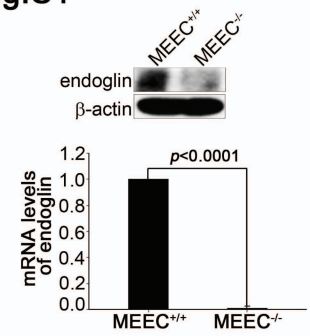
Supplementary data 1-7

Fig.S1



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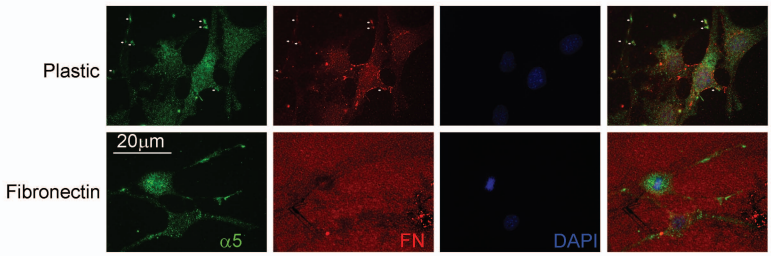
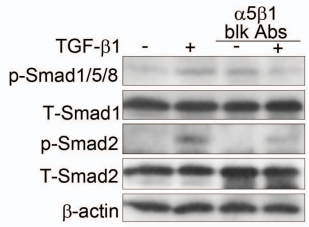


Fig.S3



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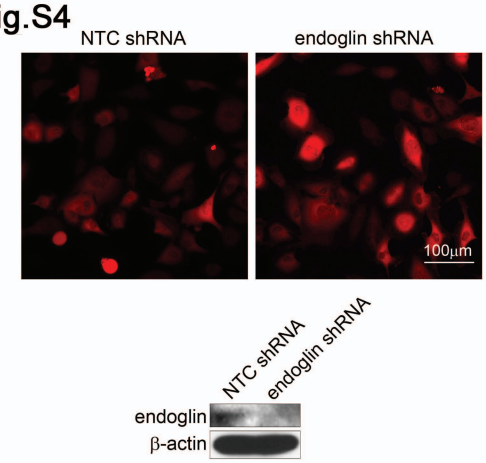
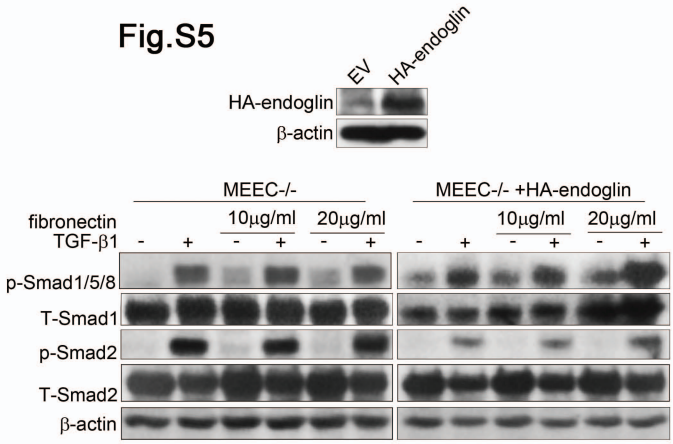


Fig.S5



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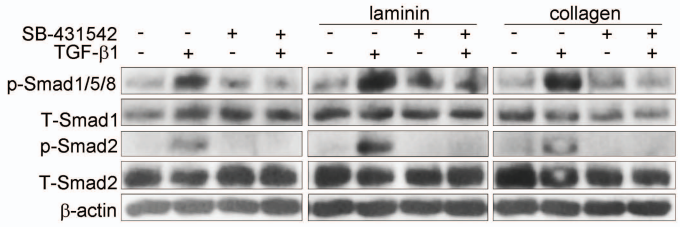

