

# Endoglin Regulation of Smad2 Function Mediates Beclin1 Expression and Endothelial Autophagy\*

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**Background:** Endoglin is a key regulator of TGF- $\beta$  signaling in endothelial cells but its role in autophagy during angiogenesis is unknown.

**Results:** Endoglin relieves Smad2-dependent BECN1 repression.

**Conclusion:** Endoglin promotes autophagy during angiogenesis by regulating Smad2 function.

**Significance:** Endoglin-mediated autophagy may be an effective vascular target.

Autophagy is the targeted degradation of proteins and organelles critical for homeostasis and cell survival. Transforming growth factor  $\beta$  (TGF- $\beta$ ) differentially regulates autophagy in a context-specific manner, although the precise intracellular mechanisms remain less clear. Importantly, how TGF- $\beta$  controls autophagic responses in endothelial cells (EC) during angiogenesis is unknown. Here we identified endoglin, an EC-specific TGF- $\beta$  co-receptor essential for angiogenesis, as a key determinant of autophagy. Among the two opposing TGF- $\beta$  Smad pathways in the EC system (Smad1/5/8 and Smad2/3), we found Smad2 as the major transcriptional regulator of autophagy that targets beclin1 (BECN1) gene expression. Smad2, but not Smad3, acts as a repressor upstream of the BECN1 promoter region. Overall, endoglin promotes autophagy by impeding Smad2 transcriptional repressor activity. Notably, increased beclin1 levels upon Smad2 knock-down directly correlated with enhanced autophagy during angiogenesis. Taken together, these results establish endoglin as a critical mediator of autophagy and demonstrate a new transcriptional mechanism by which Smad2 inhibits angiogenesis.

Autophagy is a catabolic process in which cellular components are sequestered, degraded, and then recycled to maintain cellular homeostasis. In addition to removal of long-lived proteins and damaged organelles under basal conditions, autophagy is highly up-regulated during nutrient deprivation, hypoxia, and other unfavorable conditions to promote cell survival (1). Given these cytoprotective functions, defects in autophagy contribute to a number of human pathologies including cancer and neurodegenerative conditions (2).

Many of the integral components of the autophagic machinery have been characterized, including autophagy-related gene (Atg)<sup>2</sup> proteins and microtubule-associated protein light chain 3 (LC3) that undergo cleavage and lipid modifications (1). In particular, beclin1 (Atg6) is considered a key activator of early autophagosome assembly (3). Beclin1 binds to Vsp34, a class III phosphoinositide-3-kinase (PI3K), which mediates PIP3-dependent recruitment of additional Atg proteins (1, 3–5). Numerous transcriptional and post-translational mechanisms are in place to tightly regulate beclin1 function. Several transcription factors such as FoxO3, NF $\kappa$ B, HIF1 $\alpha$ , c-Jun, and E2F1 have been shown to drive beclin1 (BECN1) gene expression, whereas calpain-mediated degradation and caspase-dependent cleavage control its function (6–11).

Despite recent progress, the functional role of autophagy remains unclear in many biological contexts. In the case of angiogenesis, previous studies have shown that autophagy can exert angiogenic or angiostatic effects under various conditions (12–15). Similarly, mechanisms governing autophagy appear to be highly context-dependent, as illustrated by the dichotomous effects of transforming growth factor  $\beta$  (TGF- $\beta$ ). Previous studies have shown that TGF- $\beta$  induces autophagy in certain epithelial and cancer cell types, whereas it counteracts this process in fibroblasts (16, 17). Importantly, TGF- $\beta$  can simultaneously activate a number of distinct cellular pathways associated with pro- and anti-autophagic responses, raising the question of how different cell systems integrate these divergent signals to regulate autophagy. For instance, TGF- $\beta$  has been linked to autophagy induction through TGF- $\beta$  activated kinase 1 (TAK1), and Jun N-terminal kinase (JNK) in epithelial and tumor cells, whereas TGF- $\beta$ -induced AKT and mTOR activation has been shown to strongly inhibit autophagy in fibroblasts (16, 18, 19). As canonical effectors of TGF- $\beta$  signaling, Smads have also been shown to control autophagy, although whether they have direct transcriptional effects remains less clear (19).

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<sup>2</sup> The abbreviations used are: Atg, autophagy-related gene; TGF, transforming growth factor; LC, light chain; TAK, TGF- $\beta$  activated kinase.

Little is known regarding the role of TGF- $\beta$  in endothelial autophagy, prompting us to investigate how endoglin contributes to this process during angiogenesis. Endoglin is an integral component of the TGF- $\beta$  receptor complex in the vasculature essential for angiogenesis during embryonic development and tumor vascularization (20–23). Functional endoglin deficiency, whether due to mutations or genetic ablation, gives rise to defective angiogenesis and arteriovenous malformations (20–24). Endoglin is selectively expressed in proliferating endothelial cells to modulate the critical balance between canonical Smad1/5/8 (pro-angiogenic) and Smad2/3 (anti-angiogenic) transcriptional responses (22, 25, 26). Similar to the pleiotropic downstream signals induced by TGF- $\beta$  in other cell types, endoglin has the capacity to regulate non-Smad pathways including AKT and ERK (27, 28). Therefore, in the present study we examined the potential role of endoglin-based mechanisms in autophagy in the endothelial system.

## 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 (29). ECs were maintained in MCDB-131 medium (Invitrogen) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 15% fetal bovine serum, 100  $\mu$ g/ml heparin, 25  $\mu$ g/ml endothelial cell growth supplement. Human umbilical vein endothelial cells (HUVECs) were maintained in EBM-2 basal medium (Lonza) supplemented with EGM-2 SingleQuot Kit Suppl. and Growth Factors (Lonza). Transfections were achieved by using Lipofectamine 2000 as described according to manufacturer's protocol (Invitrogen). shRNA constructs for mouse and human Smads Smad2 were all purchased from Sigma. The shRNA targeting sequence for mouse Smad2 were: 5'-CCGGTGGTGTTCAATCGCA-TACTATCTCGAGATAGTATGCGATTGAACACCATT-TTTG-3' and 5'-CCGGCTAAGTGATAGTGCAATCT-TTCTCGAGAAAGATTGCACTATCACTTAGTTTTTG-3'. The shRNA targeting sequence for mouse Smad3 was 5'-CCGGCTGTCCAATGTCAACCGGAATCTCGAGATT-CCGGTTGACATTGGACAGTTTTTTG-3'. The shRNA targeting sequences for human Smad2 were: 5'-CCGGGCGTTG-CTCAAGCATGTCATACTCGAGTATGACATGCTTGAG-CAACGCTTTTTG-3' and 5'-CCGGCAAGTACTCCTT-GCTGGATTGCTCGAGCAATCCAGCAAGGAGTACTTG-TTTTTG-3'. The shRNA targeting sequence for human Smad3 was: 5'-CCGGGATTGAGCTGCACCTGAATGCT-CGAGCATTCAAGTGCAGCTCAATCCTTTTTG-3'. Stable Smad2 knockdown MEECs were generated by transfecting each mouse shRNA construct into Eng $^{+/+}$  ECs and selecting with puromycin (3  $\mu$ g/ml). Individual puromycin-resistant colonies were isolated and scaled up as clones upon validation of endogenous > 80% Smad2 depletion. Although different clones of sh-Smad2 MEECs were used interchangeably, the experimental data shown is based on ECs originally derived from transfection with the construct: 5'-CCGGTGGTGTTCAATCGCA-TACTATCTCGAGATAGTATGCGATTGAACACCATT-TTTTTG-3'. Basement matrigel matrix was obtained from BD Biosciences. TGF- $\beta$ 1 and BMP-9 were obtained from R&D Sys-

