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Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction

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Endoglin is a transmembrane accessory receptor for transforming growth factor-β (TGF-β) that is predominantly expressed on proliferating endothelial cells in culture and on angiogenic blood vessels *in vivo*. Endoglin, as well as other TGF-β signalling components, is essential during angiogenesis. Mutations in endoglin and activin receptor-like kinase 1 (ALK1), an endothelial specific TGF-β type I receptor, have been linked to the vascular disorder, hereditary haemorrhagic telangiectasia. However, the function of endoglin in TGF-β/ALK signalling has remained unclear. Here we report that endoglin is required for efficient TGF-β/ALK1 signalling, which indirectly inhibits TGF-β/ALK5 signalling. Endothelial cells lacking endoglin do not grow because TGF-β/ALK1 signalling is reduced and TGF-β/ALK5 signalling is increased. Surviving cells adapt to this imbalance by downregulating ALK5 expression in order to proliferate. The ability of endoglin to promote ALK1 signalling also explains why ectopic endoglin expression in endothelial cells promotes proliferation and blocks TGF-β-induced growth arrest by indirectly reducing TGF-β/ALK5 signalling. Our results indicate a pivotal role for endoglin in the balance of ALK1 and ALK5 signalling to regulate endothelial cell proliferation.

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Introduction

Endoglin (CD105) is a homodimeric transmembrane glycoprotein that is predominantly expressed in vascular endothelial cells (ECs) (Gougos and Letarte, 1988). Other sites of expression include syncytiotrophoblasts of full-term placenta (Gougos *et al*, 1992), stromal cells (St-Jacques *et al*, 1994; Robledo *et al*, 1996) and certain haemopoietic cells (Lastres *et al*, 1992). The importance of endoglin in vascular morphogenesis is illustrated by the death *in utero* of mice deficient in endoglin from defects in angiogenesis (Bourdeau *et al*, 1999; Li *et al*, 1999; Arthur *et al*, 2000) and by the association of mutations in endoglin with an autosomal dominant disorder termed hereditary haemorrhagic telangiectasia type I (HHT1) (McAllister *et al*, 1994). This disease is characterized by bleeding through haemorrhage from vascular malformations.

Endoglin is an accessory receptor for transforming growth factor-β (TGF-β) (Cheifetz *et al*, 1992). TGF-β is a multifunctional cytokine that is part of a larger superfamily of proteins, which also include activins and bone morphogenetic proteins (BMPs) (Piek *et al*, 1999). TGF-β family members elicit cellular responses via specific type I and II serine/threonine kinase receptors and their downstream nuclear effectors, termed Smads (Heldin *et al*, 1997; Shi and Massagué, 2003). Type I receptors act downstream of type II receptors and determine the signalling specificity within the receptor complex (Carcamo *et al*, 1995; Wieser *et al*, 1995). In most cells, TGF-β signals via TGF-β type II receptor (TβR-II) and activin receptor-like kinase (ALK) 5 to induce Smad2 and Smad3 phosphorylation (Nakao *et al*, 1997). However, in ECs, TGF-β has recently been shown to activate two distinct type I receptor pathways, that is, ALK5-inducing Smad2/3 phosphorylation and ALK1-promoting Smad1/5 phosphorylation. Activation of ALK1 stimulates cell proliferation and migration, whereas activation of ALK5 inhibits these responses (Goumans *et al*, 2002). Endoglin only binds ligand while in association with the TGF-β type II receptor (TβR-II) and can bind TGF-β1 and TGF-β3 efficiently but not TGF-β2 (Letamendia *et al*, 1998; Barbara *et al*, 1999). In addition, endoglin has been shown to bind other members of the TGF-β superfamily, including activin and BMPs, and in that case to interact with activin type II receptors (Barbara *et al*, 1999). The physiological role of endoglin in signalling by activin and BMP is unclear. Furthermore, endoglin can bind to type I or II receptors even in the absence of ligand (Lux *et al*, 1999; Guerrero-Esteo *et al*, 2002). Both extracellular and intracellular domains interact with TβR-II and ALK5. The cytoplasmic domain of endoglin lacks an obvious enzymatic motif but is rich in serine and threonine amino-acid residues that can become phosphorylated by ALK5 or TβR-II (Guerrero-Esteo *et al*, 2002).

Endoglin is thought to modulate TGF-β signalling. Mutations in the *acvrl1* gene, coding for ALK1, causes HHT2, which has clinical manifestations similar to HHT1 (Johnson *et al*, 1996), suggesting that endoglin and ALK1 may act in the same pathway. Ectopic expression of endoglin has been shown to inhibit TGF-β-induced growth inhibition in monocytes (Letamendia *et al*, 1998) and myoblasts

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(Lastres *et al*, 1996). Treatment of ECs with antisense oligonucleotides for endoglin was found to counteract the inhibitory effect of TGF-β on EC migration (Li *et al*, 2000). These TGF-β responses are mediated via ALK5 (Goumans *et al*, 2002) and thus suggest that endoglin is a negative regulator of TGF-β/ALK5 signalling. However, recently, Guerrero-Esteo *et al* (2002) proposed a model in which endoglin promotes TGF-β/ALK5-induced responses. Ectopic endoglin expression altered the phosphorylation status of TβR-II and ALK5, increased Smad2 phosphorylation and promoted Smad2-driven reporter activity.

To gain more insight into the molecular mechanisms by which endoglin regulates EC function, we investigated the role of endoglin in EC proliferation and examined the contribution of endoglin to TGF-β/ALK signalling in ECs. Our results reconcile previously published reports and indicate a key positive role for endoglin in TGF-β/ALK1-induced EC proliferation.

Results

Endoglin is needed for efficient TGF-β/ALK1 signalling

Previously, TGF-β has been shown to activate two distinct type I receptor pathways in ECs, that is, ALK5-inducing Smad2/3 phosphorylation and ALK1-inducing Smad1/5 phosphorylation (Goumans *et al*, 2002). To examine whether endoglin has a regulatory role in TGF-β/ALK signalling in ECs, we first analysed the effect of siRNA-mediated knockdown of endoglin on TGF-β-induced Smad activation in ECs. siRNA-mediated downregulation of endoglin expression was confirmed by reverse-transcription polymerase chain reaction (RT–PCR) (Figure 1A) and FACS analysis (Figure 1B). Endoglin was found to be a critical determinant of TGF-β-induced Smad1/5 phosphorylation but not of TGF-β-induced Smad2 phosphorylation (Figure 1C). Consistent with these results, we found that TGF-β-induced activity of BRE_2-Luc, a specific reporter for Smad1/5 activation (Korchynskyi and

