associated with endoglin internalization. Throm-
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bin, for instance, has been shown to inhibit endoglin serine phosphorylation and induce receptor endocytosis via protease-activated receptor 1 and protein kinase C activation (23). Moreover, β-arrestin2 is thought to bind a phosphorylated threonine residue at position 650 to cause receptor internalization (19). Still, while endoglin serine/threonine phosphorylation appears to have direct roles in endocytic trafficking and downstream signaling, endoglin expression level remains unaffected (19, 23).

Interestingly, in addition to the many serine/threonine phosphate and phosphorylation and adaptor-binding sites interspersed throughout the C-terminal tail, there is an evolutionarily conserved peptide sequence containing two key tyrosine residues at positions 612 and 614 (612YIY614), located immediately distal to the transmembrane segment for which no functional role has been assigned. Given such a close proximity to the plasma membrane, we hypothesized that these tyrosine residues, if phosphorylated, could induce important local conformational changes that affect endoglin function, or serve as platform for tyrosine kinase signal transduction complexes.

Here we present evidence that the conserved tyrosine motif is a critical determinant of endoglin stability and angiogenesis, and further demonstrate that TRC105 requires this motif to efficiently down-regulate endoglin from the cell surface.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, Transfections, and Antibodies (Abs)**—Mouse embryonic endothelial cells (ECs) (Eng+/+ and Eng−/−) were derived from wild type and endoglin knock-out mice at E9 as previously described (19, 24, 25). ECs were maintained in MCDB-131 medium (Invitrogen) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 15% fetal bovine serum, 100 µg/ml heparin, 25 µg/ml endothelial cell growth supplement. HMEC-1 was maintained in MCDB-131 medium supplemented with 10% fetal bovine serum, 1 µg/ml hydrocortisone (Sigma), 10 ng/ml endothelial cell growth factor (Sigma), and 2 mM L-glutamine. COS7 cells were maintained in DMEM with 10% fetal bovine serum, 100 µM NaCl, 2 mM EDTA, 10 mM NaF, 10% (w/v) glycerol, 1% Nonidet P-40 and supplemented with protease inhibitors (Sigma protease inhibitor mixture) and phosphatase inhibitors (Sigma phosphatase inhibitor mixture). The lysates were pre-cleared by centrifugation and incubated with appropriate primary Abs for 2–4 h, and then with protein agarose G/A for 1–2 h at 4 °C. The immunoprecipitates were collected by centrifugation; pellets were washed with lysis buffer, and stored in 2X sample buffer before Western blot analyses. For soluble endoglin, endoglin was immunoprecipitated from the conditioned media with extracellular-targeting antibody (TRC105 or P3D1).

**Immunoprecipitation**—Cells were washed and 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) glycero, 1% Nonidet Nonidet P-40) and supplemented with protease inhibitors (Sigma protease inhibitor mixture) and phosphatase inhibitors (Sigma phosphatase inhibitor mixture). The lysates were pre-cleared by centrifugation and incubated with appropriate primary Abs for 2–4 h, and then with protein agarose G/A for 1–2 h at 4 °C. The immunoprecipitates were collected by centrifugation; pellets were washed with lysis buffer, and stored in 2X sample buffer before Western blot analyses. For soluble endoglin, endoglin was immunoprecipitated from the conditioned media with extracellular-targeting antibody (TRC105 or P3D1).

**Immunofluorescence**—Eng+/− ECs or COS7 cells grown overnight on coverslips were transiently transfected with appropriate constructs using Lipofectamine 2000 (Invitrogen) as described. For ubiquitin staining, cells were treated with MG132 (10 µM) for 2 h prior to fixation. For TRC105-induced endoglin internalization, ECs were pre-treated with TRC105 (200 ng/ml) for the indicated duration prior to fixation. 24–48 h following transfection, cells were washed with PBS and then fixed with 4% paraformaldehyde. Cells were permeabilized in 0.1% Triton X-100 in PBS for 3–10 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. Eng-WT, Eng-YE, and Eng-YF expression was detected using TRC105. EEA1, LAMP1, and ubiquitin Abs were used to detect early endosomes, lysosomes, and ubiquitin clusters, respectively. Proteasome was detected using 20 S Proteasome α6. ALK1, TβRII, and ALK5 were detected using ALK1, TβRII, and HA Abs, respectively. Following primary antibody incubation, cells were incubated with appropriate fluorophore conjugated secondary antibodies (Alexa-Fluor) at room temperature for 30 min. Cells were coated with DAPI (Sigma) immediately before immunofluorescence microscopy analyses (Olympus FV1000 confocal system). Pearson correlation coefficient analysis was used with ImageJ to measure co-localization.