tems. Inhibitors of ALK1 (LDN193189), ALK5 (SB431542), PI3K (LY294002), TAK1 ((5Z)-7-Oxozeanol), JNK (SP600125), the lysosome (chloroquine), proteasome (MG132), and protein translation (cyclohexamide) were obtained from Sigma Aldrich. Abs used in this study were: TRC105 (TRACON Pharmaceuticals), endoglin (H-300, Santa Cruz Biotechnology), flag (Sigma-Aldrich), and  $\beta$ -actin (Sigma-Aldrich). The following abs were all purchased from Cell Signaling: total Beclin1 (no. 3738), total Atg5 (no. 12994), total Atg12 (no. 4180), total LC3 A/B (no. 4108), total p62 (no. 5114), phospho-Smad1/5 (no. 9516), total Smad1 (no. 6944), phospho-Smad2/3 (no. 9510), total Smad2 (no. 3122), total Smad2/3 (no. 8685), phospho-Akt (no. 13038), phospho-JNK (no. 9255), and GAPDH (no. 2118).

**Immunofluorescence**—Eng $^{+/+}$  ECs or Eng $^{-/-}$  ECs were grown overnight on coverslips and transiently transfected with appropriate constructs using Lipofectamine 2000 (Invitrogen) as described. 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. Flag-Smad2 expression was detected using anti-flag Ab. LC3 A/B Ab was used to detect autophagosome clusters. Following primary antibody incubation, cells were incubated with appropriate fluorophore conjugated secondary antibodies (Alexa-Fluor) at room temperature for 30 min. Cells were co-stained with DAPI (Sigma) immediately before immunofluorescence microscopy analyses (Nikon Eclipse Ti). Autophagy was quantified by counting the number of autophagosome positive cells and the number of autophagosome vesicles per cell. Statistical significance is presented as mean  $\pm$  S.E.

**Endothelial Tube Formation**—Eng $^{+/+}$  ECs stably expressing non-targeting control vector and Eng $^{+/+}$  ECs stably expressing Smad2 knockdown (sh-Smad2) were plated on a 24-well plate coated with 200  $\mu$ l of matrigel basement matrix (Corning) at 140,000 cells/well. 30 min following plating, growth medium was removed and 200  $\mu$ l of matrigel was added. 30 min following the addition of matrigel, 500  $\mu$ l of growth medium was added. Endothelial tubes were digitally imaged and quantified by counting the number of branches per node. Following imaging, endothelial tubes were lysed using 2 $\times$  sample buffer and immunoblotted for Beclin1 and LC3A/B.

**Reverse Transcriptase, Real-time PCR**—Eng $^{+/+}$  ECs were grown overnight on 6-cm dishes and transiently transfected with appropriate constructs using Lipofectamine 2000 (Invitrogen) as described. 24 h following transfection, cells were treated with ALK5 inhibitor (SB431542, 30  $\mu$ M) for 6 h. Total RNA was extracted from the cells with Trizol reagent (Invitrogen), and 2  $\mu$ g RNA was converted to cDNA through the use of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). BECN1 was quantified by real-time reverse transcriptase PCR (Applied Biosystems) using SYBR green assay reagent and gene specific primers (Forward: 5'-GGCCAATAAGATGGTCTGA-3'. Reverse: 5'-GCTGCACACAGTCCAGAAAA-3'). Primers for MMP-2 were: (Forward: CTTTCGCTCGTTT-CCTTCAAC) and (Reverse: AGAGTGAGGAGGGGAAC-CATR). Relative amplification was quantified by normalizing