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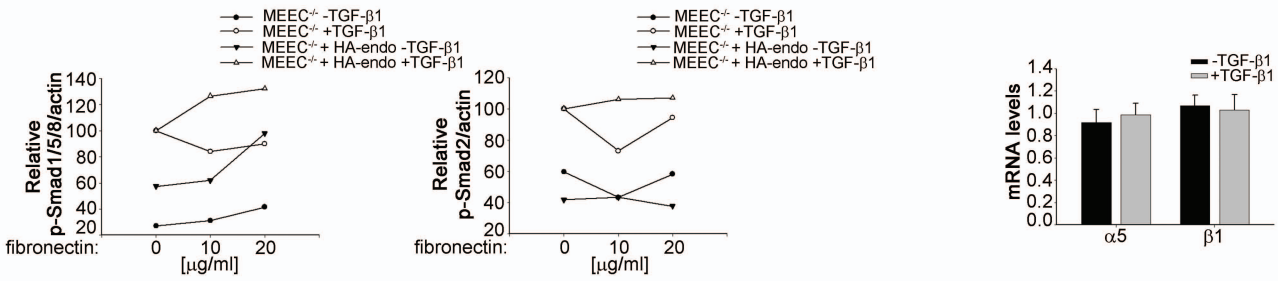
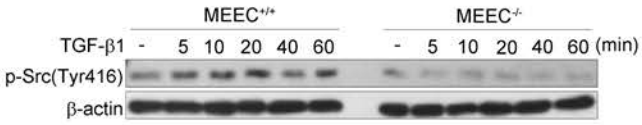


Supplementary data 8-16

Fig. S8



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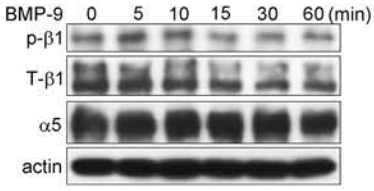

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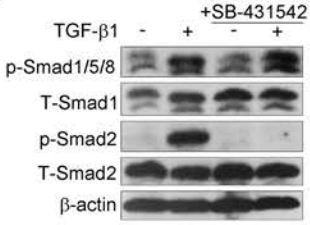

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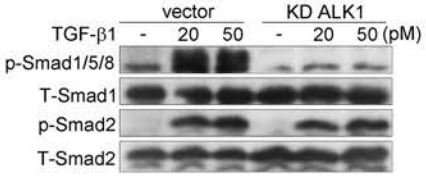

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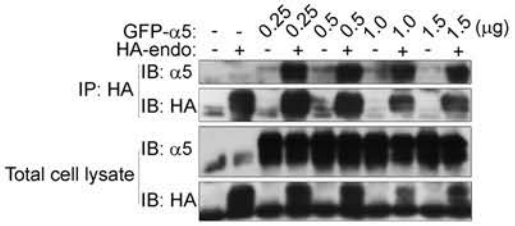

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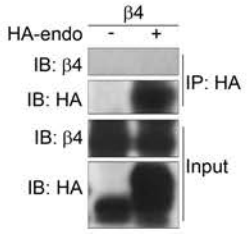

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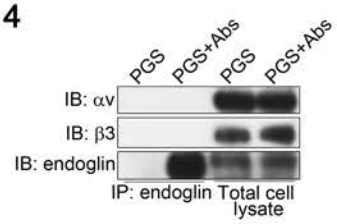

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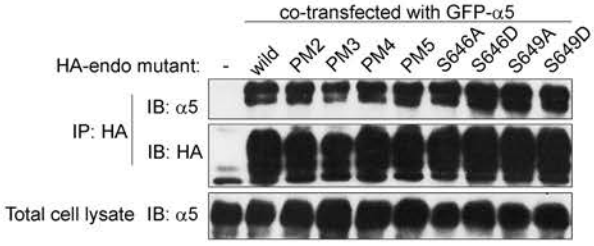