**Transwell Migration Assays**—Eng+/+ and Eng−/− ECs were transfected with appropriate endoglin constructs. 24 h following transfection, cells were seeded in the upper chamber of a transwell filter in complete growth media, coated both at the top and bottom with gelatin. Cells were allowed to migrate for 12 h toward the lower chamber containing growth media alone or growth media containing EGF (5 × 10⁻⁴ mg/ml) or VEGF (5 × 10⁻⁴ mg/ml). Cells that migrated to the bottom surface of the filter were fixed, stained, and then digitally imaged and counted.
Crystal Violet Cell Growth Assay—Eng−/− ECs were plated at 15,000 in 12-well plates and transfected with the appropriate endoglin constructs. Following transfection, cells were fixed at different time points (4% paraformaldehyde in PBS for 15 min). Following fixation, cells were washed with 1× water and stained with 0.1% crystal violet for 20 min. Cells were washed 3× with water and allowed to air dry for 30 min. Cells were destained using crystal violet destaining solution (10% acetic acid, 50% methanol, 40% H2O) for 20 min, and the optical density was read at 590 nm in a microplate reader.

Endothelial Tube Formation—Eng−/− ECs were transfected with the appropriate endoglin constructs. 24 h following transfection, cells were trypsinized and plated on a 24-well plate coated with 200 μl of matrigel basement matrix (BD Biosciences) at 160,000 cells/well. 1 h following plating, growth medium was removed and 200 μl of matrigel basement matrix was added. 30 min following the addition of the matrigel base-
(both Tyr to Phe; Eng-YF), and were transiently expressed in previously described endoglin-null (Eng−/−) ECs (Fig. 1C) (19, 25). Interestingly, Eng-YE expression was strikingly reduced compared with Eng-WT or Eng-YF, suggesting that this phosphomimetic mutant either failed to express properly, or was constitutively degraded (Fig. 1C).

Immunofluorescence microscopy revealed that both Eng-WT and Eng-YF had membrane and cytoplasmic distribution typically observed for TGF-β receptors including endoglin, whereas Eng-YE displayed an atypical punctate endocytic profile (Fig. 1D). To rule out the possibility of protein aggregation of Eng-YE due to structural perturbations, we tested the expression of endoglin that harbors dual alanine substitutions at the YIY motif (both Tyr to Ala; Eng-YA). The Eng-YA expression pattern was quite similar Eng-WT and Eng-YF, indicating that the phosphomimetic mutation likely alters Eng-YE expression. Given that the membrane-bound endoglin can undergo ectodomain cleavage by the membrane-anchored matrix metalloproteinase-1 (MMP-14) to release soluble endoglin (sEng) (26), we compared sEng production derived from Eng-WT, Eng-YE, and Eng-YF-expressing Eng−/− ECs to determine whether Eng-YE is capable of properly folding and trafficking to the cell surface. Here, sEng production was detected from the conditioned media of all three forms, albeit to a slightly lesser extent from Eng-YE-expressing cells (Fig. 1E). As no secreting isoforms of endoglin exist, these results indicated that Eng-YE is capable of trafficking to the cell surface for ectodomain cleavage despite a large localized subset in endosomal compartments.

To determine the subcellular characteristics of the Eng-YE-containing endosomes, we co-stained Eng-YE with a panel of endosomal markers including EEA1, late endosome (Rab9), lysosome (LAMP1), ubiquitin, and the proteasome (26 S) (Fig. 2). Here, the Eng-YE-containing endocytic vesicles showed prominent co-localization with various markers using Image J and Pearson's correlation coefficient analysis. Two to three random regions (ROI) away from the nuclear regions were chosen from each Eng-YE-expressing cell for correlation analyses with the indicated markers. Error bars represent the standard error of the mean of correlation coefficients between Eng-YE and each marker. Data are based on the analyses of at least 15 Eng-YE-positive cells (3 ROIs each) from three independent experiments. 

