Defective paracrine signalling by TGFβ in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia

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Summary

Hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominant disorder in humans that is characterised by multisystemic vascular dyplasia and recurrent haemorrhage. Germline mutations in one of two different genes, endoglin or *ALK1* can cause HHT. Both are members of the transforming growth factor (TGF) β receptor family of proteins, and are expressed primarily on the surface of endothelial cells (ECs). Mice that lack endoglin or activin receptor like kinase (ALK) 1 die at mid-gestation as a result of defects in the yolk sac vasculature. Here, we have analyzed TGFβ signalling in yolk sacs from endoglin knockout mice and from mice with endothelial-specific deletion of the TGFβ type II receptor (TβRII) or ALK5. We show that TGFβ/ALK5 signalling from endothelial cells to adjacent mesothelial cells is defective in these mice, as evidenced by reduced phosphorylation of Smad2. This results in the failure of vascular smooth muscle cells to differentiate and associate with endothelial cells so that blood vessels remain fragile and become dilated. Phosphorylation of Smad2 and differentiation of smooth muscle can be rescued by culture of the yolk sac with exogenous TGFβ1. Our data show that disruption of TGFβ signalling in vascular endothelial cells results in reduced availability of TGFβ1 protein to promote recruitment and differentiation of smooth muscle cells, and provide a possible explanation for weak vessel walls associated with HHT.

Key words: HHT, TGFβ, Endoglin, Yolk sac, ACVRL1

Introduction

Hereditary haemorrhagic telangietasia (HHT), also known as Rendu-Osler-Weber syndrome, is an autosomal dominant vascular dysplasia that is characterised by epistaxis, telangiectases, gastrointestinal bleeding and arteriovenous malformations (AVM). The first symptom of HHT is generally epistaxis in the nasal mucosa (Guttmacher et al., 1995; Shovlin and Letarte, 1999), whereas telangiectases appear later and are known to occur in the lung, brain and liver of individuals with HHTs. There are two distinct genes responsible for HHT, endoglin (McAllister et al., 1994), which is mutated in HHT1, and *ALK1* (*ACVRL1* – Human Gene Nomenclature Database) (Johnson et al., 1996), which is mutated in HHT2. Both genes encode receptors for the transforming growth factor β (TGFβ) superfamily of ligands.

The TGFβ superfamily controls cell proliferation, differentiation, migration and adhesion. The prototype of the family, TGFβ1, has multiple roles in development and homeostasis of the vascular system. It regulates endothelial cell proliferation, production of extracellular matrix (ECM), vascular tone and interactions between endothelium and smooth muscle cell layers in the vessel wall (Pepper, 1997). It is also implicated in vascular remodelling.

TGFβ signalling involves heteromeric complex formation of type I and type II receptors, initiated by ligand binding, and subsequent phosphorylation of downstream target proteins known as Smads (R-Smads), which then complex with the common mediator Smad (Smad4). This complex is then imported to the nucleus and activates target genes. In most cells types TGFβ signals via the ALK5 receptor, phosphorylating Smad2 and Smad3. In contrast, TGFβ appears to signal through two pathways in endothelial cells: either via ALK1 through activation of Smad1 and Smad5, or via the conventional ALK5 mediated activation of Smad2 and Smad3 (Goumans et al., 2002). In addition to the TGFβ serine/threonine kinase receptors, TGFβ also binds an accessory receptor, endoglin, in endothelial cells. It is believed that the function of endoglin involves alteration in TGFβ signalling, as

it has been show to modulate TGFβ responses in endothelial cells and rat myoblasts (Barbara et al., 1999; Lebrin et al., 2004; Letamendia et al., 1998; McAllister et al., 1994; Pece et al., 1997).

Multiple studies in mice have implicated TGFβ as a potent mediator of angiogenesis (Goumans et al., 1999). Mice deficient in TGFβ1, ALK5 or TβRII die at E10.5 as a result of an inadequate yolk sac capillary network with poor adhesiveness between endothelial and mesothelial cell layers (Dickson et al., 1995; Larsson et al., 2001; Oshima et al., 1996). Targeted inactivation of ALK1 causes severe arteriovenous malformations resulting from fusion of major arteries and veins and loss of arterial-specific hematopoiesis (Urness et al., 2000), while in endoglin (*Eng*) mutant embryos the primary abnormality appears to be defective remodelling of the primary vascular plexus that results in abnormal yolk sac and embryonic blood vessel development (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999). It has been postulated that Eng haploinsuficiency is the mechanism underlying HHT1 (Marchuk, 1998; Pece et al., 1997; Shovlin, 1997). The heterozygous mice show clinical signs of HHT, such as nose bleeds and cutaneous telangiectases (Bourdeau et al., 1999; Torsney et al., 2003). However, this phenotype is highly dependent on the genetic background of the inbred strain as additional modifier genes, contributed by the 129/Ola strain are necessary to generate the vascular anomalies associated with HHT (Bourdeau et al., 2000).

TGFβ signalling is required for both the first stage of vascular development, vasculogenesis, when the primary capillary network is formed (Dickson et al., 1995) as well as the second stage, angiogenesis, that involves remodelling the primary endothelial network into a mature circulatory system (Folkman and D'Amore, 1996; Pepper, 1997). During blood vessel assembly, endothelial cells recruit mesenchymal progenitors and induce their differentiation into vascular smooth muscle cells (VSMC) or pericytes via contact-dependent TGFβ activation. However, the molecular basis of interactions between endothelial and mesenchymal cells that lead to TGFβ activation and smooth muscle cell differentiation are not well understood.