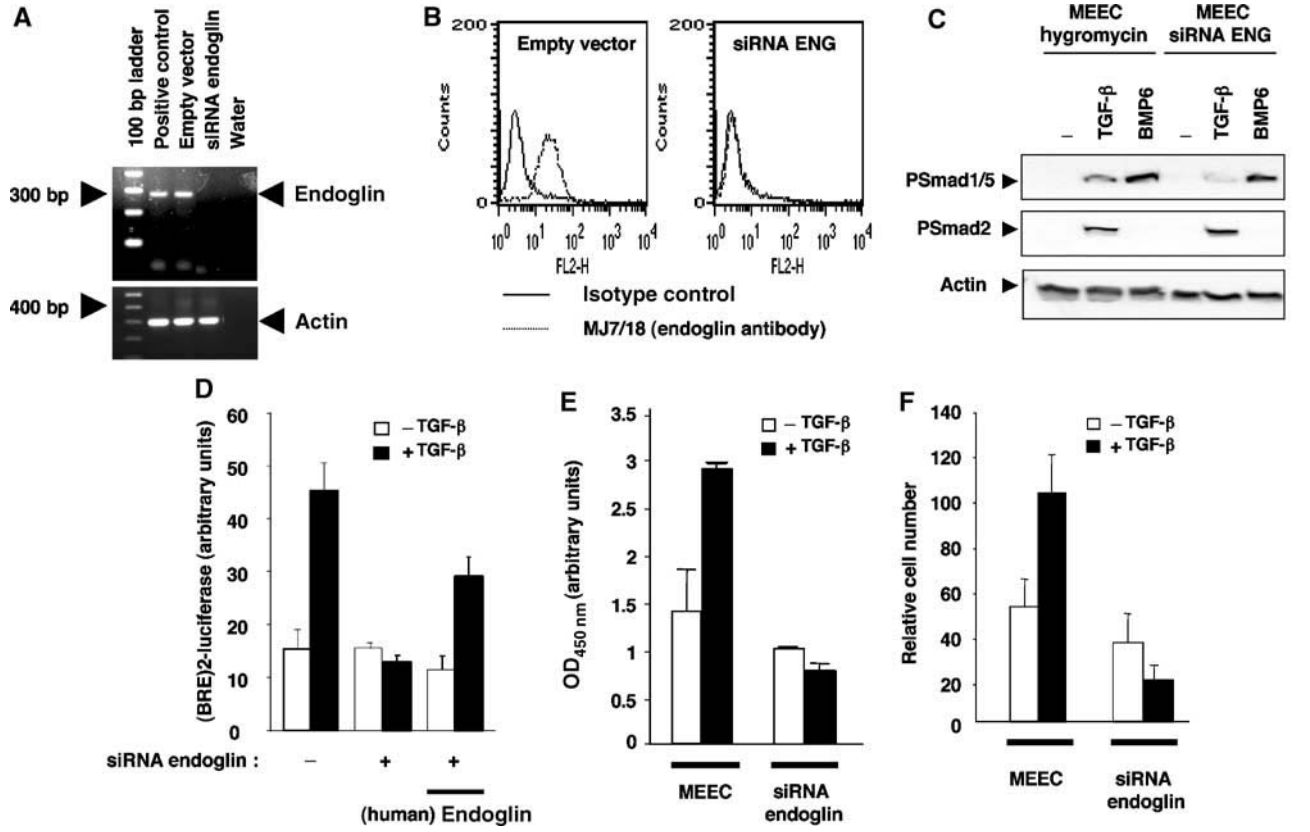

Figure 1 Endoglin is needed for efficient TGF-β/ALK1 signalling. (**A**) The expression of endoglin in mouse embryonic endothelial cells (MEECs) stably transfected with siRNA endoglin was analysed by RT–PCR. The PCR products were loaded on a 1% agarose gel and stained with ethidium bromide. Amplified products of endoglin and β-actin are indicated on the right of the figure. (**B**) Flow cytometric analysis of endoglin expressed at the cell surface using a PE-conjugated MJ17/8 antibody (solid line) or a PE-conjugated isotype-matched irrelevant antibody as a control (dotted line). (**C**) Endoglin downregulation specifically inhibits Smad1/5 phosphorylation induced by TGF-β. MEECs stably transfected with an empty vector or with siRNA endoglin were stimulated with 5 ng/ml TGF-β or 50 ng/ml BMP6 for 45 min at 37°C before lysis. Whole-cell extracts were fractionated by SDS–PAGE and blotted. The filter was incubated with PSmad2 antibody, which specifically recognizes phosphorylated Smad2, PSmad1 antibody, which specifically recognizes phosphorylated Smad1/5, and β-actin antibody. (**D**) siRNA endoglin leads to reduced TGF-β-induced $(BRE)_2$-luciferase transcriptional activity, which is rescued by ectopic expression of human endoglin. MEECs transfected with $(BRE)_2$-luciferase reporter in the presence or absence of siRNA endoglin were unstimulated or stimulated with 1 ng/ml TGF-β, and luciferase activity was measured. A representative experiment using triplicate samples corrected for transfection efficiently is shown. (E, F) Downregulation of endoglin expression inhibits proliferation and migration of MEECs induced by 'low-dose' TGF-β. (**E**) MEECs stably expressing siRNA endoglin were seeded and stimulated or not by 0.25 ng/ml of TGF-β. After 5 days, the quantity of viable cells was determined using the WST-1 assay. (**F**) MEECs stably transfected with siRNA endoglin were seeded into Transwells and unstimulated or stimulated by 0.25 ng/ml of TGF-β. After 6 h, the cells that had migrated to the other side of the filter were counted after staining with crystal violet.

ten Dijke, 2002), was blocked when endoglin was decreased in ECs (Figure 1D). This response is specific as the effect obtained with the siRNA construct targeting the mouse endoglin sequence could be rescued by ectopic expression of human endoglin (Figure 1D).

Other TGF-β/ALK1 responses in ECs, including 'low-dose' TGF-β-induced proliferation and migration (Goumans *et al*, 2002), were also inhibited upon siRNA-mediated knockdown of endoglin expression (Figures 1E and F). Finally, previous reports obtained by high overexpression of endoglin and BMP receptors have suggested a functional link (Barbara *et al*, 1999). However, BMP-induced Smad1/5 phosphorylation did not change upon siRNA-mediated knockdown of endoglin expression (Figure 1C), arguing against a physiological role for endoglin in BMP signalling.

Ectopic expression of endoglin promotes EC proliferation via TGF-β/ALK1 signalling

Endoglin is predominantly expressed in activated ECs, and elevated expression in tumour cells has been correlated with proliferation markers *in vivo* (Miller *et al*, 1999). To analyse whether overexpression of endoglin interferes with cell cycle progression, we monitored DNA replication by measuring BrdU incorporation in transiently transfected ECs. For these experiments, cotransfection of green fluorescent protein (GFP) was used as a marker to identify transfected cells. GFP appears not to interfere with cell growth and function (Zeng *et al*, 1997). Ectopic endoglin expression increased the number of positive BrdU labelled cells in a dose-dependent manner, indicating that endoglin promotes EC proliferation (Figure 2A). This effect could be blocked using a neutralizing antibody that recognizes all TGF-β isoforms, suggesting that