FIGURE 2. Phosphomimetic mutation of the tyrosine endocytic motif mediates endoglin trafficking to the lysosome. A, Eng-YE expression in Eng−/− ECs is detected with TRC105 (green) and co-stained for EEA1 (red). The numbered squares within each image represent typical ROIs used for the quantification of co-localization. B, Eng-YE expression (red) is shown to co-localize with Rab9-GFP (green). C, Eng-YE expression (green) is shown to co-localize with LAMP1 (red). D, Eng-YE expressing ECs (green) are pretreated with MG132 (4 h) prior to fixation and counterstaining for ubiquitin (red), and the proteasome (blue). E, quantification of Eng-YE co-localization with various markers using Image J and Pearson's correlation coefficient analysis. Two to three random regions (ROI) away from the nuclear regions were chosen from each Eng-YE-expressing cell for correlation analyses with the indicated markers. Error bars represent the standard error of the mean of correlation coefficients between Eng-YE and each marker. Data are based on the analyses of at least 15 Eng-YE-positive cells (3 ROIs each) from three independent experiments. F, immunoblot of Eng-YE expression in Eng−/− ECs treated with either chloroquine (100 μM, 200 μM) or MG132 (10 μM) for 9 h. G, COS7 cells expressing Eng-YE (green) are pretreated with chloroquine (100 μM) for 18 h and stained for LAMP1 (red).
chboroquine treatment produced appreciably enlarged Eng-YE-containing vesicles that strongly co-localized with LAMP1 upon accumulation in lysosomal compartments (Fig. 2G). Taken together, these findings indicated that Eng-YE down-regulation occurs primarily through the lysosomal degradation.

Given that endoglin can form a heteromeric complex with ALK1 and TβRII, we next examined whether the endoglin YIY motif influences their subcellular trafficking. As expected, the typical membrane and cytoplasmic ALK1 localization was not significantly altered upon co-expression with either Eng-WT or Eng-YF, nor did ALK1 accumulate in LAMP1-containing vesicles (Fig. 3A and B). In sharp contrast, ALK1 was recruited into endocytic vesicles by Eng-YE where they co-localized with late endosomal markers such as Rab9 (Fig. 3C, D, F). Importantly, the global ALK1 expression was selectively reduced when co-expressed with Eng-YE but not with Eng-WT or Eng-YF, supporting the specific role of the YIY motif in regulating endoglin and ALK1 stability (Fig. 3E). Similar outcomes were observed for TβRII where its membrane and diffuse cytoplasmic expression was unaltered upon co-expression with Eng-WT or Eng-YF, and was not targeted for lysosomal degradation (Fig. 4, A and B). However, TβRII co-localized with Eng-YE in endocytic vesicles in late endosomal compartments (Fig. 4, C and D). Interestingly, while ALK5 is also capable of interacting with endoglin, this receptor sustained its normal cytoplasmic distribution when expressed alone (Fig. 4F), or when co-expressed with Eng-YE (Fig. 4G). Taken together, these findings indicate that the endoglin phosphomimetic motif selectively mediates ALK1 and TβRII degradation but not ALK5.

To specifically test whether the YIY endocytic motif becomes tyrosine phosphorylated, we first immunoprecipitated for proteins that are tyrosine phosphorylated upon expression of Eng-WT, Eng-YE, or Eng-YF in endoglin-null background (Fig. 5A). Here tyrosine phosphorylation was only detected for Eng-WT despite the extracellular tyrosine residues present in both Eng-YE and Eng-YF, indicating that the YIY motif is the major site of endoglin tyrosine phosphorylation (Fig. 5A; lanes 1–4). To identify the major kinase responsible for endoglin tyrosine phosphorylation, we searched for YIY-containing peptide substrate motifs recognized by various protein tyrosine kinases. Previous studies have shown that c-Src and related family kinases recognize a diverse set of tyrosine motifs as substrates including the YIY sequence with a hydrophobic residue in position