Here, we have examined vasculogenesis in the yolk sacs of *Eng* mutant mice and in mice in which TGFβ signalling, via TβRII or ALK5, has been disrupted specifically in endothelial cells. The yolk sac is a particularly useful model for studying blood vessel formation, as it is composed of only a limited number of cell types, is easily accessible and amenable to short-term culture. We show that the lack of an intact TGFβ pathway in endothelial cells of the yolk sac results in reduced phosphorylation of Smad2 in the adjacent mesothelial layer, by affecting accessible TGFβ protein levels. These cells then fail to differentiate to vascular smooth muscle cells, a process that can be rescued in part by short term culture of the yolk sac in the presence of TGFβ1. Our data provide a molecular basis for understanding HHT and could explain the development of the weak-walled and fragile vessels that are characteristic of this disease.

Materials and methods

Transgenic mice

All the mice examined in this study were kept on a mixed genetic background. Endoglin mutant mice containing a β-galactosidase (β-gal) reporter cassette in the disrupted locus (Arthur et al., 2000), floxed *Alk5* (*Tgfbr1* – Mouse Genome Informatics) mice (Larsson et al., 2001), floxed TβRII mice (Leveen et al., 2002) have been described. Floxed *Alk5* and *TβRII* (*Tgfbr2* – Mouse Genome Informatics) mice were crossed with Gtrosa26^{tm1Sor} (Zambrowicz et al., 1997) and subsequently crossed with *tie-1*-Cre transgenic mice (Gustafsson et al., 2001) to delete ALK5 and TβRII in endothelial cells. Because of the floxed Rosa26 locus, successful recombination was evidenced by β-gal positive cells. Floxed *Tgfbr2* mice were also crossed with *PGK*-Cre transgenic mice (Lallemand et al., 1998) to delete TβRII ubiquitously. The presence of a vaginal plug was designated as embryonic day (E) 0.5.

lacZ staining and immunohistochemistry

After isolation, E9.5 yolk sacs were divided in half, cultured in Dulbecco's minimum essential medium (DMEM) with or without TGFβ1 (1 or 5 ng/ml) or BMP2 (10 ng/ml), for 1, 3 or 8 hours, as indicated, at 37°C and then fixed for 30 minutes at room temperature in 2% paraformaldehyde (PFA). Prior to immunostaining, *lacZ* reporter expression was visualised using β-gal staining, as described by Nagy et al. (Nagy et al., 2003). For whole-mount immunohistochemistry, yolk sacs from E9.5 embryos were dehydrated serially to 100% methanol, treated for 5 hours with 5% hydrogen peroxide in methanol, rehydrated to PBS at room temperature, permeabilised with 0.1% Triton X-100 (Merck) in PBS for 8 minutes. After washing with PBS, the yolk sacs were blocked for 1 hour, at room temperature in TNB blocking solution from the Tyramide Signal Amplification (TSA) Biotin System (PerkinElmer, Life Sciences). After a 1 hour incubation with anti-PECAM1 (1:100, BD Biosciences), anti-endoglin (1:100, USBiological), Flk1 (1:100, SantaCruz), affinity purified anti-Phosphorylated Smad2 (PSmad2) (Persson et al., 1998), anti-TGFβ1 (1:100, recognizing total TGFβ1, SantaCruz), anti-smooth muscle actin (1:400, Sigma), anti-caldesmon (1:500, Sigma), anti-fibronectin (1:100, Sigma) or anti-collagen type I (1:100, Rockland) in TNB blocking solution, yolk sacs were washed in 0.05% Tween/PBS and treated for 1 hour with the secondary antibody (biotin-conjugated rabbit anti-rat IgG for PECAM1 and endoglin, biotin-conjugated swine anti-rabbit for PSmad2, TGFβ1, fibronectin and collagen type I or biotin-conjugated goat anti-mouse IgG for Flk-1 and α-sma, DAKO, 1:250) in blocking solution, at room temperature. Embryos were then treated with ABComplex/HRP (DAKO), followed by 10 minutes incubation in biotinil tyramide diluted 1:50 in tyramide diluent supplied in TSA Biotin System. After washing in 0.05% Tween/PBS, yolk sacs were treated for 1 hour, at room temperature, with strepavidin-HRP (1:250) in TNB. Peroxidase activity was detected using 3,3′-diaminobenzidine tablet set (Fast DAB, Sigma), according to manufacturer's instructions. Stained yolk sacs were fixed overnight in 2% PFA/0.1% gluteraldehyde in PBS at 4°C, embedded in plastic and sectioned.

Paraffin wax embedded sections of E9.5 embryos and yolk sacs were treated as above but using 1.2% hydrogen peroxide for 15 minutes. For antigen retrieval for PSmad1 paraffin sections were treated by boiling in Tris/EDTA pH 9.0 buffer while for PSmad2 paraffin section were treated by boiling in 10 mM citrate buffer (pH 6.0). Anti-PSmad1 and anti-PSmad2 were used as primary antibodies and biotin-conjugated goat anti-rabbit IgG (1:250, DAKO) as secondary antibody.

Score of positive endothelial cells in the yolk sac

The number of PSmad1/5/8 and PSmad2 positive endothelial cells was determined as described by Lebrin et al. (Lebrin et al., 2004).