## Endoglin Promotes Endothelial Autophagy

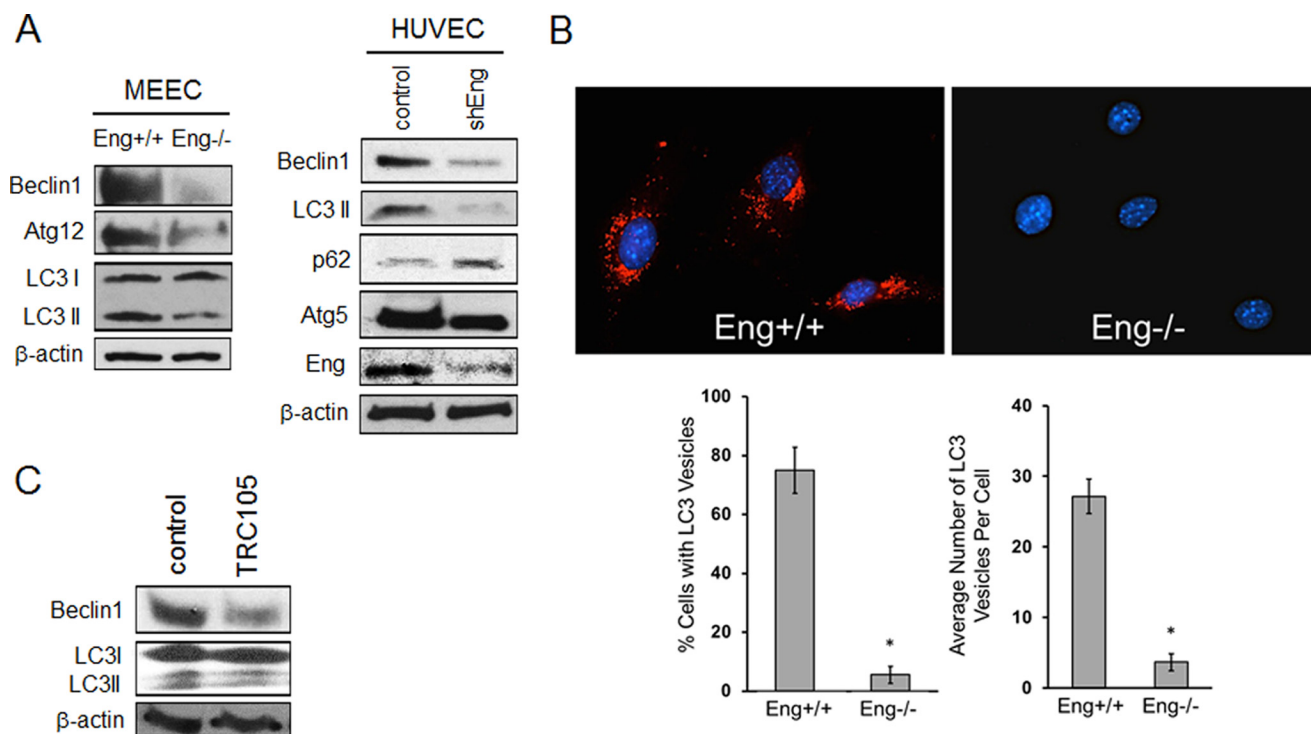


FIGURE 1. **Endoglin promotes autophagy.** *A*, Western analysis of autophagic markers in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> MEECs, and in HUVECs transiently expressing a scrambled, non-targeting shRNA (control) or shEng. *B*, LC3 A/B staining in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> MEECs followed by the percentage of LC3 A/B positive cells and number of autophagosome vesicles in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> MEECs. Bars indicate mean  $\pm$  S.E. \*,  $p$  value =  $1.1 \times 10^{-6}$ ; \*\*,  $p$  value = 0.01. *C*, Western analysis of Beclin1 levels and LC3 cleavage in HUVECs treated with TRC105 (200 ng/ml) for 24 h.

gene-specific amplification to that of 18S rRNA (5'-GCTCTA-GAATTACCACAGTTATC-3') and Reverse (5'-AAATCAG-TTATGGTTCCTTTGGT-3') in each sample. Changes in mRNA abundance were calculated using  $2^{(-\Delta\Delta C_T)}$  method. Quantitative PCR were run in triplicates. Statistical significance is presented as mean  $\pm$  S.E.

**Chromatin Immunoprecipitation Assay**—Eng<sup>+/+</sup> ECs stably expressing Smad2 knockdown were grown overnight in 10-cm dishes and transiently transfected with either control vector or Flag-Smad2. 20 h following transfection, cells were serum starved using MCDB-131 medium (Invitrogen) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate for 3 h and treated with 200 pM TGF $\beta$  for 30 min. Following treatment, formaldehyde was added directly to the media at a concentration of 0.75% v/v and rotated at room temperature for 10 min. Following rotation, glycine was added to the media to a final concentration of 125 mM and rotated at room temperature for 5 min. Cells were washed two times with PBS, harvested into a 50 ml tube by scraping, and centrifuged for 5 min at 1,000  $\times$  g. The resulting pellet was resuspended in 750  $\mu$ l of ChIP lysis buffer. ChIP lysis buffer and other buffers were made according to the recipes provided by Abcam. Samples were sonicated using 20-s bursts 9 times at 30% power. After sonication, samples were centrifuged for 30 s, 4  $^{\circ}$ C, 8,000  $\times$  g. Supernatants were harvested and immunoprecipitated using anti-flag (Sigma) and Protein G Plus-Agarose Suspension (Invitrogen) at 4  $^{\circ}$ C. Following immunoprecipitation, beads were washed three times with ChIP wash buffer and one time with ChIP final wash buffer. 120  $\mu$ l of elution buffer was added to the beads and rotated for 15 min. at room temperature. Following elution,

beads were centrifuged for 1 min at 2000  $\times$  g. Supernatants were harvested and incubated at 65  $^{\circ}$ C overnight.

### Results

While the effects of TGF- $\beta$  on autophagy have been well studied in many cell types, its actions in the vascular endothelium remain poorly defined. Given that endoglin is an essential component of the endothelial TGF- $\beta$  signaling receptor complex, we tested its role in endothelial autophagy by utilizing two types of endothelial cells (EC)- the previously characterized endoglin knock-out (Eng<sup>-/-</sup>) and control MEECs and primary human umbilical vein EC (HUVEC) (29). A panel of cellular autophagy markers was used to assess changes in autophagy in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs. Here Eng<sup>+/+</sup> ECs exhibited notably elevated levels of beclin1, Atg12, and cleaved LC3 product, LC3II, relative to Eng<sup>-/-</sup>, suggesting that endoglin expression is closely associated with increased basal autophagy (Fig. 1A: MEEC). To further test whether these are endoglin-specific effects, we knocked down endogenous endoglin in HUVECs and observed a substantial decrease in autophagy mediators such as beclin1, LC3II, and Atg5, whereas accumulation of p62 indicated the lack of autophagy (Fig. 1A: HUVEC). In addition, immunofluorescence LC3II staining in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs revealed a striking difference in autophagosome activity, as evidenced by significantly greater percentage of LC3-positive cells and number of LC3 aggregates per cell for Eng<sup>+/+</sup> ECs (Fig. 1B). In our previous studies we demonstrated that TRACON105 (TRC105), the first humanized monoclonal endoglin antibody being used in clinical trials for treatment of metastatic tumors, neutralizes