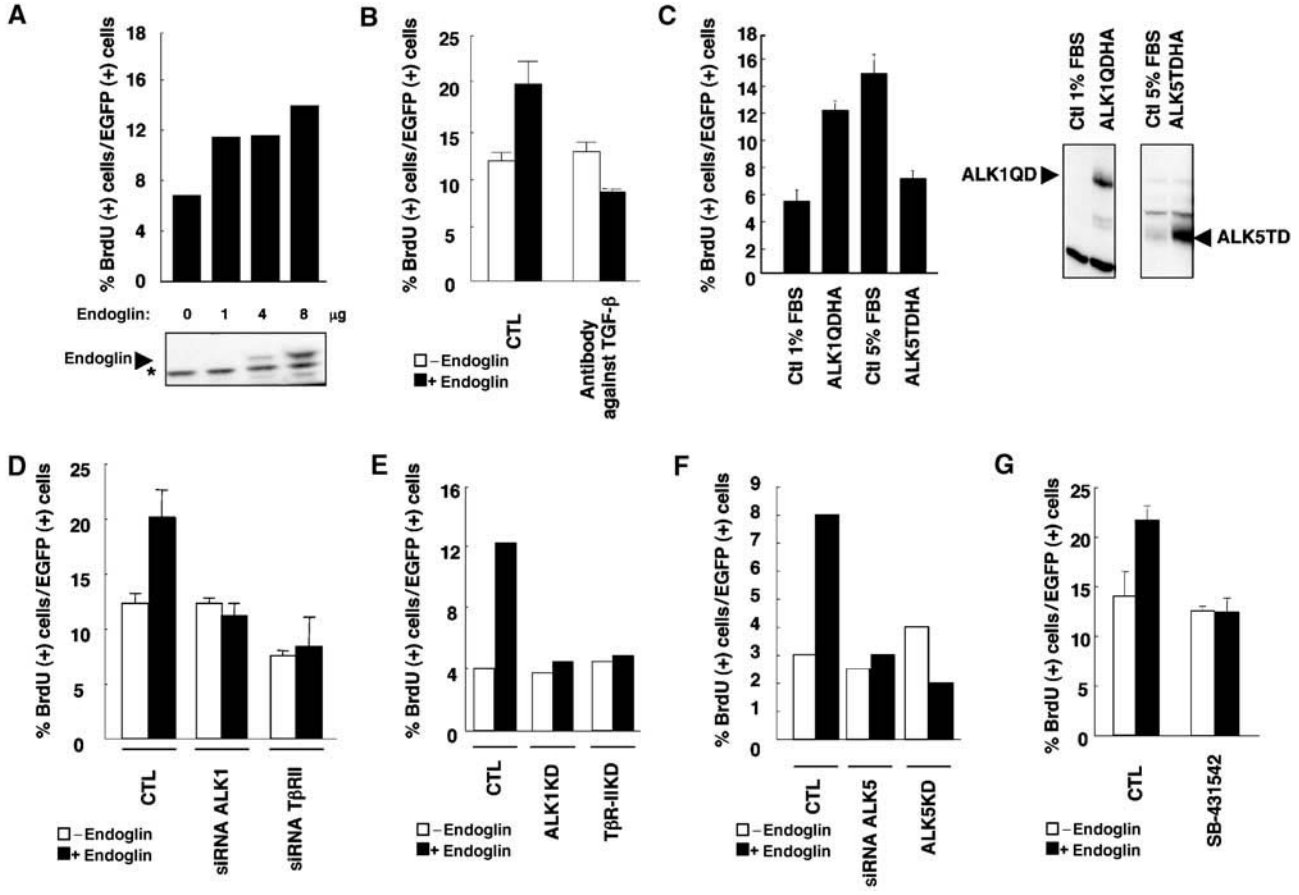

Figure 2 Ectopic expression of endoglin promotes EC proliferation via TGF-β/ALK1 signalling. MEECs were transiently transfected by calcium phosphate precipitation (**A**) with pEGFP alone or together with different doses of HA tagged endoglin. MEECs transfected were cultured for 48 h in 1% FBS, with BrdU being added for the last 3 h. The transfected cell population was identified by EGFP fluorescence. EGFP (+) cells were examined for BrdU incorporation. BrdU (+) cells were identified using indirect immunofluorescence (monoclonal anti-BrdU antibody). BrdU (+) cells represent the fraction of EGFP (+) cells with active DNA synthesis. The graphs shown are representative of three independent experiments. Endoglin expression was detected by Western blot analysis with a haemagglutin A (HA) antibody (asterisk: nonspecific band). (**B**) Neutralizing antibody against TGF-β blocks endoglin-mediated growth of ECs. MEECs transfected with pEGFP alone or together with (h) endoglin were cultured for 48 h in 1% FBS in the presence of neutralizing antibody against TGF-β. Proliferation rate was measured as described above. (**C**) Overexpression of (ca)TGF-β type I receptors on EC proliferation. MEECs were transfected with pEGFP alone or in the presence of ALK1QDHA or ALK5TDHA. Cells were cultured for 48 h in 1 or 5% FBS for ALK1QDHA and ALK5TDHA transfected cells, respectively. Proliferation rate was measured as described above. Expression of (ca)TGF-β type I receptors was determined by Western blot analysis using an HA antibody. (**D–G**) Inhibition of the TGF-β/ALK1 signalling pathway blocks the promoting effect of endoglin on EC proliferation. MEECs transfected with pEGFP alone or together with (human) endoglin were cultured for 48 h in 1% FBS in cotransfection with (D) siRNA ALK1, siRNA TβR-II; (E) with ALK1 kinase-inactive (ALK1KD) or TβR-II kinase-inactive (TβR-IIKD); (F) with siRNA ALK5 or ALK5 kinase-inactive (ALK5KD) and in the presence or absence of 10 μM of SB-431542 component, an inhibitor of ALK5 kinase activity. Proliferation rate was measured as described above. The results represent determinations for one experiment that is representative of three independent experiments.

TGF-β present in serum and/or TGF-β produced by ECs is sufficient to stimulate EC proliferation via endoglin (Figure 2B). In parallel, we confirmed that the TGF-β antibody had no effect on basal EC proliferation (Figure 2B). It has already been shown that expression of constitutively active (ca) ALK1 promotes EC proliferation, while expression of caALK5 inhibits EC proliferation (Figure 2C; Goumans *et al*, 2002). This suggests that ectopic expression of endoglin may potentiate the ALK1 pathway and/or inhibit the ALK5 pathway. To assess whether endoglin-induced EC proliferation is dependent on ALK1 and TβR-II function, we carried out siRNA-mediated ALK1 knockdown and siRNA TβR-II knockdown in ECs. We found that downregulation of ALK1 or TβR-II expression is sufficient to block endoglin-induced EC proliferation as measured by the BrdU incorporation (Figure 2D). This was confirmed by inhibiting endoglin-induced EC proliferation by ectopic expression of a dominant-negative ALK1 as well as a dominant-negative mutant of its upstream activator TβR-II (Figure 2E). Finally, we have recently shown that ALK5 activity is necessary for ALK1 signalling (Goumans *et al*, 2003). Therefore, as expected, siRNA-mediated knockdown of ALK5, ectopic expression of a dominant-negative ALK5 and addition of the SB-431542 component, an inhibitor of ALK5 kinase activity, block the promoting effect of ectopic expression of endoglin on EC growth (Figures 2D–F). Taken together, these results suggest that enforced expression of endoglin promotes EC proliferation via the TGF-β/TβR-II/ALK1 pathway.

Endoglin blocks TGF-β-induced growth inhibition of ECs and indirectly inhibits ALK5 signalling

TGF-β/ALK5 signalling induces Smad2/3 phosphorylation, activates Smad3/Smad4-driven $CAGA_{12}$-Luc reporter activity and for 'high doses of TGF-β' inhibits EC proliferation (Goumans *et al*, 2002). We examined the effect of ectopic expression of endoglin on TGF-β-induced EC growth inhibition. In agreement with previously published data (Letamendia *et al*, 1998), we found that ectopic endoglin expression potently inhibits TGF-β-induced inhibition of EC proliferation (Figure 3A). siRNA-mediated knockdown of endoglin was found to have no effect on TGF-β-induced Smad2 phosphorylation (Figure 1B). However, siRNA-mediated knockdown of endoglin significantly enhanced TGF-β-induced $CAGA_{12}$-Luc activity in ECs (Figure 3B). This effect is specific as it can be rescued by ectopic expression of human endoglin (Figure 3B). Previously, we and others have shown that ALK1 inhibits TGF-β/ALK5 signalling downstream of Smad phosphorylation (Oh *et al*, 2000; Goumans *et al*, 2003). We therefore tested whether stimulation of the ALK5 pathway by endoglin knockdown could be due to reduced ALK1 activity. Coexpression of ALK1QD was found to block enhanced $CAGA_{12}$-Luc activity induced by endoglin knockdown (Figure 3C). The regulatory effects of overexpression or siRNA-mediated knockdown of endoglin on ALK5 signalling may thus be indirectly caused through the modulation of TGF-β/ALK1 signalling.