Western blotting

Protein isolation for western blotting has been described previously (Fauré et al., 2000). Analysis was as described by Larsson et al. (Larsson et al., 2001), using the total lysate of two yolk sacs. Anti-

TGFβ1 (1:200, SantaCruz) was used as primary antibody and HRP-conjugated goat anti-rabbit IgG as secondary antibody (1:10000, BD Biosciences).

In situ hybridization

Whole-mount in situ hybridisation was performed as described before (Roelen et al., 2002). The digoxigenin-labelled (Boehringer Mannheim) TGFβ1 antisense probe was a T3 polymerase transcript from a 600 bp *Kpn*I-*Apa*I fragment (Millan et al., 1991).

Separation of yolk sac mesoderm and endoderm

The yolk sac mesoderm and endoderm of E9.5 embryos were mechanically separated after incubating in trypsin/pancreatin as described by Roelen et al. (Roelen et al., 1994).

RNA extraction and real time PCR analysis

One half of each E9.5 yolk sac was cultured with TGFβ1 (1 ng/ml) for 3 hours, the other without as before, and were collected in 100 μl Ultraspec (Biotecx). RNA was extracted according to the manufacturer's protocol; 10 μg PolyI (Sigma) was added as a carrier. Samples were DNase I treated to eliminate genomic DNA and 1 μg RNA was reversed transcribed as described before (Roelen et al., 1994). RNA samples which had not been reverse transcribed served as negative controls for genomic DNA contamination and RNA isolated from the endoderm layer was used as an additional negative control.

Real-time PCR was performed in a MyiQ^{TM} single-color real time detection system (BioRad). The PCR primers were tested for both primer dimer formation and efficiency. To normalise for the amount of mRNA used as starting material, cDNA of β-actin was amplified. Routinely, a three-step program, followed by melt curve, was used. Data was collected and real-time analysis carried out during the extension period. Primers were as follows: β-actin forward primer, 5′-CCTGAACCCTAAGGCCAACCG-3′ and reverse 5′-GCTCATAG-CTCTTCTCCAGGG-3′, annealing temperature 60.2°C; smooth muscle actin forward primer, 5′-CAGAGCAAGAGAGGGATCCT-GA-3′ and reverse 5′-TAGATAGGCACGTTGTGAGTCACA-3′, annealing temperature 60.2°C; TGFβ forward primer, 5′-ATGGAG-CTGGTGAAACGGAA-3′ and reverse primer 5′-ACTGCTTCC-CGAATGTCTGA-3′, annealing temperature 61.2°C.

Results

Although the endoglin mutant mice were first described several years ago (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999), the molecular mechanism causing the HHT-like phenotype is still unclear. As this is essential for understanding vascular pathology in individuals with HHT, we have examined vasculogenesis/angiogenesis in the yolk sac of mutant mice in more detail.

Crucial to the present study was the unambiguous identity of ECs in the yolk sac. We therefore first used β-gal staining to identify *Eng*-positive cells and immunohistochemistry for PECAM1, Flk1 and Eng to show that ECs were present as an extremely thin layer surrounding the developing vessels. The EC-specific immunostaining was clearer in individual cells that had been sectioned through the nucleus, although they were in all sections easily distinguishable from the adjacent mesothelial cell layer, which completely lacked these markers (Fig. 1).

Smad activation is reduced in yolk sac of Eng knockout mice

In endothelial cells, TGFβ can bind to and transduce signals via ALK5 and ALK1 (Goumans et al., 2002; Oh et al., 2000).

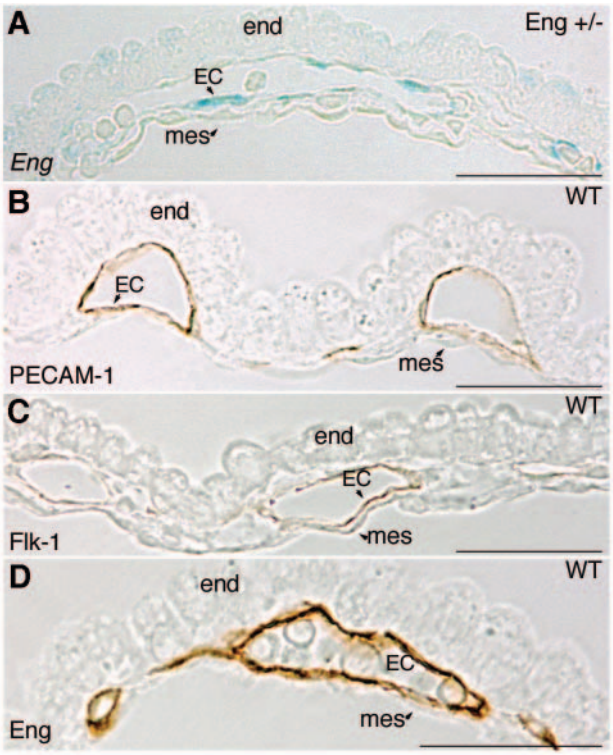

Fig. 1. Expression of endothelial cell markers in the yolk sacs. (A) *lacZ* staining for *endoglin*, in *Eng*$^{+/-}$ mice with *β-gal* knocked into the endoglin locus. (B-D) Immunohistochemistry for PECAM1 (B), Flk1 (C) and endoglin (D) in wild-type yolk sacs. Yolk sac was sectioned in plastic following staining as whole mounts in all cases. Scale bar: 0.5 mm. Abbreviations: EC, endothelial cell layer; end, endoderm; mes, mesothelial cell layer.