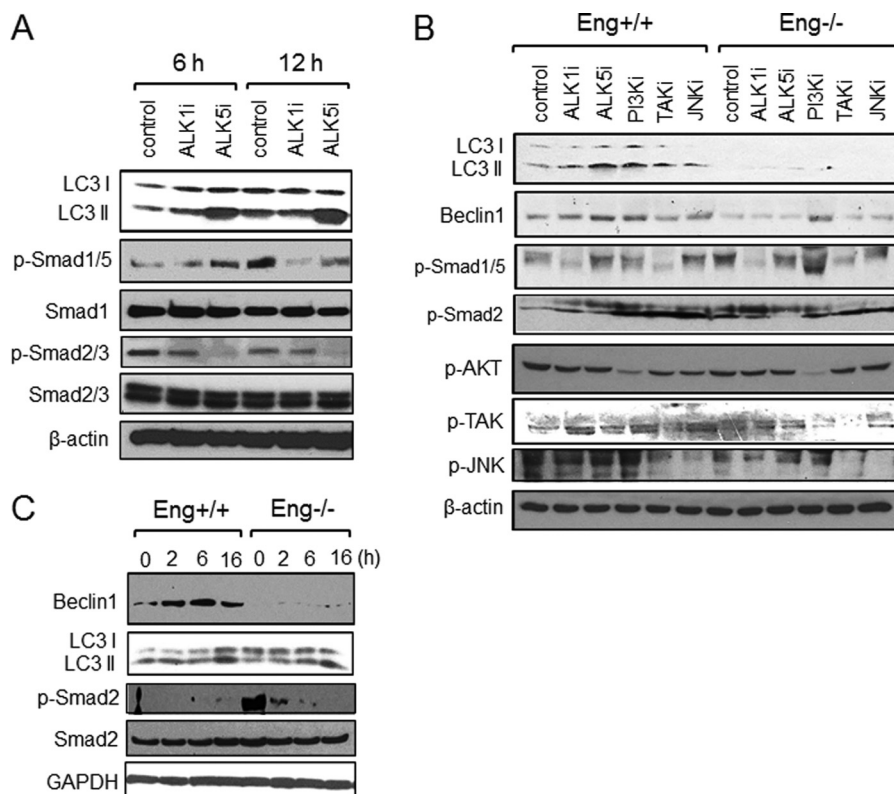


FIGURE 2. **ALK5 inhibition enhances autophagy.** *A*, Western analysis of LC3 cleavage in HUVECs treated with ALK1 inhibitor (LDN193189, 1  $\mu\text{M}$ ) and ALK5 inhibitor (SB431542, 30  $\mu\text{M}$ ) for 6 h and 12 h. *B*, Western analysis of LC3 cleavage and Beclin1 levels in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs treated with ALK1 inhibitor (LDN193189, 0.5  $\mu\text{M}$ ), ALK5 inhibitor (SB431542, 15  $\mu\text{M}$ ), PI3K inhibitor (LY294002, 15  $\mu\text{M}$ ), TAK inhibitor ((5Z)-7-Oxozeaenol, 5  $\mu\text{M}$ ), and JNK inhibitor (SP600125, 15  $\mu\text{M}$ ) for 16 h. *C*, Western analysis of LC3 cleavage and Beclin1 levels in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs treated with ALK5 inhibitor (SB431542, 30  $\mu\text{M}$ ) for 2, 6, and 16 h.

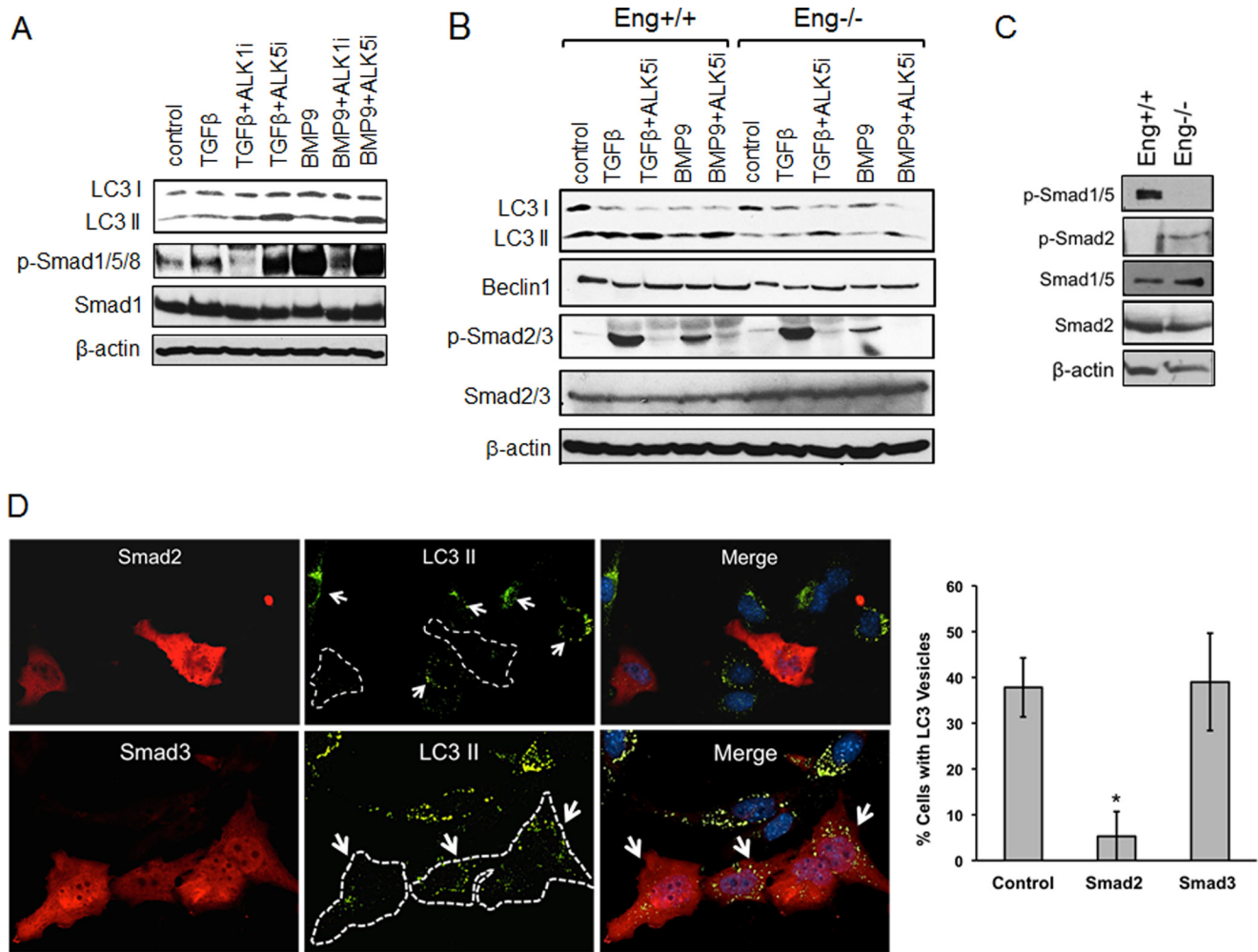
endoglin function by promoting its intracellular degradation and extracellular domain shedding (30). Here, treatment of HUVECs with TRC105 markedly reduced beclin1 levels and the cleaved LC3II (lower band), further demonstrating that endoglin is a key mediator of endothelial autophagy (Fig. 1C).

It is established that endoglin expression is dramatically elevated during angiogenesis to modulate TGF- $\beta$  signaling toward endothelial proliferation and migration via the ALK1-induced Smad1/5/8 pathway while simultaneously attenuating growth-inhibiting ALK5/Smad2/3 transcriptional responses. To test these pathways as mediators of endothelial autophagy, we first employed ALK1 and ALK5 inhibitors to inactivate Smad1/5 and Smad2/3 in HUVECs over the course of 12 h (Fig. 2A). Interestingly, a significant increase in LC3II levels was observed only upon ALK5 inhibition and subsequent Smad2/3 inactivation over the course 6–12 h, suggesting that ALK5-induced Smad2/3 signaling may down-regulate autophagy in ECs (Fig. 2A). However, as TGF- $\beta$  signaling through ALK5 also mediates the activation of a number of autophagic regulators including JNK, PI3K/AKT, and TGF- $\beta$ -activated kinase 1 (TAK1) in different cell systems, we tested for their potential influence on endothelial autophagy by pharmacologic screening in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> MEECs (Fig. 2B). Consistent with the results in HUVECs, greater levels of LC3II and beclin1 were present in Eng<sup>+/+</sup> relative to Eng<sup>-/-</sup> MEECs under no treatment and upon ALK5 inhibition. Surprisingly, minimal changes were

observed following TAK1 or JNK inactivation, suggesting that these autophagy inducers may have a minor role in the EC system. In contrast, PI3K inhibition caused a marked increase in LC3II and beclin1 levels, confirming its canonical inhibitory role in autophagy through AKT and mTOR signaling (Fig. 2B). Lastly, a time-course experiment showed a greater and more sustained expression beclin1 and LC3 cleavage in Eng<sup>+/+</sup> than Eng<sup>-/-</sup> ECs over time (Fig. 2C). Taken together, our results strongly suggested a distinct role for endoglin regulation of the ALK5/Smad2/3 activity to promote EC autophagy.