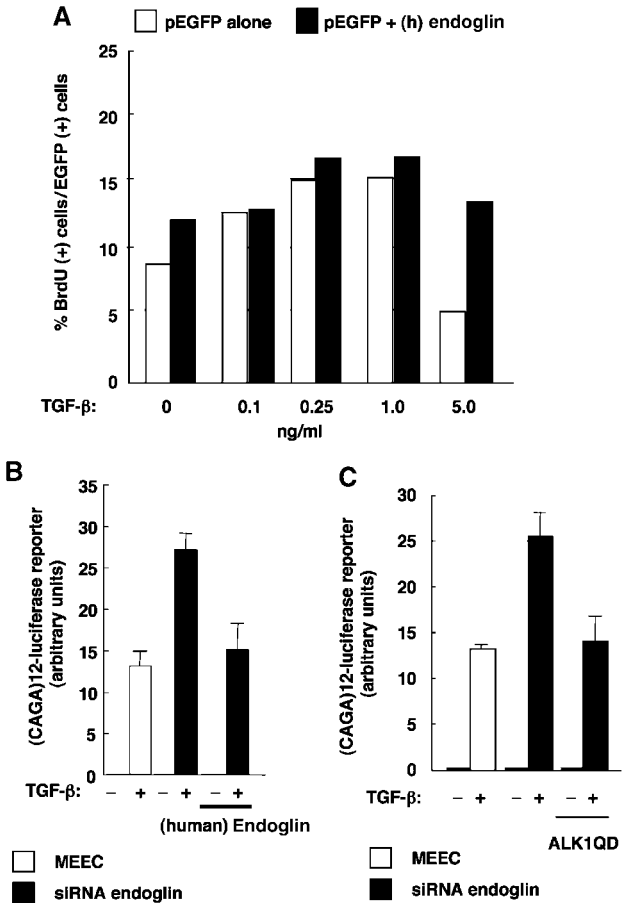

Figure 3 Endoglin indirectly inhibits TGF-β/ALK5 signalling. (**A**) Endoglin overexpression blocks the inhibitory effect of a high dose of TGF-β on EC proliferation. MEECs transfected with pEGFP alone (grey bar) or together with (human) endoglin (solid bar) were cultured for 48 h in 1% FBS and different concentrations of TGF-β. Proliferation rate was measured as described above. (B, C) TGF-β-induced transcriptional activation of $(CAGA)_{12}$-luciferase reporter is upregulated in ECs transfected by siRNA endoglin construct. MEECs were transfected and unstimulated or stimulated for 16 h with 5 ng/ml of TGF-β followed by measurement of the luciferase activity in the cell lysates. (**B**) Human endoglin was cotransfected to restore the TGF-β-induced transcriptional activity. (**C**) Expression of a caALK1 receptor (ALK1QD) rescues the effect of loss of endoglin expression on TGF-β transcriptional activity.

Defective EC proliferation upon RNAi-mediated knockdown of endoglin can be rescued through interference with ALK5 signalling

To analyse the effect of siRNA-mediated knockdown of endoglin on basal EC proliferation, we performed a colony formation assay by transfecting ECs with hygromycin expression vector in the absence or presence of siRNA endoglin. After selection for 10 days in the presence of hygromycin, the cells that survived and formed colonies were visualized by crystal violet staining. We observed a dramatic reduction in the number of colonies upon knockdown of endoglin expression (Figure 4A). Mock-transfected ECs did not survive the hygromycin treatment (Figure 4A). This result suggests that endoglin is a strong contributor to basal EC growth. We further investigated this finding by measuring BrdU incorporation in ECs transiently transfected or not with siRNA endoglin construct. In ECs with reduced endoglin expression, there was a significantly lower proliferation rate compared to wild-type ECs (Figure 4B). The inhibitory effect of siRNA endoglin on proliferation was found to be specific as it could be rescued by ectopic expression of human endoglin (Figure 4B). To consolidate these findings even further,

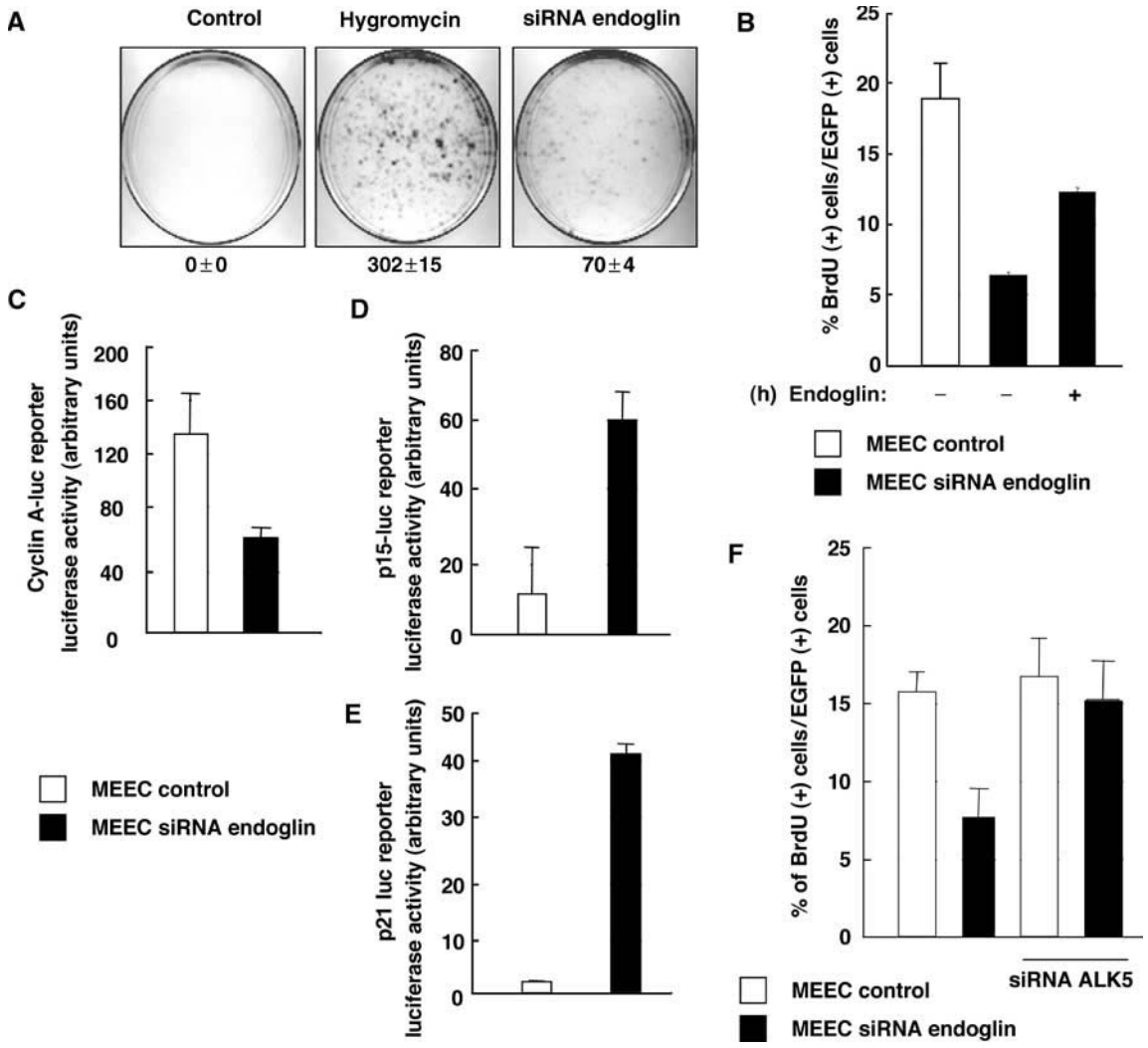

Figure 4 Defective EC proliferation upon RNAi-mediated knockdown of endoglin can be rescued through interference with TGF-β/ALK5 signalling. (**A**) Colony formation assay of ECs stably transfected with a hygromycin expression vector in the presence or absence of siRNA endoglin. A total of 1000 cells were seeded on a 10 cm dish and cultured for 10 days in the presence of hygromycin before staining with crystal violet. (**B**) BrdU incorporation in EGFP (+) cells. MEECs were transiently transfected by using lipofectamine with pEGFP alone, together with siRNA endoglin alone and in cotransfection with (human) endoglin. Cells were cultured for 57 h in the presence of 5% FBS, with BrdU being added for the last 3 h. Proliferation rate was measured as described in Figure 1. (C–E) siRNA-mediated knockdown of endoglin expression on the transcriptional activity of specific cell cycle targets. MEECs were transiently transfected by lipofectamine with different luciferase reporter gene constructs. (**C**) For (cyclin-A)-luciferase reporter, cells were starved for 36 h and stimulated by 10% FBS for 24 h before luciferase activity was read. (**D, E**) For (p15)-luciferase and (p21)-luciferase reporters, cells were cultured after transfection in the presence of 2.5% FBS for 36 h before luciferase activity was read. Values were corrected for differences in transfection efficiency. (**F**) Inhibition of ALK5 expression rescues the growth inhibitory effect of ECs induced by siRNA-mediated knockdown of endoglin. MEECs were transiently transfected with pEGFP together with siRNA endoglin alone or in the presence of siRNA ALK5. Active DNA synthesis was determined as indicated in Figure 1.

analysis of transcriptional activity of genes involved in cell cycle regulation, that is, cyclin A, $p15^{ink4A}$ and $p21^{waf/cip}$ cyclin CDK inhibitors, using promoter-driven luciferase reporter constructs was performed in wild-type ECs and after siRNA-mediated knockdown of endoglin expression. Consistent with the results described above, cyclin A promoter activity was found to be decreased (Figure 4C), but $p15^{ink4A}$ and $p21^{waf/cip}$ promoter activities were increased upon reducing endoglin expression (Figures 4D and E). Thus, endoglin expression controls the basal proliferation rate of ECs. To test whether the effect observed was dependent on ALK5 signalling, we examined the effect of siRNA-mediated knockdown of ALK5 on the inhibitory effect of growth induced by loss of endoglin expression. Consistent with our expectation, we observed that defective EC proliferation upon siRNA-mediated knockdown of endoglin could be rescued by reducing ALK5 signalling (Figure 4F).