ALK5 is widely expressed and induces phosphorylation of Smad2 and Smad3, while ALK1, which is predominantly expressed in ECs, stimulates Smad1 and Smad5 phosphorylation. To establish how loss of endoglin affected TGFβ signalling, we first examined phosphorylation of these downstream targets of TGFβ using antibodies that specifically recognize the phosphorylated forms of Smads (Faure et al., 2000; Sousa Lopes et al., 2003). Whole-mount immunohistochemistry was used on yolk sacs isolated at E9.5 of development. Each yolk sac half was treated with TGFβ1 or BMP2 as indicated. PSmad1 expression was observed in endothelial and mesothelial layers of E9.5 yolk sacs from wild-type embryos (Fig. 2A,K). Addition of BMP2 led to increased Smad1 phosphorylation in both mesothelial and endothelial layers, while TGFβ increased Smad1 phosphorylation in ECs only (not shown). In yolk sacs from *Eng* mutants, phosphorylation of Smad1 was detected in mesothelial cells, as in wild type, but was not observed in ECs of untreated yolks sacs (Fig. 2B,K), although this was restored after BMP2 (Fig. 2C,K) and TGFβ1 (Fig. 2D,K) treatment. This was expected as mesothelial cells express BMP receptors but no TβRII/ALK1 complexes, while ECs express both TβRII/ALK1 and BMP receptor complexes (Valdimarsdottir et al., 2002).

PSmad2 expression was observed in the endothelial and mesothelial layers of E9.5 yolk sacs from wild-type embryos (Fig. 2E,K,L) and this was increased by treatment with TGFβ1