Mutant	Mutant site	Sequence
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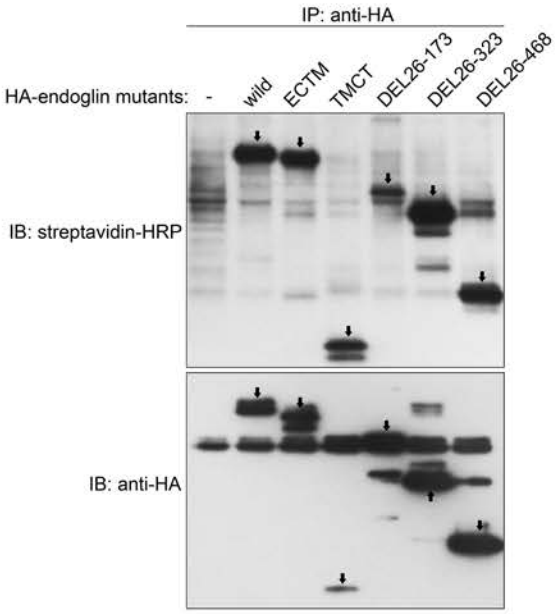
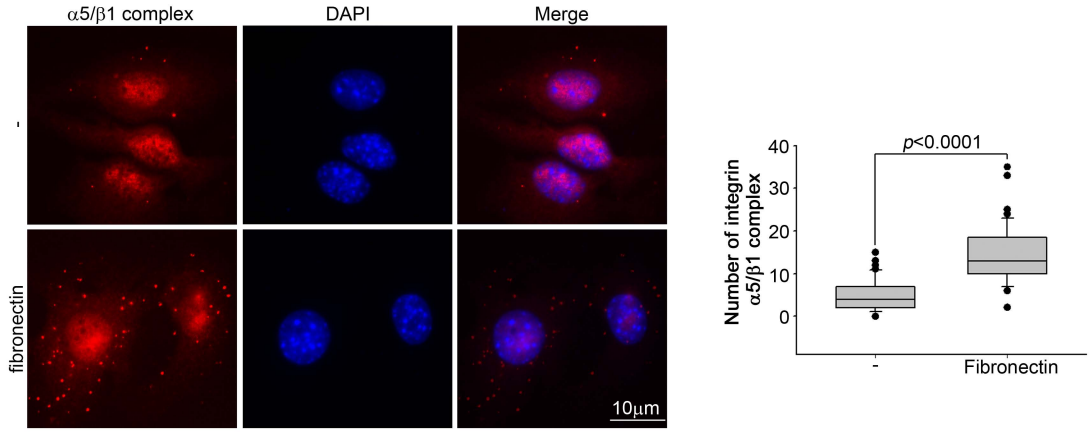
Fig.S16



Supplementary data 17-18

Fig.S17



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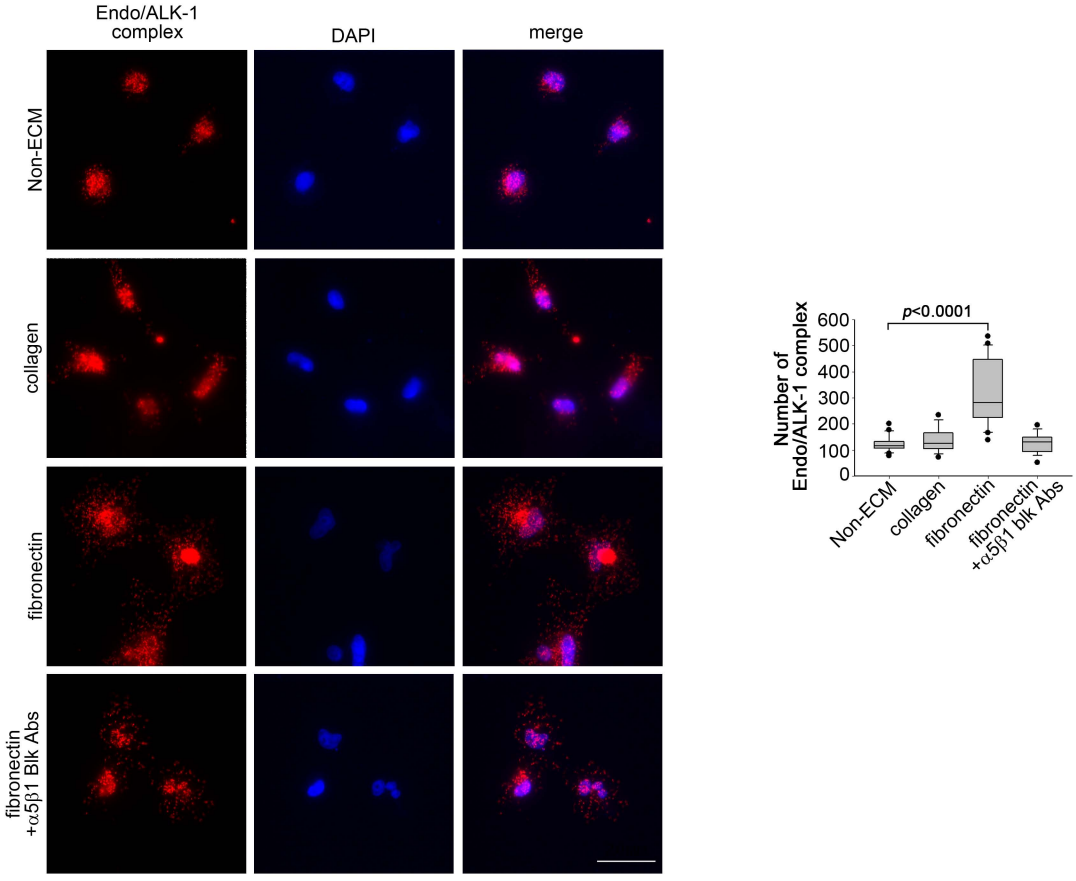
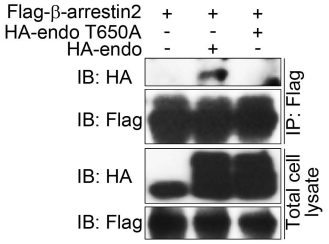