FIGURE 3. Endoglin tyrosine motif regulates ALK1 trafficking to the lysosome. A, Eng−/− ECs expressing either Eng-WT (green; upper panels) or Eng-YF (green; lower panels) and HA-ALK1 (red) and stained for LAMP1 (blue). B, quantification of Eng-WT or Eng-YF with ALK1 and LAMP1 using ImageJ and Pearsons correlation coefficient analysis. Random regions were chosen from each Eng-WT or Eng-YF-positive Eng−/− EC and analyzed for correlation with ALK1 and LAMP1. Data are based on the analyses of at least 5 Eng-WT and Eng-YF-positive cells (2 ROIs each) from three independent experiments. C, ALK1 (red) co-localization with Eng-YE (green) in punctate vesicles in Eng−/− ECs. D, Eng−/− ECs expressing Eng-YE, ALK1, and Rab9-GFP (green) and stained for ALK1 (red). E, Western analysis of Eng−/− ECs expressing HA-ALK1 alone, HA-ALK1 with Eng-WT, HA-ALK1 with Eng-YE, HA-ALK1 with Eng-YF. F, quantification of Eng-YE co-localization in a complex with ALK1 and Rab9 using ImageJ and Pearsons correlation coefficient analysis. Random regions of each ALK1-positive cell were analyzed for correlation with ALK1 and Rab9. Data are based on the analyses of at least 15 Eng-YE-positive cells (3 ROIs each) from three independent experiments.
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2 and a basic amino acid in position 7 (27, 28). Given that endoglin contains this YIY peptide sequence followed by a conserved basic residue arginine at position 7 (Fig. 1B), we tested c-Src as a potential kinase by inhibiting its catalytic activity in the presence of Eng-WT tyrosine phosphorylation relative to control (Fig. 5A; lane 2 versus 5). Next, to determine whether Src regulates endoglin expression through tyrosine phosphorylation, Eng-WT and Eng-YF were expressed in the presence of increasing Src expression (Fig. 5B). Indeed, Eng-WT stability inversely correlated with increased Src expression, whereas Eng-YF remained constant, indicating that the tyrosine motif is a key determinant of endoglin stability and turnover.

To determine whether endoglin is a Src substrate, we tested for their endogenous interaction by co-immunoprecipitation. Here, interaction between endogenous endoglin and Src was detected in ECs when immunoprecipitated with an antibody targeting the endoglin extracellular domain, but not upon endoglin knockdown or immunoprecipitation with a control IgG (Fig. 5C). We next tested for Src interaction with the endoglin mutants to determine whether Src binding specifically required the YIY motif. Similar co-immunoprecipitation studies revealed that Src interacted with Eng-WT and Eng-YF, but not Eng-YE, strongly suggesting that the bulky dual aromatic side chains of the WY612IY614 motif serve as a structural recognition motif for the Src catalytic domain (Fig. 5D; third panel). More importantly, the Eng-WT/Src interaction resulted in endoglin tyrosine phosphorylation whereas the Eng-YF/Src complex did not, hence further supporting endoglin Y612Y614 as a novel Src phosphorylation motif and not an SH2 domain-binding site (Fig. 5D; lane 2 versus 4 of second panel).

We next screened for various Src-activating cytokines and growth factors that mediate endoglin tyrosine phosphorylation and degradation (29, 30). While 5 ligands tested induced Src activation to varying degrees compared with no treatment, only VEGF and EGF caused notable endoglin turnover during a 2-h stimulation, indicating a selective Src-dependent response (Fig. 5E; first and middle panel). To further test Src as a mediator of this process, we examined the effects of VEGF and EGF stimulation on endogenous endoglin expression in the presence or absence of Src inhibition (Fig. 5F). Consistent with our previous results, both VEGF and EGF-induced endoglin turnover was completely blocked upon Src inhibition. Taken together, the results provide novel evidence for Src-mediated endoglin down-regulation following stimulation by pro-angiogenic mitogens VEGF and EGF.