ECs derived from endoglin heterozygous embryos exhibit a reduced TGF-β signalling

To investigate how reduced levels of endoglin lead to HHT, we isolated ECs from mouse embryos from intercrosses of mice heterozygous for endoglin. We immortalized ECs from embryos at embryonic day 9.5 (E9.5) with retrovirus expressing polyoma middle T (PmT) as previously described (Larsson *et al*, 2001). PmT specifically immortalizes ECs and not any other cell types (Williams *et al*, 1989; Garlanda *et al*, 1994). This allows pure EC lines to be obtained even

if starting from a mixed population (Garlanda *et al*, 1994). Interestingly, ECs could be readily established from heterozygous and wild-type embryos, but ECs from knockout embryos died in culture, suggesting that endoglin is essential for EC survival (Figure 5A). A PECAM staining has been performed and confirmed the homogeneous character of the EC lines obtained (Figure 5B). Finally, expression of endothelial markers, that is, VEGF, PECAM and FLT-1, confirmed the EC identity of the lines generated from wild-type and heterozygous embryos (Figure 5C). The endoglin expression level was strongly reduced in endoglin heterozygous cell lines compared to wild-type cell lines derived from embryos (Figure 5C). When we examined TGF-β-induced Smad responses in these cell lines, we observed that TGF-β-induced Smad1/5 phosphorylation is absent in endoglin heterozygous lines but occurs normally in EC lines derived from wild-type

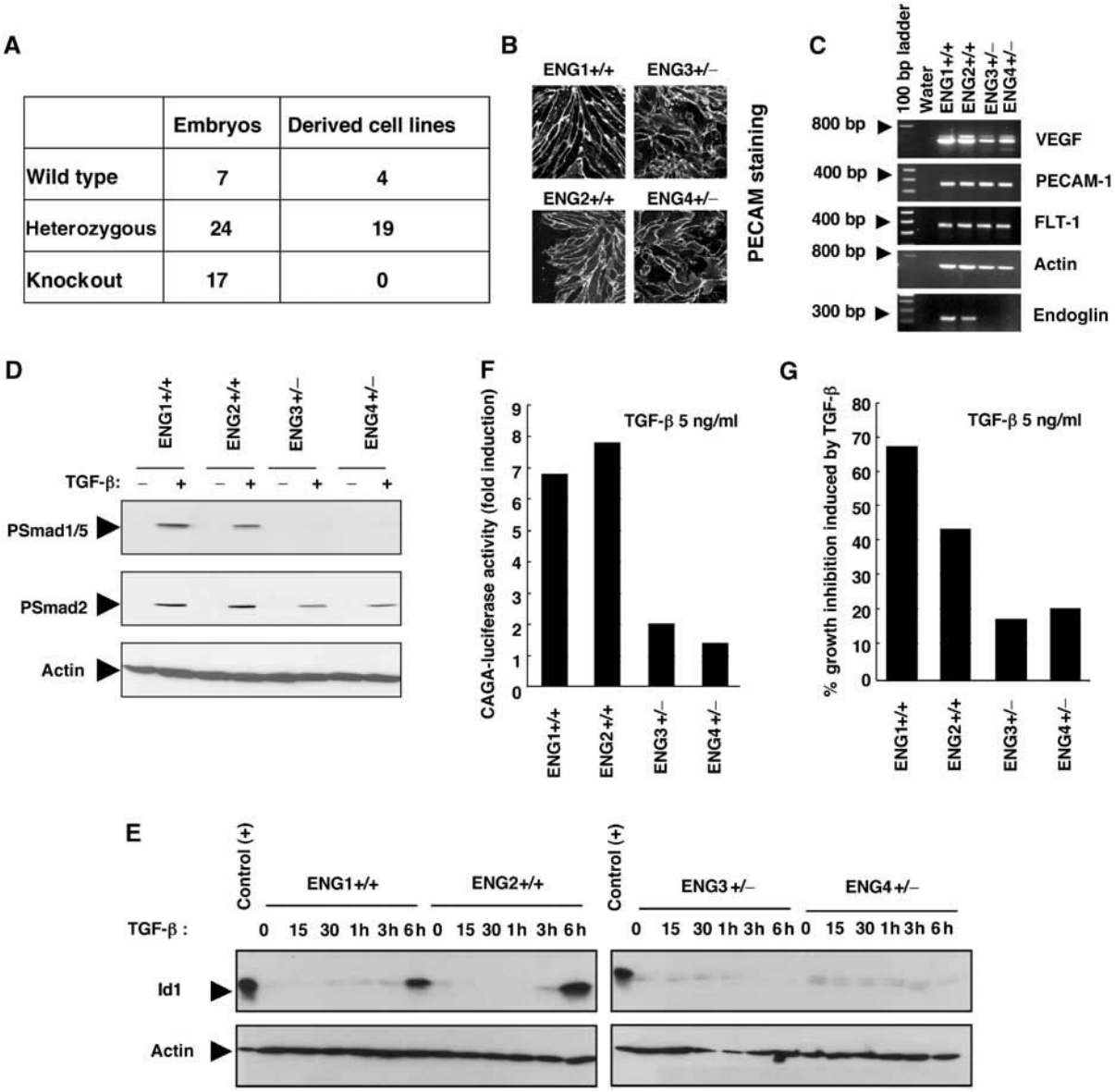

Figure 5 ECs derived from endoglin heterozygous embryos exhibit reduced TGF-β signalling. Establishment of ECs derived from endoglin wild-type, heterozygous or deficient mice. (**A**) Summary of cell lines obtained after isolation and immortalization of ECs obtained from embryos of heterozygous crosses. (**B**) PECAM staining of the different cell lines generated. (**C**) Expression of endothelial specific markers in the immortalized cells obtained was analysed by RT–PCR. The PCR products were loaded on a 1% agarose gel and stained with ethidium bromide. Amplified products of VEGF, PECAM-1, FLT-1, endoglin and β-actin are indicated on the right of the figure. (**D**) ECs derived from endoglin heterozygous embryos show a reduction of Smad1/5 and Smad2 phosphorylation induced by TGF-β. MEECs wild type or heterozygous for the gene ENG were stimulated with 5 ng/ml of TGF-β for 45 min at 37°C before lysis. Whole-cell extracts were fractioned by SDS–PAGE and blotted with PSmad2, PSmad1/5 or β-actin antibody. (**E**) Loss of TGF-β-induced Id1 expression in ECs heterozygous for the gene ENG. MEECs wild type or heterozygous for the gene ENG were treated with 0.25 ng/ml of TGF-β for different times before lysis. Whole-cell extracts were fractioned by SDS–PAGE and blotted with an Id1 antibody. (**F**) Reduction of $(CAGA)_{12}$-luciferase reporter transcriptional activity in ECs heterozygous for the gene ENG. ECs wild type and heterozygous for the gene ENG were transfected with $(CAGA)_{12}$-luciferase reporter and unstimulated or stimulated for 16 h by TGF-β before the luciferase activity was measured. (**G**) Endoglin heterozygous cell lines are less sensitive to TGF-β-induced growth inhibition. ECs were seeded in DMEM containing 5% FBS in the presence or absence of 5 ng/ml of TGF-β for 3 days and cell numbers were determined using an electronic counter. The graph is representative of three independent experiments.