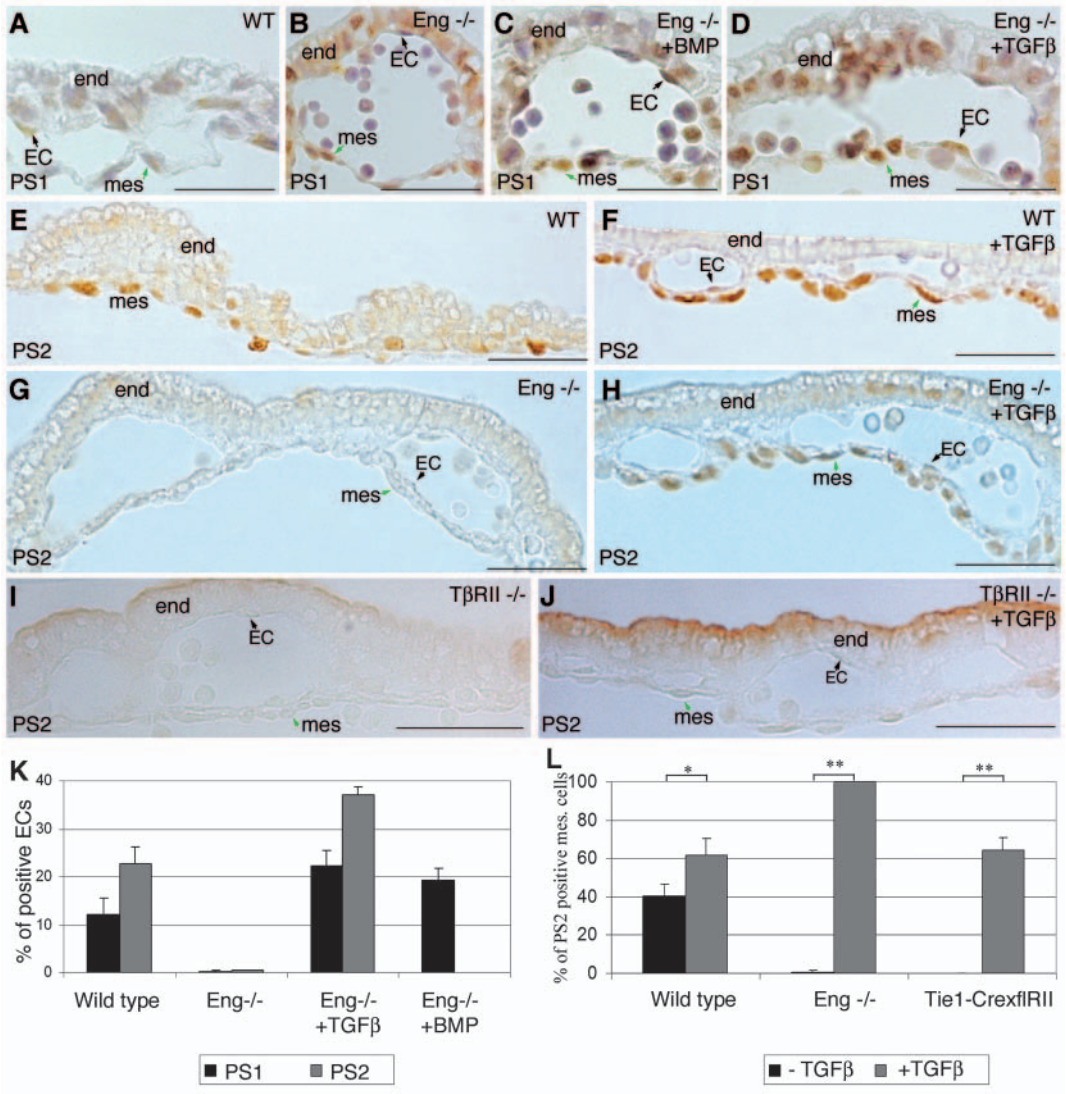

Fig. 2. TGFβ induces Smad2 phosphorylation in the yolk sacs of wild-type and *Eng* mutant embryos. PSmad1/5/8 (PS1) was detected by immunohistochemistry in both endothelial and mesothelial cells of wild-type yolk sacs (A). In *Eng* mutant yolk sacs, Smad1 was phosphorylated in mesothelial cells (green arrow) but not in endothelial cells (B); after a 1 hour treatment with BMP2 (C) or TGFβ1 (D) PSmad1 was detected in endothelial cells. PSmad2 (PS2) was detected by immunohistochemistry in the mesothelial layer of both untreated (E) and TGFβ1-treated (F) wild-type yolk sacs. In the *Eng* null yolk sac, PSmad2 was not detectable in the mesothelial layer (G) but phosphorylation of Smad2 was restored after TGFβ1 treatment (green arrow, H) for 1 hour. To confirm the specificity of PSmad2 antibody, *Tgfbr2* null yolk sac were analysed. PSmad2 was not present in either endothelial or mesothelial cell layers (I) and its phosphorylation was not restored after TGFβ1 treatment (J). (K) Percentage of endothelial cells expressing PSmad1/5/8 and PSmad2 in sections from wild-type and *Eng* mutant treated with BMP2 or treated with TGFβ1. Bars represent the mean±s.e.m. of five independent experiments. (L) Percentage of mesothelial cells expressing PSmad2 in sections from wild-type, *Eng* mutant and *tie-1*-Cre/*TβRII*$^{fl/fl}$ yolk sacs with or without 1 hour TGFβ1 treatment. Bars represent the mean±s.e.m. of cells from four independent experiments (*P<0.05; **P<0.01). Scale bar: 0.5 mm. Abbreviations: EC, endothelial cell layer; end, endoderm; mes, mesothelial cell layer.

for 1 hour (Fig. 2F,K,L). This demonstrated the presence of active TβRII and ALK5 receptor complexes in ECs and mesothelial cells as they would be required for TGFβ-induced phosphorylation of Smad2. However, in *Eng* knockout mice, neither the endothelial cells nor the mesothelial cells of the yolk sac showed phosphorylation of Smad2 (Fig. 2G,K,L), but treatment with high concentrations of TGFβ1 (1 ng/ml) resulted in Smad2 phosphorylation in both ECs and mesothelial cells (Fig. 2H,K,L).

To confirm the specificity of PSmad2 staining, we examined yolk sacs from *Tgfbr2* mutant embryos resulting from intercrosses of *PGK*-Cre transgenics with floxed *Tgfbr2* mice (Lallemand et al., 1998; Leveen et al., 2002). As expected, PSmad2 staining was absent in both endothelial and mesothelial layer of the yolk sac (Fig. 2I) and could not be restored by incubation with TGFβ1 (Fig. 2J).

This demonstrated that TβRII/ALK5 receptor complexes on the surface of both cell types in the *Eng* mutants can be activated by exogenous TGFβ1 in short-term culture. The mesothelial cell layer, which does not express *Eng* (Fig. 1A,D),