Supplementary data 19-25

Fig.S19



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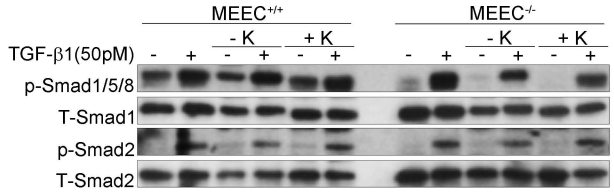


Fig.S21

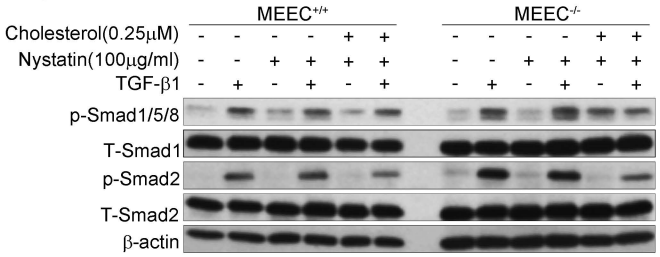
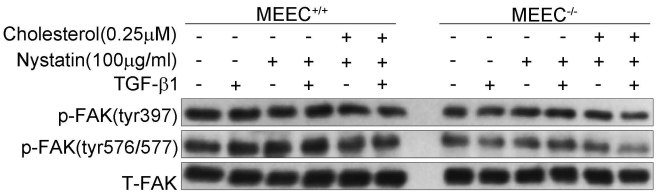


Fig.S22



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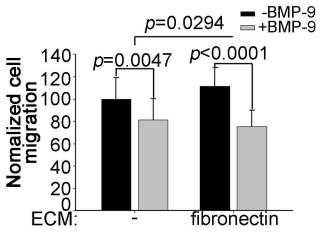

Fig.S24

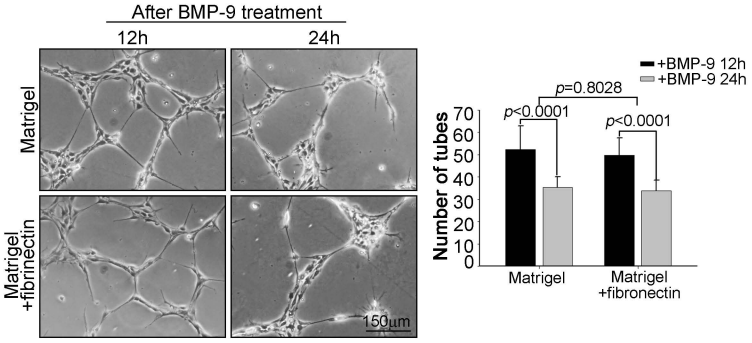

Fig.S25

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IQTKCADDAMTLVLKKELVAHLKCTITGLTFWDPSCEAEDRGDKFVLRSAYSSCGMQVSA  418   A8K2X4_HUMAN
IQPRCSDDVMTLVLRKDLISTLGCTITSLTFWDPSCQAEDTDDKFVLRGTYSSCGMKVAE  417   EGLN_PIG
**.:*.::.***.*.*. :  * ****.*:***.**:*:* ..::** .:******:*:
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Supplementary Figure Legends