embryos (Figure 5D). Consistent with these results, we observed that TGF-β-induced Id1, a specific downstream target gene of the TGF-β/ALK1 pathway, is induced in endoglin wild-type ECs, but not in endoglin heterozygous cell lines (Figure 5E). Thus, these data confirmed our findings using siRNA-mediated knockdown of endoglin that endoglin promotes ALK1 signalling. However, we were initially surprised to find that TGF-β-induced Smad2 phosphorylation is reduced by approximately half in endoglin heterozygous ECs (Figure 5D). Consistent with this, TGF-β-induced $CAGA_{12}$-Luc response is only weakly induced (Figure 5F) and TGF-β-induced growth inhibition is reduced in these cell lines compared to endoglin wild-type ECs (Figure 5G). The decreased TGF-β-induced $CAGA_{12}$-Luc activity in cell lines with reduced levels of endoglin expression contrasts with the potentiating effect of siRNA-mediated knockdown effects of endoglin on $CAGA_{12}$-Luc activity (Figure 3B). The difference may be caused by the fact that the decrease in endoglin expression by siRNA-mediated knockdown occurs quickly, whereas in the heterozygous cell lines, ECs might have had time to adapt to compensate for decreased endoglin expression.

ALK5 expression is reduced in endoglin heterozygous embryos

To investigate how these cells adapt to reduce ALK5 signalling, we next examined by RT–PCR the expression of ALK5 and TβR-II in endoglin wild-type and heterozygous EC lines. We noted a substantial decrease of ALK5 expression in endoglin heterozygous ECs compared to wild-type ECs, whereas the expression of TβR-II was not affected (Figure 6A). An ^{35}S-methionine labelling of the different cell lines followed by an immunoprecipitation of ALK5 was performed. We observed a decrease of 75% of the ALK5 receptor expressed by the endoglin heterozygous compared to the wild-type cell lines (Figure 6B). Taken together, these

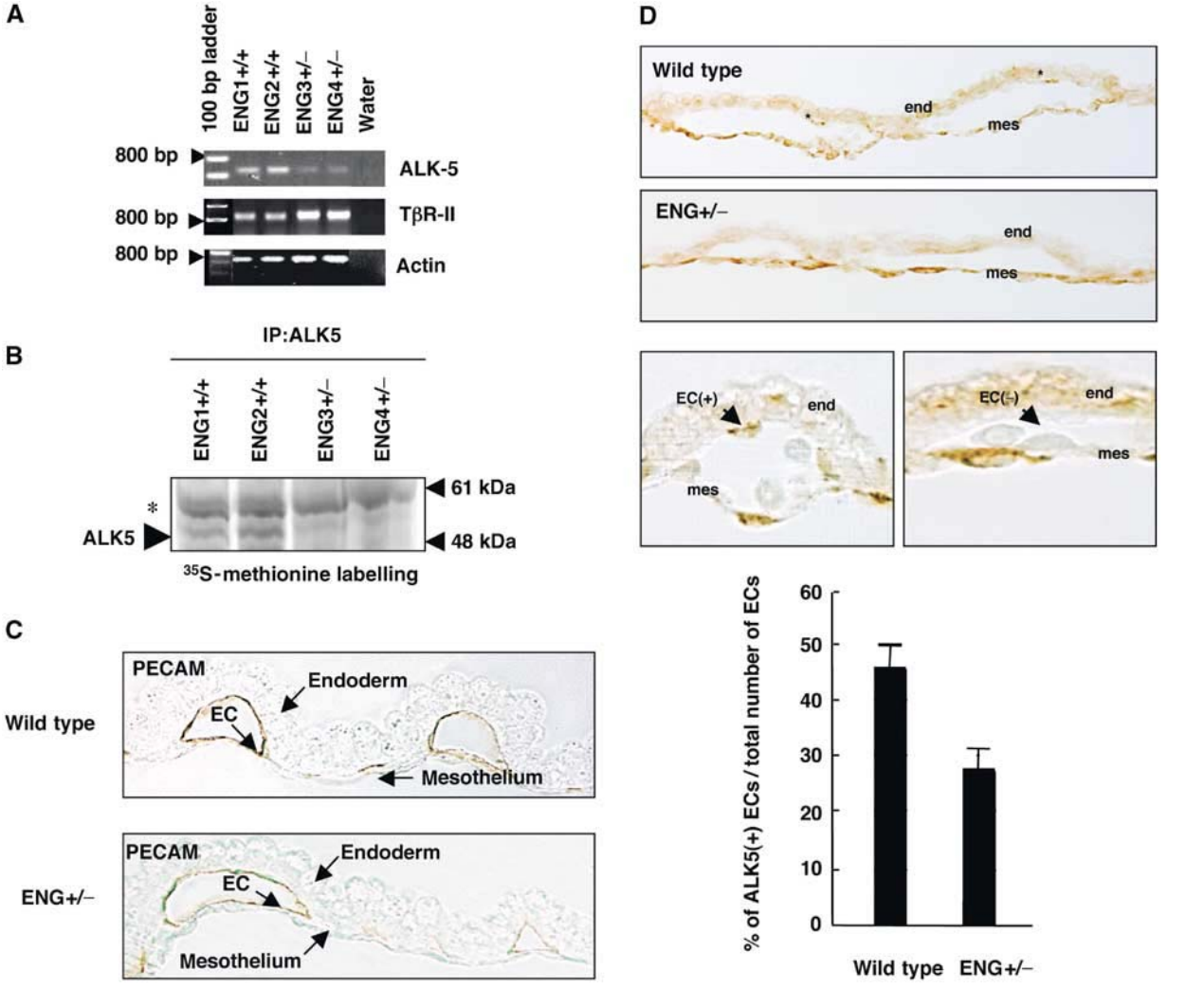

Figure 6 ALK5 expression is reduced in embryos heterozygous for endoglin. (**A**) RT–PCR analysis of ALK5 and TβR-II expressed in EC lines derived from embryos obtained from heterozygous intercrosses. The PCR products were loaded on a 1% agarose gel and stained with ethidium bromide. Amplified products and β-actin are indicated on the right of the figure. (**B**) EC lines were metabolically labelled with methionine for 6 h. The cell lysates were then subjected to precipitation using an anti-ALK5 antibody and precipitates were analysed by SDS-gel electrophoresis and autoradiography. (**C**) Wild-type and ENG + /− yolk sacs (E9.5) immunostained with PECAM showing that the number of ECs is similar in wild-type and ENG + /− yolk sacs. (**D**) Wild-type and ENG + /− yolk sacs (E9.5) immunostained for ALK5. The histogram shows the average proportion of ALK5-positive ECs in five wild-type and five heterozygous embryos obtained from different littermates.

results confirm our previous finding that endoglin heterozygous cell lines only weakly respond to TGF-β-induced $CAGA_{12}$-luc reporter and growth arrest (Figures 5F and G). We next analysed the expression of ALK5 in yolk sacs isolated from endoglin heterozygous embryos. No difference was observed in the total number of ECs present in endoglin wild-type and heterozygous yolk sacs as measured by a PECAM staining (Figure 6B). However, the number of ECs positive for ALK5 in endoglin heterozygous yolk sacs was reduced to half that of wild-type yolk sacs (Figure 6C). Thus, endoglin heterozygous ECs might have adapted to the potentiating effect on the ALK5 pathway, which inhibits EC proliferation, by downregulating ALK5 expression.

Discussion

Angiogenesis is a carefully regulated process, which can be viewed as two separate, but balanced phases, activation and resolution, that lead to blood vessel formation from a preexisting vascular network (Risau, 1997; Carmeliet, 2000). TGF-β plays a pivotal role during angiogenesis and has been shown either to inhibit or promote blood vessel formation (Pepper, 1997). A recent study has provided a framework to understand the pro- and antiangiogenic properties ascribed to TGF-β. In contrast to most cells, TGF-β can activate two receptor/Smad cascades with opposite effects in ECs: TGF-β/ALK5-induced Smad2/3 phosphorylation that inhibits proliferation and migration and TGF-β/ALK1-induced Smad1/5 phosphorylation that promotes both processes (Goumans *et al*, 2002). How ALK1 and ALK5 signalling pathways are regulated within ECs to coordinate responses to TGF-β remains to be elucidated.