A distinct feature of TGF- $\beta$  signaling in ECs is its ability to activate the ALK1/Smad1/5/8 and ALK5/Smad2/3 pathways simultaneously (22, 31). Endoglin modulates the balance of these opposing pathways, favoring ALK1/Smad1/5/8 signaling and suppressing ALK5/Smad2/3 signaling during EC activation through TGF- $\beta$  and bone morphogenetic protein 9 (BMP9), a structurally related ligand previously characterized to bind selectively to endoglin and ALK1 (22, 32, 33). To test the role of endoglin ligands in EC autophagy, HUVECs were treated with TGF- $\beta$  or BMP9 in the presence or absence of ALK inhibitors (Fig. 3A). Here LC3II levels increased only upon ALK5 inhibition irrespective of TGF- $\beta$  or BMP9-induced Smad1/5/8 activation, suggesting that autophagy is Smad1/5/8-independent (*second panel*). Next, to test for ALK5/Smad2/3-mediated changes in autophagy, we subjected Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs to different ligand treatment in the presence or absence of ALK

## Endoglin Promotes Endothelial Autophagy

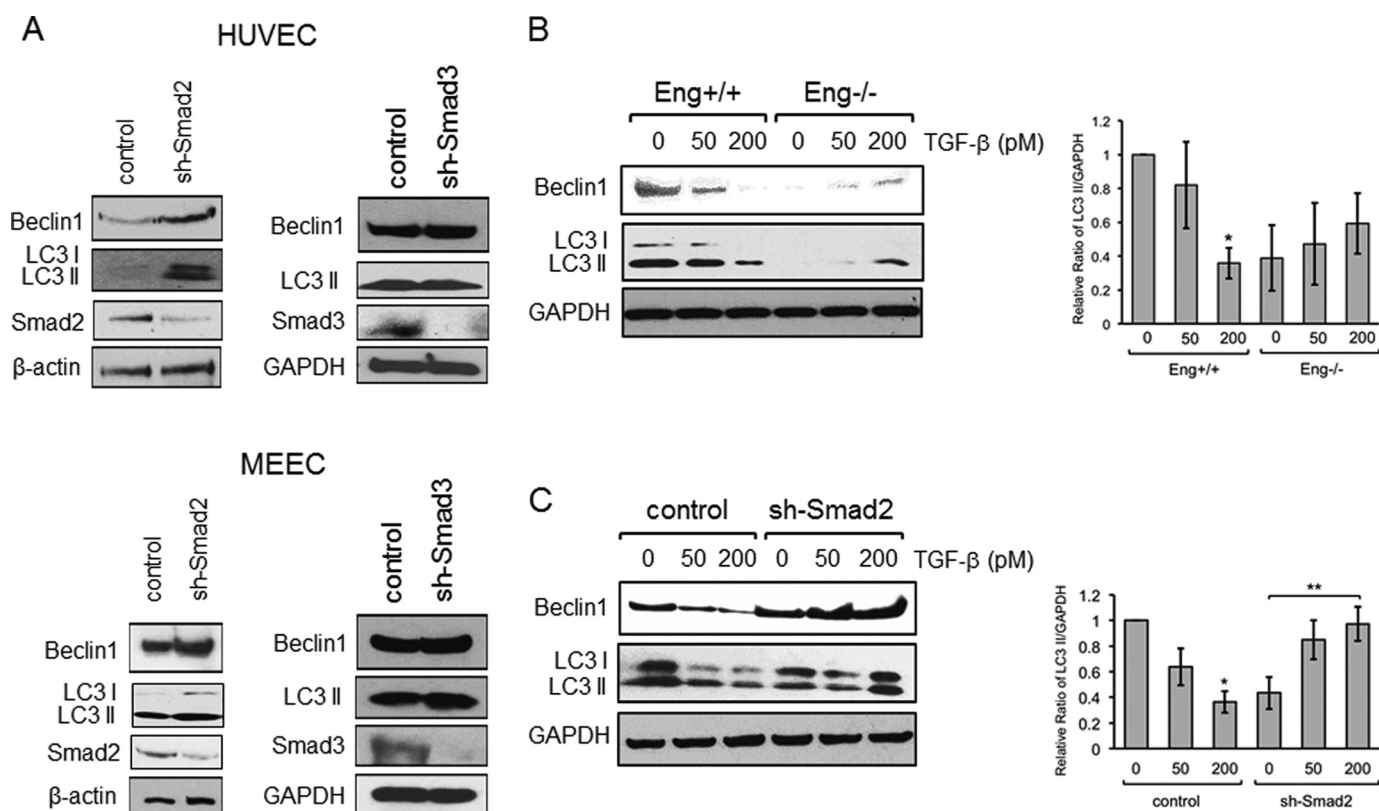


**FIGURE 3. Endoglin promotes autophagy through suppression of Smad2 activity.** *A*, Western analysis of LC3 I/II, p-Smad1/5/8 and Smad1 in HUVECs treated with TGFβ (100 pM) and BMP9 (1 nM) in the presence or absence of ALK1 inhibitor (LDN193189, 1 μM) and ALK5 inhibitor (SB431542, 30 μM). *B*, Western analysis of LC3 cleavage and Beclin1 levels in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs treated with TGFβ (100 pM) and BMP9 (1 nM) in the presence or absence of ALK1 inhibitor (LDN193189, 0.5 μM) and ALK5 inhibitor (SB431542, 15 μM). *C*, comparison of Smad1/5 versus Smad2/3 activation in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> MEECs in complete growth medium. *D*, immunofluorescence studies of Smad2, Smad3, and LC3II in Eng<sup>+/+</sup> MEECs transiently transfected with Flag-Smad2/3 and stained for Flag (red) and endogenous LC3 II (green), and graph quantification (\*, *p* value = 0.0007).