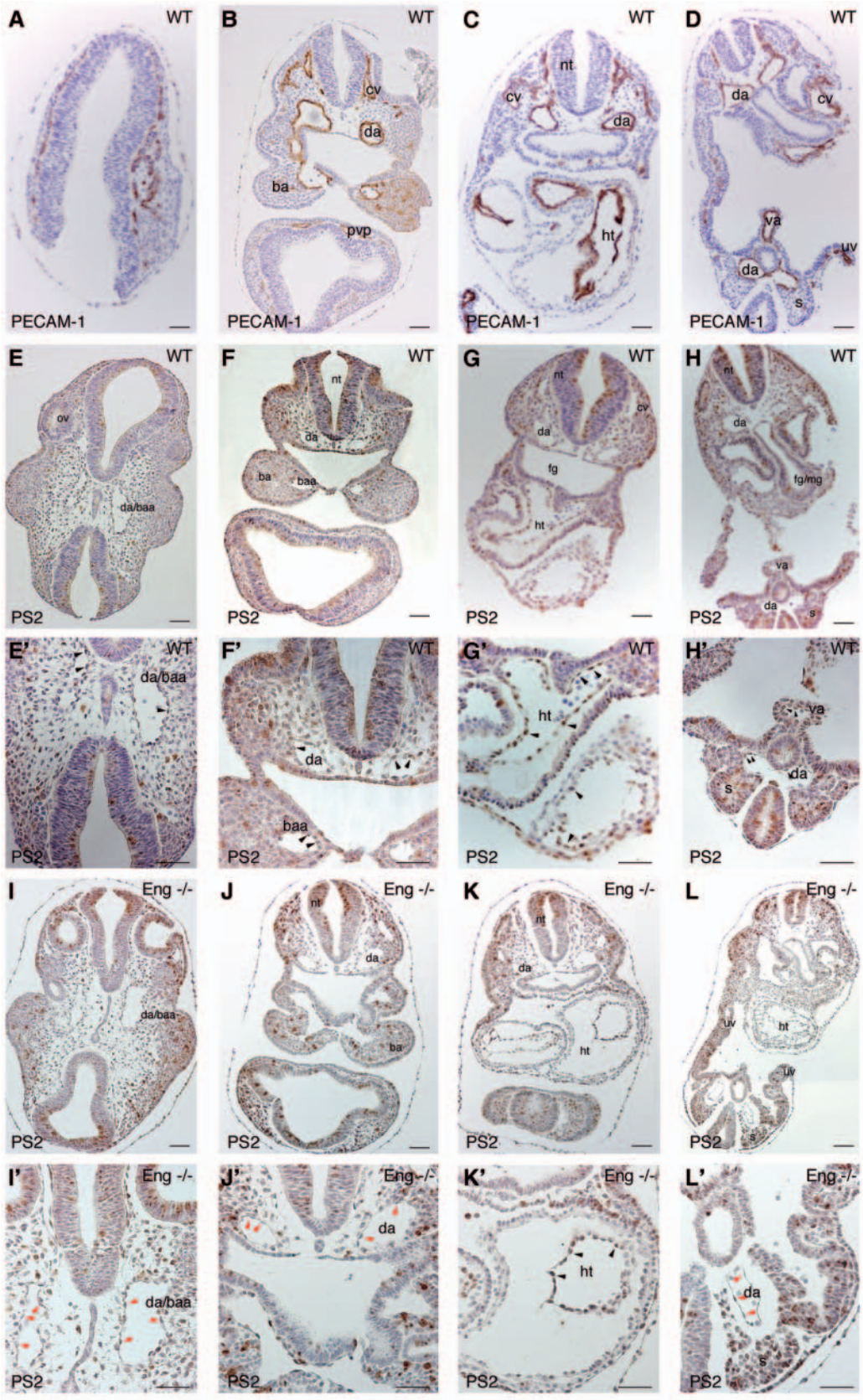

Fig. 3. Smad2 phosphorylation is absent in endothelial cells of *Eng* mutant embryos. Transverse sections of E9.5 embryos stained with PECAM1 antibody showing endothelial cells in the brain (A), dorsal aorta and cardinal vein (B), heart (C), and vitelline artery and umbilical vein (D). Transverse sections of E9.5 wild-type embryos stained with PSmad2 (PS2) antibody showing positive endothelial cells in the brain (E,E′), dorsal aorta and branchial arch artery (F,F′), endothelium of the heart (G,G′), dorsal aorta and vitelline artery (H,H′). In the endoglin null embryos, PSmad2 was not detectable in endothelial cells of arteries and veins (I,J,I′,J′) but was observed in the endothelium of the heart (K,K′). (L,L′) Scale bars: 0.5 mm. Red arrowheads in I′,J′,L′ indicate negative ECs. Black arrowheads in K′ indicate PS2-positive ECs. Abbreviations: bba, branchial arch artery; cv, cardinal vein; da, dorsal aorta; da/baa, communication between da and baa; fg, foregut, ht, heart; nt, neural tube; pvp, perioptic vascular plexus; s, somite; uv, umbilical vein; va, vitelline artery.

showed reduced PSmad2 levels when loss of endoglin disrupted TGFβ signalling in the endothelial layer. We also observed downregulation of ALK5 expression in ECs from $Eng^{+/-}$ embryos (Lebrin et al., 2004). This genetic adaptation

might occur in order for ECs to survive the potentiation of ALK5-induced growth arrest (Goumans et al., 2003).

Smad2 activation is absent in endothelial cells of Eng knockout embryos

To investigate whether loss of PSmad2 in *Eng* knockout mice was restricted to the yolk sac vasculature or was a more general feature of the *Eng* mutant mice, we also examined the embryo proper. Previously, we have shown PSmad2 scattered throughout the embryonic mesenchyme, somites, body wall, surface ectoderm, blood vessels, blood, fore- and hindgut, endo- and myocardium of the heart at E8.5 but by E10.5 most endothelial cells of arteries veins and capillaries had become PSmad2 negative (Sousa Lopes et al., 2003).

We again used PECAM1 staining in parallel with PSmad2 staining to distinguish embryonic ECs unequivocally from adjacent cells (Fig. 3A-D). As expected, at E9.5 PSmad2 was observed in endothelial cells of both arteries and veins of wild-type embryos at this stage. However, activated Smad2 was no longer detected in any $Eng^{-/-}$ ECs throughout the embryonic blood vasculature (Fig. 3). These results are in agreement with the observation in the extra-embryonic yolk sac (Fig. 2D,F). Interestingly, Smad2 was still phosphorylated in the endocardium of the heart of endoglin mutant embryos (Fig. 3K,K′), probably via ALK4 or ALK7, although the expression of these two receptors has not been documented at these stages.

Smooth muscle cell marker expression is reduced in Eng mutant yolk sacs but extracellular matrix proteins are normal

The extracellular matrix is a major constituent of blood vessels and provides a framework in which the various cell types are attached and embedded. TGFβ is a key regulator of ECM assembly and remodeling (Verrecchia and Mauviel, 2002). We therefore investigated whether ECs behaviour was affected through altered expression of ECM proteins, such as fibronectin and collagen that regulate adhesion and are both known TGFβ target genes. In both wild-type and $Eng^{-/-}$ embryos, fibronectin and collagen were localized between the cell layers of the yolk sac and neither their production nor deposition appeared affected by lack of Eng (Fig. 4).