In the present study, we show that endoglin, an accessory receptor of TGF-β and highly expressed in activated ECs, is a necessary component for efficient ALK1 signalling. Moreover, the level of endoglin expressed determines EC growth capacities. Our data implicate endoglin as a modulator of the balance between TGF-β/ALK1 and TGF-β/ALK5 signalling pathways. In the absence of endoglin, ECs do not proliferate and ALK1 signalling is abrogated, whereas ALK5 signalling is stimulated. Our results favour a model, in which endoglin stimulates TGF-β/ALK1-induced Smad1/5 responses and indirectly inhibits TGF-β/ALK5 signalling pathway, thereby promoting the activation state of the endothelium (Figure 7). In addition, ECs from embryos with reduced endoglin expression exhibit a downregulation of ALK5 expressed at the cell surface. This regulatory adaptation might occur in order for ECs to survive the potentiation of ALK5-induced growth arrest that would otherwise occur and may provide an explanation for similarity in angiogenesis defects in TGF-β1-, TβR-II-, ALK1-, endoglin- and ALK5-deficient mice.

Mutations in endoglin or ALK1 cause the autosomal dominant bleeding disorder HHT types I and II respectively, suggesting that both receptors act to the same signalling pathway. However, biochemical data to support this are still lacking. In fact, a model has been proposed in which endoglin promotes TGF-β/ALK5 signalling (Guerrero-Esteo *et al*, 2002). Here, we report that endoglin is a necessary component of TGF-β/ALK1 signalling. Several lines of evidence support this conclusion. Firstly, ECs lacking or with decreased expression of endoglin have a greatly attenuated TGF-β/ALK1 signalling as measured by Smad1/5 phosphorylation, BRE-luc activity and Id1 protein expression. Secondly, ECs lacking endoglin are impaired in 'low-dose' TGF-β-induced cell proliferation and migration, which are biological responses of TGF-β mediated by ALK1 signalling pathway (Goumans *et al*, 2002). We demonstrate that ectopically expressed endoglin is sufficient to induce EC proliferation, a result consistent with previous observations describing that elevated endoglin protein levels are often correlated with highly proliferating ECs *in vitro* and *in vivo* (Fonsatti *et al*, 2001). Moreover, we show that endoglin-mediated stimulation of EC proliferation is dependent on TGF-β/ALK1 signalling as demonstrated by blocking antibody against TGF-β and siRNA or kinase-inactive mutant forms of ALK1 or TβR-II, which all interfere with this endoglin effect. Taken together, our results indicate that endoglin/ALK1 signalling stimulates EC proliferation and migration.

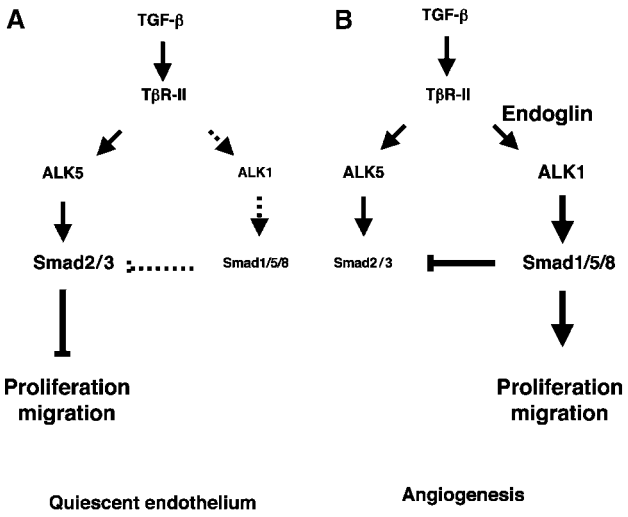

Figure 7 Model of regulation of angiogenesis by the ancillary receptor endoglin. TGF-β can stimulate two distinct type I receptor/Smad signalling pathways with opposite effects in ECs. The TGF-β/ALK5 signalling pathway leads to inhibition of cell proliferation and migration, whereas the TGF-β/ALK1 pathway induces EC proliferation and migration. Endoglin, an accessory TGF-β receptor, highly expressed during angiogenesis, is essential for ALK1 signalling. In the absence of endoglin, the TGF-β/ALK5 signalling is predominant and maintains quiescent the endothelium. High endoglin expression stimulates the ALK1 pathway and indirectly inhibits ALK5 signalling, thus promoting the activation state of angiogenesis.

Previous studies, using transiently expressed endoglin and BMP receptors, have suggested that endoglin could act as an accessory protein of BMP signalling (Barbara *et al*, 1999). We failed to observe any effect of siRNA-mediated knockdown of endoglin on BMP signalling, arguing against a physiological role of endoglin on BMP signalling. Our results favour a model, in which endoglin is a necessary accessory receptor of ALK1 signalling, rather than a modulator of multiple ALK signalling pathways.

Consistent with previous observations, we demonstrate that ectopic expression of endoglin blocks TGF-β-induced EC growth arrest (Lastres *et al*, 1996; Li *et al*, 2000; She *et al*, 2004). Moreover, in the present study, we demonstrate that TGF-β/ALK5 pathway is potentiated after siRNA-mediated knockdown of endoglin expression. This results

in a more potent TGF-β-induced growth inhibition, even at low doses of TGF-β that are known to promote proliferation (Goumans *et al*, 2002). The mechanism by which TGF-β inhibits EC growth is probably through upregulation of $p15^{ink4A}$ and $p21^{waf/cip}$ expression. We failed to detect TGF-β-induced Smad2 phosphorylation after siRNA-mediated knockdown of endoglin expression, arguing against a direct effect of endoglin on ALK5 receptor activity as proposed by Guerrero-Esteo *et al* (2002). Previous studies have suggested that the ALK1 cascade antagonizes ALK5 signalling responses (Oh *et al*, 2000; Goumans *et al*, 2003). Consistent with this notion, we found that ectopic expression of caALK1 kinase rescues siRNA-mediated knockdown of endoglin effect on ALK5 signalling. Taken together, these results suggest that the inhibitory effect of endoglin on TGF-β/ALK5 signalling is caused indirectly by promoting ALK1 signalling. The mechanism of how endoglin/ALK1 signalling pathway inhibits ALK5 signalling remains to be elucidated, but might occur at the Smad level. In fact, we have recently observed that TGF-β induces heteromeric complex formation between Smad2/3 and Smad1/5 in ECs. The interaction of Smad1/5 with Smad2/3 may thus interfere with their specific DNA binding and/or recruitment of cofactor resulting in inhibition of ALK5-dependent gene transcription (M Thorikay and P ten Dijke, unpublished data).

Interestingly, we observe that EC lines established from heterozygous mice with dramatically reduced endoglin expression also have a significant decrease in TGF-β/ALK5 signalling. This adaptation already occurs in the embryos as demonstrated by the decreased ALK5 expression in ECs from endoglin heterozygous embryos compared to wild-type embryos. Our result is consistent with a recent study that has examined the effect of VEGF hyperstimulation on brain microvessels in adult endoglin heterozygous mice. These authors reported vascular defects associated with a reduced level of ALK5 expression (Xu *et al*, 2004). Thus, ECs with decreased endoglin expression may adapt by downregulating ALK5 signalling. Consistent with this, simultaneous knockdown of endoglin and ALK5 rescued the defective EC proliferation that occurs when endoglin expression alone is inhibited. Our results provide a possible explanation of why phenotypes of ALK5- and endoglin-deficient mice are similar (Arthur *et al*, 2000; Larsson *et al*, 2001) and might provide a framework for understanding the clinical manifestations of HHT. ENG +/− mice used as a model for HHT type I show extremely dilated, tortuous vessels with weak-walled, disorganized and sparsely distributed elastin fibres and smooth muscle cells. This phenotype relates to the formation of structurally incompetent basement membrane (Torsney *et al*, 2003). In postcapillary venules from human, an increase in vessel wall size resulting from excessive pericyte recruitment and excessive numbers of SMC layers has been reported (Braverman *et al*, 1990). Both clinical manifestations result from a defect during the maturation phase of angiogenesis and relate to ALK5 signalling.