inhibitors and monitored LC3II and beclin1 levels (Fig. 3*B*). Similar to results in HUVECs, there was an endoglin-dependent increase in beclin1 and LC3II levels largely upon ALK5 inhibition, suggesting that endoglin promotes autophagy by selectively suppressing Smad2/3 activation (Fig. 3*B*). To test this, we first assessed the basal levels of Smad1/5 and Smad2 activation between Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs (Fig. 3*C*). As expected, more Smad2/3 activation occurred in Eng<sup>-/-</sup> ECs than Eng<sup>+/+</sup>, while the opposite held true for Smad1/5/8 activity (Fig. 3*C*). Interestingly, immunofluorescence studies revealed a notable loss of LC3II staining in Smad2, but not Smad3 overexpressing cells, indicating that Smad2 is the major inhibitor of endothelial autophagy (Fig. 3*D* and graph quantification). Similar results were observed in a reciprocal experiment where a notable increase in beclin1 and LC3II levels were associated with knockdown of Smad2 but not Smad3 in both HUVECs and MEECs (Fig. 4*A*). Next, the effects of TGF-β in this process were first determined by treating Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs with increasing TGF-β concentrations (Fig. 4*B*). Here, we observed a dramatic TGF-β concentration-depen-

dent decrease in beclin1 and LC3II levels for Eng<sup>+/+</sup> but not Eng<sup>-/-</sup> ECs, supporting a key role of TGF-β-induced Smad2 activation (Fig. 4*B* and graph quantification). In a parallel experiment, autophagic responses to TGF-β were assessed in control and a stable Smad2 knockdown ECs (sh-Smad2). As expected, TGF-β induced a significant loss of beclin1 and LC3II in control, but not sh-Smad2 ECs, indicating that Smad2 is the key regulator of EC autophagy (Fig. 4*C* and graph quantification).

Given the prominent link between endoglin and beclin1, we hypothesized that endoglin regulates beclin1 expression either by enhancing its biosynthesis or half-life. To test whether endoglin controls beclin1 turnover, Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs were treated with proteasome (MG132) and lysosome (chloroquine) inhibitors to measure the contribution of these two major degradation pathways. Here beclin1 levels following MG132 and chloroquine remained relatively constant, suggesting that endoglin has minimal impact on beclin1 stability (Fig. 5*A*). Instead, a significant loss of beclin1 was observed particularly in Eng<sup>+/+</sup> upon inhibition of protein transla-



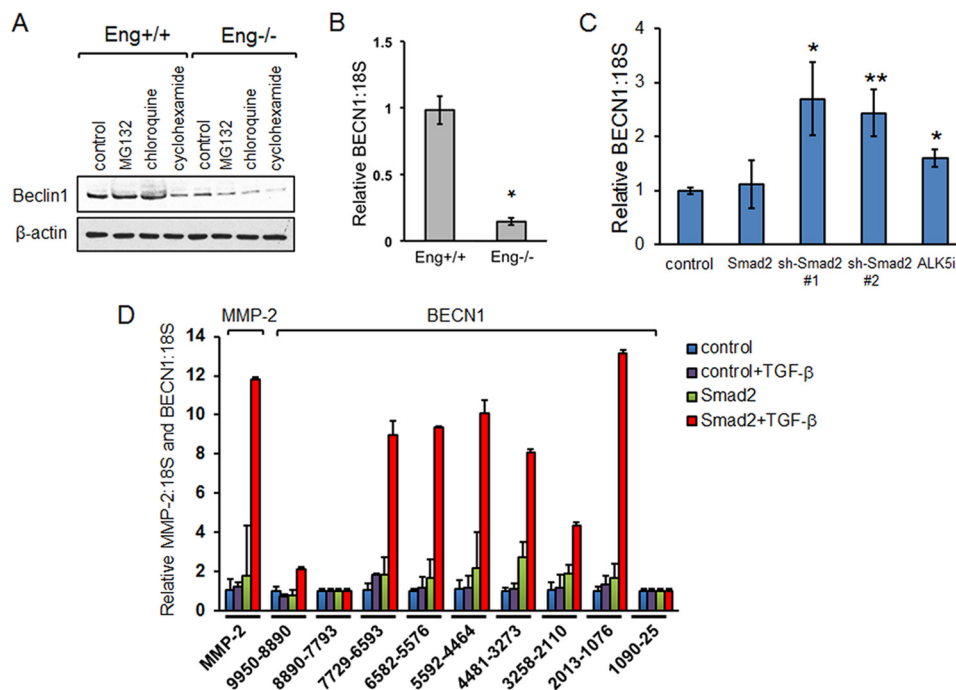
**FIGURE 4. Inhibition of autophagic responses via TGF- $\beta$ -induced Smad2 activation.** A, comparison of LC3 cleavage and Beclin1 levels upon stable Smad2 knockdown (sh-Smad2) and transient Smad3 knockdown (sh-Smad3) in Eng<sup>+/+</sup> MEECs and HUVECs. B, Western analysis of LC3 cleavage and Beclin1 levels in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs treated with increasing doses of TGF $\beta$  (50 pM, 200 pM) for 16 h, and densitometry quantification of the relative ratio of LC3 II to GAPDH for three independent experiments (\*,  $p = 0.002$ ). C, Western analysis of LC3 cleavage and Beclin1 levels upon stable Smad2 knockdown (sh-Smad2) in Eng<sup>+/+</sup> ECs treated with increasing doses of TGF $\beta$  (50 pM, 200 pM) for 16 h, and densitometry quantification of the relative ratio of LC3 II to GAPDH for three independent experiments (\*,  $p$  value = 0.001; \*\*,  $p$  value = 0.04).

tion with cyclohexamide, suggesting that endoglin controls beclin1 biosynthesis (Fig. 5A). Further substantiating this finding, real-time qPCR analysis of BECN1 levels in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> MEECs indicated a 5-fold higher mRNA levels in Eng<sup>+/+</sup> compared with Eng<sup>-/-</sup> ECs (Fig. 5B). To specifically test whether endoglin promotes BECN1 by impeding Smad2 function, we measured BECN1 levels following Smad2 overexpression, transient Smad2 knockdown using two different shRNA targeting sequences, and ALK5 inhibition. As expected, there was a notable increase in BECN1 transcription upon ALK5 inhibition relative to control (Fig. 5C). More notably, Smad2 knockdown caused an even greater up-regulation of BECN1 whereas Smad2 overexpression had statistically insignificant effect, indicating that overall Smad2 inhibits BECN1 transcription (Fig. 5C). To determine whether Smad2 serves as a direct BECN1 transcriptional repressor, we performed a chromatin immunoprecipitation (ChIP) assay in sh-Smad2 ECs transiently expressing either the full-length human Flag-Smad2 or pcDNA control (Fig. 5D). Here, cells were stimulated with or without TGF- $\beta$ , then immunoprecipitated with Flag antibody prior to RT-PCR analysis using a combination of primers that covered up to 10 kb upstream of BECN1 start site, while MMP-2 was used as control (34). There was at least a 10-fold increase in Smad2-binding near the promoter region of MMP-2 upon TGF- $\beta$  treatment, consistent with previous

findings in which MMP-2 was demonstrated as a Smad2-specific target gene (34). Importantly, we identified DNA elements spanning ~6.6 kb upstream (approximately -7700 to -1100) of the BECN1 start site as Smad2-binding elements strongly induced by TGF- $\beta$  (Fig. 5D). Not all upstream DNA elements represented Smad2-binding sites, as noted by marginal to no binding observed within the first 1.1 kb upstream (-1090 to -25) of the BECN1 start site, or the most distal DNA elements (-9950 to -7700). Overall, our results indicate that Smad2 acts as a direct transcriptional repressor by binding upstream of the BECN1 promoter region (Fig. 5, C and D).