Supplementary Figure S1. Endoglin protein and mRNA levels in MEEC +/+ and MEEC-/- cells were determined using western blot, with β-actin as a loading control (top), and real time PCR (bottom), respectively. Forward primer sequence for murine endoglin: 5'-GCTACCATCCCTTACCTCCAG-3', reverse primer sequence for murine endoglin: 5'-CTTCTGGCAAGCACAAGAATG-3'.

Supplementary Figure S2. HMEC-1 cells were cultured in non-ECM coated (plastic) or 5μg/ml fibronectin coated plastic dishes for 12h. α5 integrin and fibronectin were detected by immunofluorescent microscopy. Nuclei were stained using DAPI, and a merged image of the α5 integrin and fibronectin images is presented in the far right panels.

Supplementary Figure S3. HMEC-1 cells were cultured on plastic in the absence of exogenous fibronectin, serum starved for 5h, pretreated with 10μg/ml integrin α5β1 function-blocking antibody (blk Abs) for 1h, followed by treatment with 50pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S4. HMEC-1 cells were adenovirally infected with dsRed expressing shRNA to a non-targeted control (NTC) or endoglin for 48h. Infection efficiency (dsRed expression, top) was assessed by florescence microscopy and knockdown efficiency (bottom) relative to NTC was assessed by western blot, with β-actin as a loading control.

Supplementary Figure S5. MEEC -/- cells were nucleofected with empty vector or HA-tagged human endoglin for 24h, cultured in dishes coated with the indicated doses of fibronectin, serum starved for 6h, treated with 50pM TGF-β1 for 30min, and the cell lysates analyzed with the indicated antibodies. Data are quantitated below.

Supplementary Figure S6. HMEC-1 cells were cultured in non-coated, 10μg/ml laminin or 10μg/ml collagen coated dishes, serum starved for 5h, pretreated with 10μM ALK5 inhibitor, SB-431542 for 1h, then treated with or without 50pM TGF-β1, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S7. HMEC-1 cells were treated with or without 100pM TGF-β1 for 30min. mRNA levels of integrin α5 and β1 were assessed using real

time PCR. Forward primer sequence for human α5: 5'-TGCTCCTAGGTCTACTCATCTAC-3', reverse primer sequence for human α5: 5'-ATGGGAGTCTGAAATTGGGAG-3'; Forward primer sequence for human β1: 5'-TGTAAGGAGAAGGATGTTGACG-3'; reverse primer sequence for human β1: 5'-CAACCACACCAGCTACAATTG-3'.

Supplementary Figure S8. MEEC +/+ and MEEC -/- cells were serum starved for 6h prior to treatment with the indicated doses of TGF-β1 treatment for 30min, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S9. HMEC-1 cells were serum starved for 6h prior to treatment with 2ng/ml BMP-9 for indicated times, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S10. HMEC-1 cells were cultured on 10μg/ml fibronectin coated dishes, serum starved for 5h, pretreated with 10μM ALK5 inhibitor, SB-431542 for 1h, then treated with or without 50pM TGF-β1, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S11. HMEC-1 cells were adenovirally infected with empty virus or ALK-1 kinase dead (KD ALK-1) for 48h, treated with 50pM TGF-β1 for 30min, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S12. Anti-HA immunoprecipitates were prepared from COS7 cells expressing HA-endoglin with the indicated levels of GFP-α5 integrin. HA-endoglin and α5 integrin were detected in immunoprecipitates (IP) and total cell lysates using western blot analysis.

Supplementary Figure S13. Anti-HA immunoprecipitates were prepared from COS7 cells expressing HA-endoglin with β4 integrin. HA-endoglin and β4 were detected in immunoprecipitates (IP) and total cell lysates using western blot analysis.