In conclusion, our results show that endoglin is necessary for efficient ALK1 signalling and indirectly inhibits ALK5 signalling, thereby promoting the activation state of EC during angiogenesis. Moreover, we show that embryos defective in endoglin expression adapt to overcome the enhanced ALK5 signalling when endoglin expression decreases in ECs. Our results provide a framework to understand the previously conflicting reports between ALK1 function based upon *in vitro* studies in ECs and phenotypic analysis of heterozygous and knockout mice.

Materials and methods

Isolation of embryonic ECs and cell culture

Mouse embryos from endoglin heterozygous intercrosses were collected at E9.5 of development. Embryos were washed in Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) before enzymatic desegregation by collagenase (5 min at 37°C). Dissociated cells were plated on gelatin (0.1%) in DMEM (10% fetal bovine serum (FBS), VEGF 2.5 ng/ml, 2.5 ng/ml b-FGF) (Larsson *et al*, 2001). For selective transformation of ECs, the embryonic cells were infected with a retrovirus expressing the PmT oncogene (Williams *et al*, 1988) and then grown for a sufficient number of passages that cells with EC morphology became evident. As previously reported, PmT specifically immortalizes ECs and not any other cell types (Williams *et al*, 1989; Garlanda *et al*, 1994). This allows pure EC lines to be obtained even if starting from a mixed population (Garlanda *et al*, 1994). ECs were then cultured in DMEM supplemented with 10% FBS, nonessential amino acids, L-glutamine and penicillin/streptomycin in 5% CO_2-containing atmosphere at 37°C.

Transfection and reporter assays

Transient transfections and reporter assays were performed as previously described (Goumans *et al*, 2002). In all reporter assays, the β-galactosidase expression plasmid pCH110 (Pharmacia) served as an internal control to correct for transfection efficiency. The experiments were performed in triplicate at least three times; representative experiments are shown. For $(SBE)_4$-Luc and $CAGA_{12}$-Luc, the luciferase activity was measured 18 h after TGF-β_3 (1 ng/ml) addition. BRE_2-Luc was measured 8 h after TGF-β_3 (1 ng/ml) addition. P21 and p15-luciferase reporters were measured 36 h after transfection (Li *et al*, 1995; Pardali *et al*, 2000). Cyclin A-luciferase reporter was measured 24 h after 10% FBS addition.

RNA isolation and reverse-transcription polymerase chain reaction

Total RNA was isolated, using RNeasy columns (Qiagen) according to the manufacturer's instructions. RT–PCR was performed as previously described (Goumans *et al*, 1999; Arthur *et al*, 2000).

Metabolic labelling and immunoprecipitation

Cells were labelled in methionine-free DMEM (ICN) supplemented with 100 μCi/ml of [^{35}S]methionine for 6 h at 37°C. Thereafter, cells were washed with cold PBS and lysed with lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, NaF 50 mM, EDTA 5 mM, β-glycerophosphate 40 mM, Triton X-100 1%, 1 mM PMSF). After 15 min on ice, the cell lysate was centrifuged and the supernatant was precleared one time. Samples (500 μg of total proteins) were then incubated in the presence of a specific antiserum ALK5 for 1 h at 4°C. Immune complexes were then incubated with 50 μl of protein A–Sepharose (Pharmacia) for 30 min at 4°C. Thereafter, the beads were spun down and washed five times with the lysis buffer. The beads were analysed by SDS-gel electrophoresis and autoradiographed.

Western blot analysis and flow cytometry analysis

Western blot analysis was performed as previously described (Goumans *et al*, 2002). Detection was by ECL. PSmad1 and PSmad2 antibodies that specifically recognize phosphorylated Smad1/5 and Smad2 have been previously described (Goumans *et al*, 2002).

For FACS analysis, cells grown on 100-mm tissue culture plates were detached with dissociation buffer (GIBCO) and washed twice with PBS and 2% FBS. Cells were then incubated for 45 min with a PE-conjugated endoglin (MJ7/18) antibody (Santa Cruz) or a PE-conjugated $IgG_{2a}\kappa$ isotype antibody, used as a control (BD Pharmingen). Cells were analysed by a FACScan® apparatus (Becton-Dickinson).

BrdU incorporation

Cells were plated onto glass coverslips at a density of 350 000–500 000 cells/60 mm dish. Cells were either transfected by calcium phosphate or lipofectamine (Life Technologies Inc.) procedure with

pEGFP and the relevant constructs (ratio 1/10). After transfection, cells were cultured for 48 h in 1–5% serum in presence or absence of TGF-β with a pulse of BrdU (25 μM) for the last 3 h. The cells were then fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT), rinsed in PBS and the chromatin was rendered accessible by a 30 min treatment with HCl (2 N). Cells were extensively washed with PBS and the nonspecific sites were blocked by incubation with goat sera. The cells were incubated with the anti-BrdU monoclonal antibody (Sigma) for 1 h, washed with TBS + 0.1% Tween 20 and finally incubated with the secondary antibody goat anti-mouse cyanine-conjugated antibody (Jackson Immunotech.). The coverslips were mounted in citifluor. The proportion of BrdU (+) cells/EGFP (+) cells was determined by analysing at least 100 EGFP (+) cells in 10–15 fields for each transfection performed in triplicate, in at least three independent experiments.

Immunohistochemistry

For whole-mount immunohistochemistry, E9.5 yolk sacs were fixed for 30 min at RT in 2% PFA, dehydrated serially to 100% methanol, treated for 5 h with 5% hydrogen peroxide in methanol, rehydrated to PBS at RT and permeabilized with 0.1% Triton X-100 (Merck) in PBS for 8 min. After washing with PBS, the yolk sacs were blocked for 1 h at RT in TNB blocking solution from Tyramide Signal Amplification (TSA) Biotin System (PerkinElmer, Life Sciences). After 1 h incubation with PECAM (Pharmigen) or with anti-ALK5 (Santa Cruz) diluted (1:100) in TNB blocking solution, embryos were washed in 0.05% Tween/PBS and treated for 1 h with the second antibody (biotin-conjugated goat anti-mouse IgG, DAKO, 1:250) in blocking solution at RT. Embryos were then treated with the ABComplex/HRP (DAKO), followed by the Fast 3,3′-diaminobenzidine tablet set (DAB, Sigma), according to the manufacturer's instructions, fixed overnight in 2% PFA/0.1% gluteraldehyde in PBS at 4°C, embedded in plastic and sectioned. ECs are easily distinguishable from other cells of the yolk sac at high magnification on the basis of their position at the inner aspects of the vessels and their morphology in the plastic sections. For quantification of the number of ALK5-positive cells, serial sections were examined through the yolk sac and more than 300 cells were scored in each. ECs are typically evident as a thin layer (in parallel positive PECAM staining) around the vessel wall over several sections until a section through the nucleus is reached. The ECs can then easily be scored as ALK5 positive or negative.

EC proliferation assay and colony formation assay

ECs were seeded in DMEM containing 1 or 5% serum at a density of 2×10^4 cells per 24-well plate. The next day, TGF-β was added at 0.1 or 5 ng/ml. At the indicated time of culture, the number of cells was determined using either a Casy-1 cell counter (Schäfer Systems) or the WST-1 colorimetric assay (Roche). For colony formation assay, a total of 1000 cells were seeded on a 10 cm dish and cultured for 10 days in the presence of hygromycin before staining with crystal violet.

EC migration assay

Migration (Chemokinesis) was measured using a Boyden chamber as previously described (Goumans *et al*, 2002). The filter was coated with fibronectin and the number of migrated cells was counted 6 h after addition of cells in the upper chamber.

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