Lastly, we investigated whether Smad2 regulation of beclin1 impacts angiogenesis using the matrigel three-dimensional capillary tube formation assay. Here Smad2 knockdown produced greater branching of capillary tube structures compared with control Eng<sup>+/+</sup> MEECs, supporting a novel inhibitory function for Smad2 in angiogenesis (Fig. 6A). To correlate capillary tube formation with autophagy, we measured the levels of beclin1 and LC3I/II in control and sh-Smad2 ECs from whole cell lysates derived directly from matrigel-induced EC capillaries. As expected, sh-Smad2 ECs displayed higher beclin1 levels and LC3I/II conversion (Fig. 6, A and B). To test the contribution of Smad2-mediated angiogenic inhibition, we knocked down Smad2 in Eng<sup>-/-</sup> MEECs and observed a significant rescue of endothelial capillary tubes and branching, which also

## Endoglin Promotes Endothelial Autophagy



**FIGURE 5. Smad2 is a BECN-1 transcriptional repressor.** *A*, Western analysis of Beclin1 protein levels in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> ECs treated with MG132 (10  $\mu$ M), chloroquine (100  $\mu$ M), and cyclohexamide (3  $\mu$ g/ml) for 6 h. *B*, quantification of Beclin1 mRNA (BECN1) in Eng<sup>+/+</sup> and Eng<sup>-/-</sup> MEECs by quantitative PCR analyzed by delta-delta-CT (ddCT) methods using 18S rRNA as internal control. Fold change was calculated by setting mean fractions of Eng<sup>+/+</sup> MEECs as one. Bars indicate mean  $\pm$  S.E. \*, *p* value = 0.0029. *C*, quantification of BECN1 in Eng<sup>+/+</sup> MEECs transiently transfected with Flag-Smad2, two separate sh-Smad2 constructs, or treated with ALK5 inhibitor (SB431542, 15  $\mu$ M) for 6 h. Fold change was calculated by setting mean fractions of non-treated Eng<sup>+/+</sup> MEECs as one. Bars indicated mean  $\pm$  S.E. \*, *p* value < 0.04; \*\*, *p* value < 0.01. *D*, ChIP assay upon expression of Flag-Smad2 or control pcDNA in Eng<sup>+/+</sup> ECs treated with or without TGF- $\beta$  for 30 min following 4 h of serum deprivation. Graph of RT-PCR data represents the relative binding by Smad2 to the specified DNA binding elements.

directly correlated with beclin1 and LC3II levels (Fig. 6, C and D and graph quantification). Collectively, these findings establish a functional role for endoglin in promoting autophagy during angiogenesis.