Antibody-induced internalization is a common process by which membrane receptors are degraded. Endoglin antibodies have also been previously reported to induce receptor internalization and presumed to become degraded through an unknown mechanism (31, 32). As part of our ongoing investigation on the anti-angiogenic properties of TRC105, we tested the functional role of the tyrosine endocytic motif in TRC105-induced endoglin internalization. To this end, Eng-WT and...
Eng-YF-expressing Eng−/− ECs were incubated with TRC105 for various time intervals to allow antibody-induced endoglin internalization in live cells (0 to 6 h). Internalization of the receptor-antibody complex (TRC105-Eng) was monitored at each time point by labeling with a fluorescent secondary antibody upon cell fixation followed by permeabilization. As expected, at early time points there was minimal TRC105-induced internalization of cell surface labeled Eng-WT and Eng-YF (Fig. 6, A and B; 0 to 15 min). However, a significant level of TRC105-Eng-WT was observed in endosomes from 0.5 to 2 h relative to TRC105-Eng-YF (Fig. 6C), suggesting that the tyrosine endocytic motif plays a major role in the endocytic process. Co-staining with markers for early endosomes (EEA1) and the lysosome (LAMP1) further revealed that the receptor complex is ultimately degraded (Fig. 7A; upper and lower panels). Interestingly, there was a TRC105 concentration-dependent increase in endoglin tyrosine phosphorylation that was abrogated upon Src inhibition (Fig. 7, B and C), further supporting a general role for Src-induced tyrosine phosphorylation in endoglin degradation.

Finally, we began characterizing the cellular roles of the tyrosine motif by comparing the effects of Eng-WT, Eng-YE, and Eng-YF expression on endothelial functions. Consistent with our previous findings that indicated endoglin regulation of EC migration (33), here again endoglin inhibited cell motility by 30–40% when comparing between Eng+/+ and Eng−/− ECs, but demonstrated a further inhibition upon rescue expression of Eng-WT in Eng−/− ECs (Fig. 8A). But unlike Eng-YF and Eng-WT, Eng-YE phenocopied Eng−/− ECs in that it failed to suppress migration likely due to its constitutive turnover (Fig. 8A). In parallel studies, we examined the effects of VEGF and EGF stimulation, which normally enhance EC migration through mitogenic signaling (34). Indeed, Eng-WT-expressing ECs treated with either VEGF or EGF promoted EC migration relative to control, whereas Eng-YF expression impaired the VEGF and EGF-induced migratory response (Fig. 8, B and C). Although Src inactivation generally reduces mitogenic and migratory responses in many cell types including ECs, here Src inhibition had the greatest effect at suppressing VEGF and EGF-induced motility for Eng-WT expressing ECs but not Eng-YF (Fig. 8D), suggesting that endoglin turnover following tyrosine phosphorylation by Src is an integral process of VEGF/EGF-induced EC migration.

In addition to migration, we examined the role of the tyrosine motif in a three-dimensional capillary tube formation assay. Consistent with our previous findings, Eng−/− ECs formed unstable capillary tube-like structures that regressed over time compared with those rescued with Eng-WT expression (Fig. 9, A and B). However, the rescue expression with Eng-YE or Eng-YF only partially restored efficient capillary branching and stability relative to Eng-WT (Fig. 9B), suggesting that a proper balance in endoglin tyrosine phosphorylation is critical during capillary morphogenesis. Consistent with this notion, Src inhibition during capillary morphogenesis had minimal impact on...
the stability and branching capacity of all except Eng-WT expressing ECs (Fig. 9B). Although the similarly reduced levels of capillary formation observed in Eng-YE and Eng-YF was rather unexpected, this could be attributed to the fact that Eng-YE-expressing ECs formed branches that regressed over time, whereas Eng-YF-expressing ECs failed to efficiently sprout new branches from the initial stages (24 versus 72 h; Fig. 9C). Consistent with this notion, Eng-YF-expressing ECs not only had impaired VEGF and EGF-induced cell migration (Fig. 8), but also proliferated slower than Eng-YE expressing ECs (Fig. 9D). Overall, our results demonstrate that the phosphorylation status of the endoglin tyrosine motif and stability play critical roles in EC functions during angiogenesis.

DISCUSSION

Our investigation of the conserved endoglin tyrosine motif revealed one of the first post-translational mechanisms by which endoglin is regulated. Our study defines Src and potentially other family members as major tyrosine kinases contributing to endoglin turnover, and further identifies Src-activating pro-angiogenic mitogens (VEGF and EGF) as regulators of this process. A previous study investigated the post-translational effects of endoglin expression by tumor necrosis factor (TNF)-α (35). Here, TNF-α-induced endoglin down-regulation appeared to be a much more gradual process requiring up to 24 h, whereas Src-induced degradation was far more rapid (Fig. 4D). Nevertheless, as the underlying mechanism for TNF-α-induced endoglin turnover has yet to be defined, it will be interesting to determine whether Src and the endoglin 612YI614 motif are involved in this process.