Angiogenesis involves differential growth and sprouting of endothelial tubes and recruitment and differentiation of mesenchymal cells into VSMC and pericytes. In order to determine whether the mesothelial layer differentiates normally to smooth muscle cells in $Eng^{-/-}$ embryos, we examined the expression of α-smooth muscle actin (α-sma) and caldesmon in the yolk sac. Caldesmon is a smooth muscle cell protein that binds actin, tropomyosin, Ca(2+)-calmodulin, myosin and phospholipids (Huber, 1997). Immunohistochemical analysis revealed strikingly lower levels of both caldesmon and α-sma staining in the mesothelial cells in the *Eng* mutants (Fig. 5B,D) compared with wild-type embryos (Fig. 5A,C), suggesting that their differentiation was indeed impaired. Quantification of α-sma mRNA expression by real time RT-PCR confirmed reduced levels of α-sma expression in yolk sacs from the mutant embryos (Fig. 5H). More importantly, we were able to restore in part α-sma protein and mRNA in the mutants by exposure of the yolk sac for 3 hours to TGFβ1 (Fig. 5D,E,H). To confirm that TGFβ1 is indeed responsible for this rescue, yolk sacs were incubated for

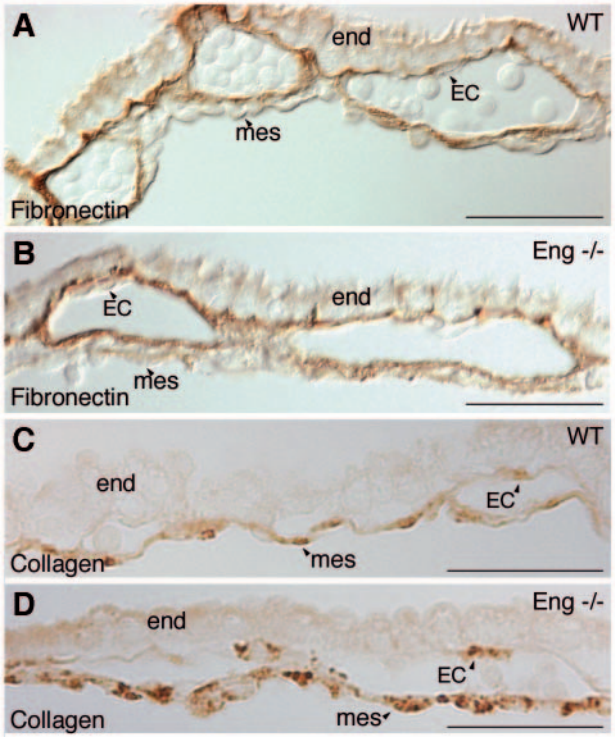

Fig. 4. Extracellular matrix components in wild-type and *Eng* mutant yolk sac. Immunostaining for fibronectin and collagen in sectioned whole mounts of yolk sacs. In wild-type (A) and *Eng* mutant (B) yolk sacs, fibronectin was observed between the layers of the yolk sac. No striking difference was observed in collagen expression in the wild-type (C) and the *Eng* mutant yolk sacs (D). Scale bar: 0.5 mm. Abbreviations: EC, endothelial cell layer; end, endoderm; mes, mesothelial cell layer.

8 hours with a higher concentration of TGFβ1 (5 ng/ml). After treatment, we observed a significant increase in α-sma expression in the knockout yolk sac compared with the untreated yolk sac (Fig. 5F,G), demonstrating that indeed the lack of endoglin affects smooth muscle cells differentiation possibly by decreased levels of TGFβ1.

The apparent staining of the endoderm cell layer of the yolk sac is nonspecific and observed with several (non relevant) primary antibodies (Goumans et al., 1999). Nevertheless, we confirmed this was also the case for the α-sma antibody by demonstrating the lack of α-sma mRNA expression by real time RT-PCR in isolated endoderm (Fig. 5H).

TGFβ expression is reduced in yolk sac of Eng knockout mice

One explanation for these findings might be that disruption of TGFβ signalling in endothelial cells by loss of Eng resulted in decreased levels of TGFβ1 in the yolk sac and, consequently, insufficient ligand to activate paracrine TGFβ signalling to neighbouring mesothelial cells. Whole-mount in situ hybridization and immunohistochemistry for TGFβ1 mRNA and protein were thus carried out on yolk sacs from wild-type and *Eng* mutant embryos. In situ hybridization showed that *Tgfb1* mRNA is not detected in the mesothelial layer but it is expressed strongly by endothelial cells in the yolk sac. Its expression appeared unaltered in *Eng* knockout yolk sacs (Fig.