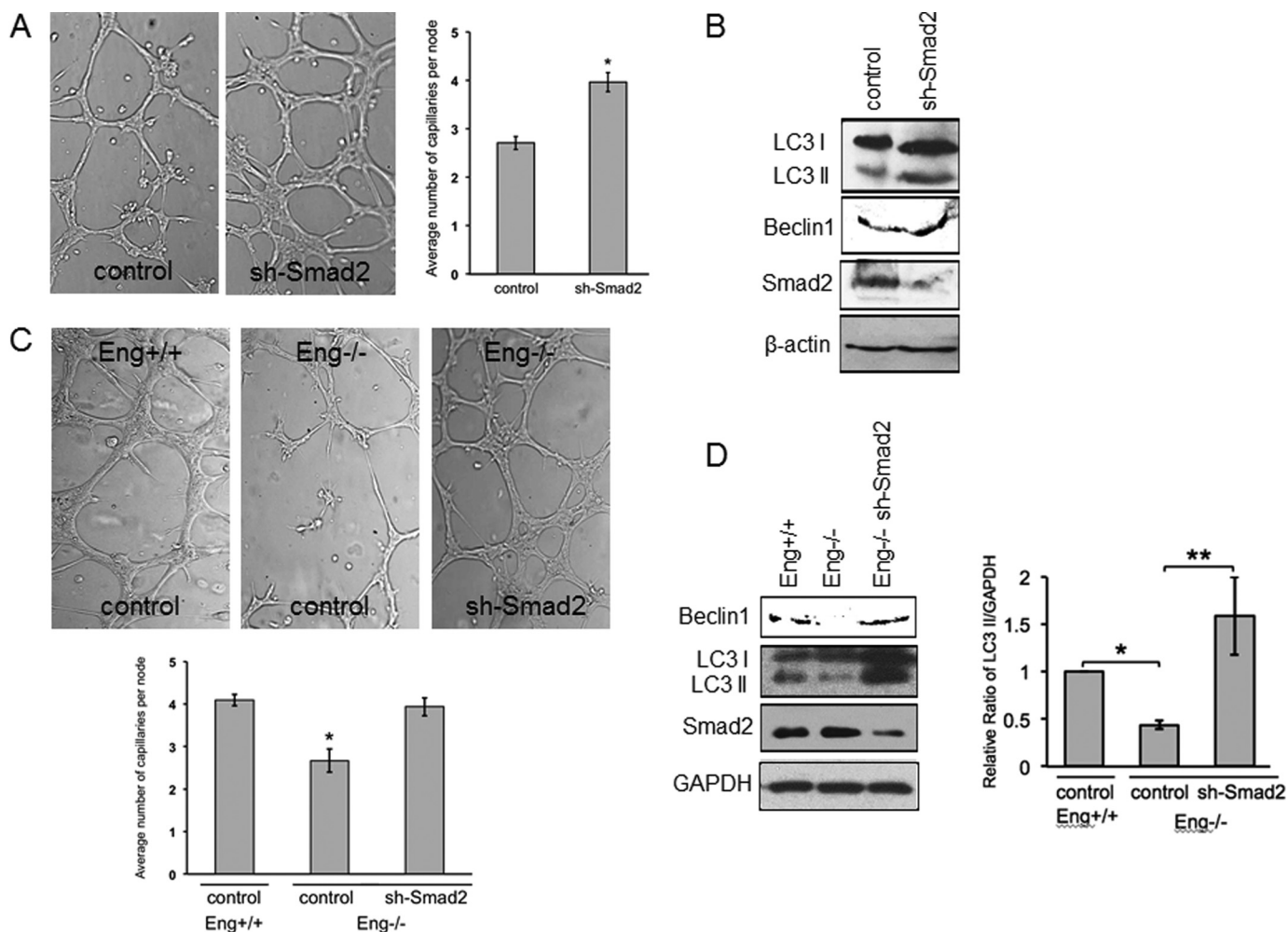
### Discussion

In the present study we examined how endoglin-mediated TGF- $\beta$  signaling regulates EC autophagy. While TGF- $\beta$  controls numerous mediators of autophagy under various cellular contexts, our experiments were performed in EC growth-promoting culture conditions to gauge how TGF- $\beta$  signaling influences autophagy during normal angiogenesis. Under these conditions, our pharmacologic screening results indicated that many of the previously reported mediators of TGF- $\beta$ -induced autophagy, such as TAK1, JNK, and Smad1/5, had surprisingly little impact on endothelial autophagy (Fig. 2B). Instead, we identified endoglin regulation of ALK5 signaling as a critical determinant. Our work further demonstrates that endoglin plays a critical role in disrupting Smad2-dependent BECN1 repression to promote autophagic responses in ECs. In our view, this finding represents an important new mechanism by which Smad2 impairs angiogenesis alongside its established inhibitory role in cellular proliferation.

Endoglin binds TGF- $\beta$  with high affinity to stimulate ALK1/Smad1/5/8 signaling while indirectly inhibiting TGF- $\beta$ /ALK5/Smad2/3 to promote the activation phase of angiogenesis (22). Given a recent study in which Smad1/5-mediated autophagy was demonstrated in Hep3B cells (18), we initially hypothesized that endoglin promotes autophagy through this same pathway. However, there was no evidence of ALK1/Smad1/5/8 involvement, as

we detected negligible effects on autophagy even upon treatment with BMP-9, a more potent activator of ALK1/Smad1/5/8 than TGF- $\beta$  (Figs. 2 and 3). Prior studies have established that Smad1/5 up-regulates Id-1 gene expression, an inhibitor of basic helix-loop-helix proteins that promotes EC proliferation and migration (35). ALK5, on the other hand, induces expression of plasminogen activator inhibitor type 1 (PAI-1), a negative regulator of EC migration *in vitro* and angiogenesis *in vivo* (36). ALK5 further transduces antiproliferative signals in most cell types via Smad2/3, which has been shown to inhibit the expression of c-Myc and cyclin-dependent kinases (CDKs) while simultaneously increasing the expression of CDK inhibitors p15<sup>INK4B</sup> and p27<sup>KIP1</sup> (35, 36). Although these anti-migratory and anti-proliferative transcriptional responses mediate the antiangiogenic effects, our work supports the inhibitory role of Smad2 in autophagy as a novel means of further suppressing angiogenesis (Fig. 6). Mechanistically, notable changes in beclin1 mRNA and protein expression, upon pharmacologic inactivation of ALK5, Smad2 knockdown, and overexpression collectively demonstrate that beclin1 is a major Smad2 regulatory target (Figs. 4, 5).

Interestingly, contrasting observations have been previously reported in certain normal epithelial and carcinoma cell lines where TGF- $\beta$  appeared to promote autophagy through a Smad-dependent increase in ATG gene transcripts (19). In particular, Kiyono *et al.* observed an overall increase in autophagy following Smad2/3 knockdown in human hepatocarcinoma cells, while Smad4 knockdown specifically abolished TGF- $\beta$ -induced BECN1 expression (19). Of note, whether these Smads



**FIGURE 6. Smad2 suppression enhances endothelial capillary sprouting by upregulating Beclin-1 levels and LC3 cleavage.** *A*, representative images and quantification of average number of capillaries per node of three-dimensional Matrigel-induced capillary tubules upon stable Smad2 knockdown (sh-Smad2) in Eng<sup>+/+</sup> ECs at 16 h. \*,  $p$  value < 0.0001. *B*, Western analysis of LC3 cleavage and Beclin1 levels in three-dimensional Matrigel-induced capillary tubules upon stable Smad2 knockdown (sh-Smad2) in Eng<sup>+/+</sup> ECs. *C*, representative images and quantification of capillaries per node of three-dimensional Matrigel-induced capillary tubules in Eng<sup>+/+</sup>, Eng<sup>-/-</sup>, and Eng<sup>-/-</sup> transiently expressing Smad2 shRNA (sh-Smad2) ECs at 16 h. \*,  $p$  value = 0.0006. *D*, Western analysis of LC3 cleavage and Beclin1 levels of three-dimensional Matrigel-induced capillary tubules in Eng<sup>+/+</sup>, Eng<sup>-/-</sup>, and Eng<sup>-/-</sup> transiently expressing Smad2 shRNA (sh-Smad2) ECs and densitometry quantification of relative ratio of LC3 II to GAPDH for three independent experiments (\*,  $p$  value = 0.0001; \*\*,  $p$  value = 0.04).

play a direct role in transcriptional regulation as an inducer or repressor has not been demonstrated by ChIP or other DNA-binding studies in these cell contexts. At least in the EC system, our results provide compelling evidence that Smad2 specifically acts as a BECN1 transcriptional repressor by binding upstream of the BECN1 promoter region (Fig. 5C). However, whether Smad2 directly interacts with the canonical Smad binding element, or requires additional co-factors, needs to be addressed in future studies.

In addition to inhibiting Smad2 function, our results suggest that endoglin engages other TGF- $\beta$ -related mechanisms that contribute to EC autophagy. Indeed, although our results clearly indicate that endoglin expression correlates with autophagy (Fig. 1), we failed to observe a proportional increase in autophagy upon endoglin rescue in Eng<sup>-/-</sup> MEECs, and instead observed a further reduction in autophagy (data not shown). This outcome, while unexpected, suggests a more complex role for endoglin in autophagy, one in which it participates in biphasic regulation of the endothelial autophagic

machinery. This notion is consistent with our previous findings in which we demonstrated that endoglin enhances BMP-9-induced AKT activation to promote EC survival. Considering that AKT is a potent inhibitor of autophagy in most cell types including ECs (Fig. 2B), ectopic endoglin overexpression may have not only failed to restore endoglin-dependent autophagy, but further suppressed this process due to AKT hyperactivation. Collectively, our work defines a novel role for endoglin in endothelial autophagy. Our data indicates that ALK5/Smad2 signaling negatively regulates EC autophagy by inhibiting BECN1 expression. Endoglin has a key role in opposing this process by inhibiting Smad2/3 function. Lastly, endoglin-mediated autophagy enhances capillary tube formation, supporting a new functional mechanism by which endoglin promotes angiogenesis.

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