Supplementary Figure S14. Immunoprecipitates were prepared from MEEC+/+ cells with P3D1 antibody to endoglin. αv or β3 integrin was detected in immunoprecipitates (IP) and cell lysates by western blot analysis.

Supplementary Figure S15. Anti-HA immunoprecipitates were prepared from COS7 cells expressing GFP-α5 integrin with HA-endoglin or different phosphorylation site mutants of HA-endoglin on as indicated in the bottom panel. HA-endoglin and α5 were detected in immunoprecipitates (IP) and total cell lysates using western blot analysis.

Supplementary Figure S16. COS7 cells were transfected with HA-endoglin or or indicated HA-endoglin mutants for 24h, labeled with Sulfo-NHS-LC-Biotin, immunoprecipitated with anti-HA antibody, resolved by SDS-PAGE, and Western blots were performed using streptavidin-horseradish peroxidase (top) or HA antibody (below). Arrows indicate the size of the different deletion constructs.

Supplementary Figure S17. MEEC+/+ cells were cultured in dishes coated with PBS, or 10μg/ml fibronectin. Interactions between endogenous integrin α5 and integrin β1 were assessed by Duolink assay. Nuclei were stained using DAPI. The data are quantitated on the right.

Supplementary Figure S18. COS7 cells expressing HA-endoglin and myc-ALK-1 were cultured in dishes coated with PBS, 10μg/ml collagen, or 10μg/ml fibronectin together with or without 10mg/ml integrin α5β1 function blocking antibody for 1h. Interactions between HA-endoglin and myc-ALK-1 were assessed by Duolink assay. Nuclei were stained using DAPI. The data are quantitated on the right. The number of endoglin/ALK-1 complexes per cell +/- SD (N=20 cells) from one representative experiment of two independent experiments is presented.

Supplementary Figure S19. Anti-Flag immunoprecipitates were prepared from COS7 cells expressing Flag-β-arrestin2 with HA-endoglin or HA-endoglin T650A mutant. HA-endoglin and Flag-β-arrestin2 were detected in IP and total cell lysates by western blot analysis.

Supplementary Figure S20. MEEC +/+ and -/- cells were serum starved for 5h. Clathrin dependent endocytosis was inhibited by potassium (K) depletion for 1h. Cells were treated with 50pM TGF-β1 for 30min, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S21. MEEC +/+ and -/- cells were serum starved for 5h. Clathrin-independent endocytosis was inhibited by adding 100μg/ml nystatin for 1h. 0.25μM cholesterol was used to rescue clathrin-independent endocytosis. Cells were treated with 50pM TGF-β1 for 30min, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S22. MEEC +/+ and -/- cells were serum starved for 5h. Clathrin-independent endocytosis was inhibited by adding 100μg/ml nystatin for 1h. 0.25μM cholesterol was used to rescue clathrin-independent endocytosis. Cells were treated with 50pM TGF-β1 for 30min, and the cell lysates analyzed with the indicated antibodies.

Supplementary Figure S23. (A) HMEC-1 cells were plated in the transwells coated with non-ECM, or 20μg/ml fibronectin, pretreated with or without 5ng/ml BMP-9 and assessed for migration after 8 h.

Supplementary Figure S24. HMEC-1 cells were cultured on the Matrigel mixed with or without 50μg/ml fibronectin with 5ng/ml BMP-9 treatment for indicated times. Images were taken at 4× microscopy, and tubules were counted using Image J.

Supplementary Figure S25. Alignment of endoglin sequences across species (rat, mouse, human and pig). The RGD sequence in human endoglin is highlighted.

Supplementary materials and methods:

Protein Overexpression and Knockdown MEECs were nucleofected with cDNA constructs using Amaxa nucleofection system as described previously(Lee & Blobe, 2007). HMEC-1s were infected with either a non-targeting vector control or shRNA endoglin-expressing adenovirus (targeting intracellular sequence) at 50 MOI and incubated for 72 h before harvest. COS7 cells were transfected using Lipofectamine™ 2000 transfection reagent as described by the manufacturer.