Whereas little is known about endoglin down-regulation, the process by which TβRII and ALK5 expression is regulated is more thoroughly characterized, with previous studies demonstrating their internalization in both clathrin-coated pits and lipid raft endosomes (1, 36–38). Furthermore, it is now known that ALK5 degradation requires the ubiquitin-associated salt-inducible kinase (SIK) and Smad7 (39, 40). While we failed to detect endoglin ubiquitination and degradation via the proteasome, it still remains plausible that a subset is degraded via the proteasome as part of heteromeric complexes with TβRII and/or ALK5, or through other endoglin binding partners. In contrast, results indicating a clear chloroquine-induced Eng-YE accumulation in the lysosomal compartments, as well as its restored overall expression (Fig. 2, F and G), strongly support a novel role for the tyrosine motif in mediating endoglin degradation through the lysosomal pathway.
The fact that endoglin down-regulation occurred mostly in response to VEGF and EGF stimulation, and not other Src-activating ligands, indicates that Src targeting of the endoglin tyrosine motif is context-specific and likely tightly regulated. Consistent with this notion, our data showed that insulin, not VEGF or EGF, yielded the highest sustained Src activation dur-
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Endoglin tyrosine phosphorylation is critical for endothelial capillary sprouting and stability. A, representative images of three-dimensional Matrigel-induced capillary tubules for Eng−/− ECs, and Eng−/− ECs expressing Eng-WT, Eng-YE, or Eng-YF for 72 h. B, quantification of overall capillary tube formation and branching by measuring the average number of branches per node at 72 h in the presence or absence of PP2 treatment (5 μM) 72 h following plating. *, p < 0.05 (Eng−/−, Eng-YE, or Eng-YF compared with Eng-WT); **, p = 0.01 (Eng-WT + SRC inhib compared with Eng-WT). C, time course measurements of capillary tube branching for Eng-WT, Eng-YE, and Eng-YF at 24 and 72 h. D, crystal violet growth assay of Eng−/− and Eng−/− ECs expressing Eng-WT, Eng-YE, or Eng-YF 12 h, 24 h, and 36 h following transfection. *, p = 0.002 (Eng−/− compared with Eng-WT at 36 h); **, p = 0.00005 (Eng-YE compared with Eng-WT at 36 h).

The defects in capillary tube formation shared by both Eng-YE and Eng-YF were initially surprising since endoglin normally enhances tube formation, and therefore a less stable vessel than proliferation, since Eng-YF ECs proliferated slightly faster than that of Eng-WT (Figs. 8C and 9D). Although the restored expression of endoglin wild type (Eng-WT) inhibited proliferation in Eng−/− just as we reported previously, currently it is unclear as to why Eng-YF expression increases proliferation relative to Eng-WT over time (Fig. 9D; 24 h versus 36 h). Given that endoglin modulates numerous signaling pathways closely associated with migration and proliferation, such as the Smads, ERK, and PI3K/Akt, it will be crucial in future studies to investigate the signaling properties governed by this novel tyrosine motif.

Defining the mechanisms by which TRC105 down-regulates endoglin is part of our ongoing investigation. Our current work demonstrates that TRC105 causes endoglin internalization and receptor trafficking toward lysosomal degradation (Fig. 6). Although the precise mechanism is unclear, the rapid, concentration-dependent effects of TRC105 on Src activation and endoglin tyrosine phosphorylation suggest that, whether induced by exogenous (i.e. VEGF/EGF), Src phosphorylation of the612Y1614 motif may be a crucial event preceding endoglin degradation.

In conclusion, this work provides critical new information on the structure and function of endoglin. Our studies indicate that the evolutionarily conserved612Y1614 motif serves as a direct substrate for Src tyrosine kinase and functions as a key determinant for endoglin down-regulation and angiogenesis. Along these lines, Src-activating mitogens such as VEGF and EGF may regulate endoglin expression as part of a negative feedback mechanism. Finally, our work provides clinically relevant data on the mechanisms by which TRC105 induces endoglin inhibition.
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