Western blot analysis: To detect phosphorylated and total Smad1,2,5,8, integrin β1 and FAK, HMEC or MEEC cells were serum starved for 6 hours, and treated with 50 pM or the indicated doses of TGF-β1, or 2μg/ml or indicated doses of BMP-9 for 30min or the indicated times. Cells were then lysed using 2×sample buffer and subjected to western blot analysis. To detect ECM substrate effects on TGF-β or BMP-9 signaling, 6-well or 12-well plates were coated with 10μg/ml or the indicated doses of ECM components for 1h, HMEC-1 or MEEC cells were cultured in the coated dishes overnight, serum starved for 6h, and treated with 50 pM TGF-β1 or 2μg/ml BMP-9 for 30min. Dishes coated with PBS were used as a negative control. Integrin α5, integrin β1, phosphospecific Smad1/5/8 and Smad2/3, as well as their respective total Smad antibodies, were all purchased from Cell Signaling. HA antibody was purchased from Roche. Phosphorylated integrin β1 antibody was purchased from Abcam.

Cell-substrate adhesion assay: 96-well dishes were coated with 100μl of the indicated concentrations of ECM substrates for 60 min. at room temperature. The wells were then blocked with 200μl of 10mg/ml heat-denatured BSA solution for 30 min. at room temperature and washed with 100μl PBS. 50μl PBS and 50μl of 5×10^5 HMEC-1 cells were then added, incubated for 15min at 37^{o}C. Nonadherent and loosely attached cells were removed by tapping the plate, and washed 3 time with 100μl PBS. Cells were then fixed with 4% parafomaldehyde for 15min in room temperature. After washing 3 times with PBS, the cells were

stained with Crystal Violet for 10min, washed with PBS 3 times, allowed to dry, and then 2% SDS was added and the plates incubated at room temperature for 30min, and read at 595μm.

Co-immunoprecipitation: MEEC cells cultured in 10 ml dishes or COS7 co-expressing 1.5 μg HA-endoglin and 0.5 μg β1 or GFP-α5 for 24h in 6 well plates were washed with PBS, then lysed on ice with lysis buffer (20Mm Tris-HCl pH8, 137mM NaCl, 10% glycerol, 1% NP-40, 2mM EDTA, 10 mM NaF, 5μg/ml leupeptin, 1mM PMSF, 1mM Na_3VO_4). The lysates were precleared by centrifugation and incubated with 30μl protein G-agarose beads for 2h, incubated with 5μg anti-HA or 10μg P3D1 antibodies (Millipore) at 4°C overnight, and then incubated with 50μl protein G-agarose beads for 1h. The immunoprecipitates were collected by centrifugation, the pellets washed with lysis buffer, and stored in 2X sample buffer prior to Western blot analysis.

Immunofluorescence: MEEC-/- cells expressing of HA-endoglin and GFP-α5 were washed with PBS, fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100/PBS for 5 min and then blocked with 5% bovine serum albumin in PBS for 1 h. HA-endoglin, GFP-α5, and Rab5 or EAA1 were detected using P3D1, anti-GFP, and anti-Rab5 or anti-EAA1 at 1:200 dilution rate, respectively, for 1 h at room temperature. Cells were washed with PBS and incubated with appropriate secondary fluorophore-conjugated antibodies for 1 h at room temperature, washed, then mounted with Prolong Anti-Fade (Sigma).

K+ depletion and Nystatin treatment:

Clathrin-dependent and independent endocytosis were inhibited by potassium (K) and cholesterol depletion, respectively (Di Guglielmo et al, 2003). Briefly, MEEC cells were incubated in MCDB131:water (1:1) for 5 min at 37°C followed by incubation in minimal media (20 mM Hepes at pH 7.5, 140 mM sodium chloride, 1 mM calcium chloride, 1 mM magnesium sulphate, 5.5 mM glucose and 0.5% BSA) for 1h at 37°C. Control cells were incubated in minimal media supplemented with 10 mM potassium chloride. Cholesterol depletion was carried

out by incubating cells in 100 μg/ml of Nystatin (Sigma) for 1 h at 37^{o}C. Control cells were co-treated with 0.25μM cholesterol.