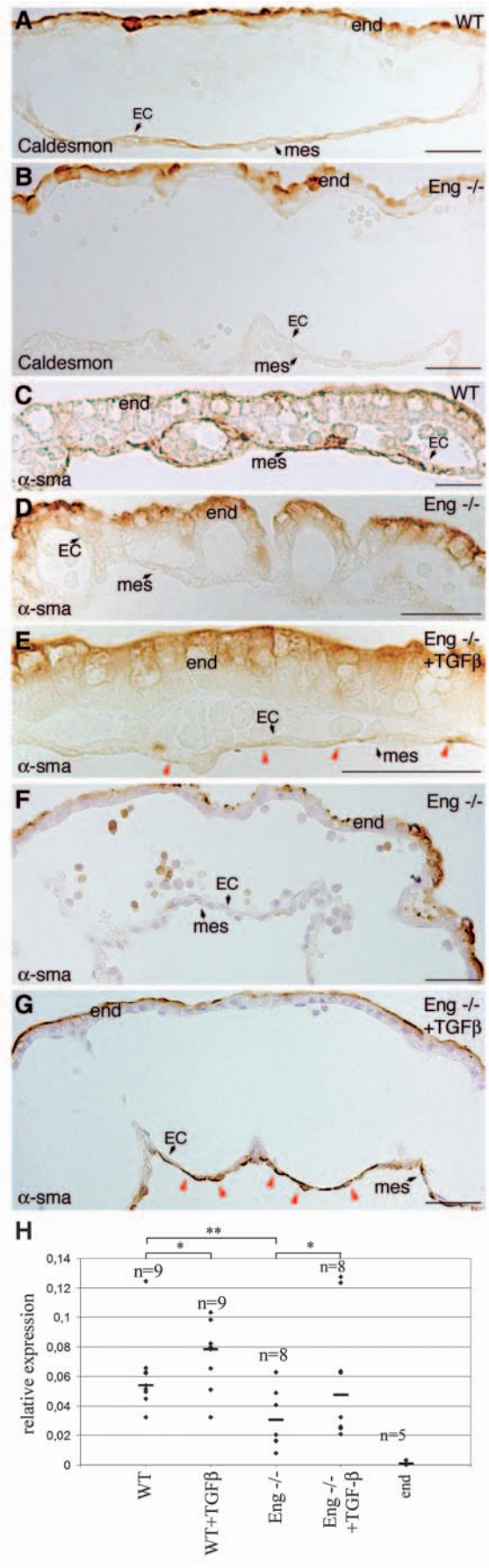

Fig. 5. Effect of TGFβ1 in smooth muscle actin expression in *Eng* mutant yolk sacs. Caldesmon and α-smooth muscle actin was detected by immunohistochemistry in sectioned yolk sacs, stained as whole mounts. (A) Caldesmon was expressed in mesothelial cells of wild-type yolk sacs but was not detected in *Eng* mutant yolk sacs (B). (C) In wild-type embryos, α-sma was observed in cells surrounding the vessels while in (D) *Eng* knockout yolk sac its expression was absent. (E) α-sma expression was partially restored after 3 hours of TGFβ treatment (red arrowheads). In *Eng* mutant yolk sacs α-sma was absent (F) while after treatment with high concentrations of TGFβ1 (5 ng/ml) for 8 hours its expression was clearly restored (G, red arrowheads). (H) Real-time RT-PCR analysis of α-sma RNA expression in wild-type and *Eng* mutant yolk sacs. Expression of *α-sma* mRNA was compared with β-actin (as loading control) in wild-type and *Eng* mutant yolk sac. E9.5 yolk sac endoderm was used as a negative control. The number of yolk sacs analysed is shown (n). Contamination with genomic DNA was not evident in samples without RT (not shown). The Wilcoxon Test was used for statistical analysis of significance; medians are shown as black horizontal bar (**P*<0.1; ***P*<0.05). Scale bar: 0.5 mm. Abbreviations: EC, endothelial cell layer; end, endoderm; mes, mesothelial cell layer.

6A,B); this was confirmed by real time RT-PCR (Fig. 6G). However, using an antibody specific for TGFβ1 we observed that TGFβ1 protein levels were significantly reduced in the mesothelium of yolks sac lacking endoglin (Fig. 6E,F) compared with wild-type yolk sacs (Fig. 6C,D). This was confirmed by western blotting (Fig. 6H). It is therefore most likely that loss of Eng reduces availability of TGFβ1 protein to the mesothelial cells and as a consequence these cells are no longer able to differentiate fully into smooth muscle cells.

In order to determine whether this disruption of TGFβ signalling in the ECs and its subsequent effect on TGFβ availability is specific for Eng or reflects a more general function of the TGFβ pathway in ECs, we used transgenic mouse embryos in which TβRII or ALK5 were specifically deleted in endothelial cells. This was achieved by intercrossing the *tie-1*-Cre transgenic mice with floxed *Tgfbr2* and *Alk5* mice, respectively (Gustafsson et al., 2001; Larsson et al., 2001; Leveen et al., 2002). Whole-mount β-gal staining of E9.5 yolk sacs confirmed the efficiency and specificity of the transgenic Cre. Interestingly, we found that endothelium-specific knockout are like *Tgfbr2* or *Alk5* null embryos, as they are also embryonic lethal at mid gestation and developed similar defects in the yolk sac (M-.J.G., R.L.C.C., Franck Lebrin, Philippe Bartolino, Peggy T. T. Sin, P.t.D., H.M.A. and C.L.M., unpublished), suggesting that the phenotype resulting from complete deletion of these genes is largely or completely attributed to the lack of TGFβ signalling in ECs.

Whole-mount immunohistochemistry for PSmad2 was carried out on half yolk sacs collected at E9.5 from these embryos treated or not with TGFβ1 for 1 hour, as for the yolk sacs from the *Eng* mutants. Phosphorylated Smad2 was again absent in the endothelial and mesothelial cells of conditional knockouts lacking either TβRII or ALK5 in ECs of the yolk sacs (Fig. 7B,C), although in wild-type yolk sacs Smad2 is clearly phosporylated (Fig. 7A,D). However, after treatment with TGFβ the nucleus of mesothelial cells of both mutants became positive for PSmad2 (Fig. 7E,F; Fig. 2L) again demonstrating the activity of intact TβRII/ALK5 complexes in the mesothelial cells even when either TβRII or ALK5 had

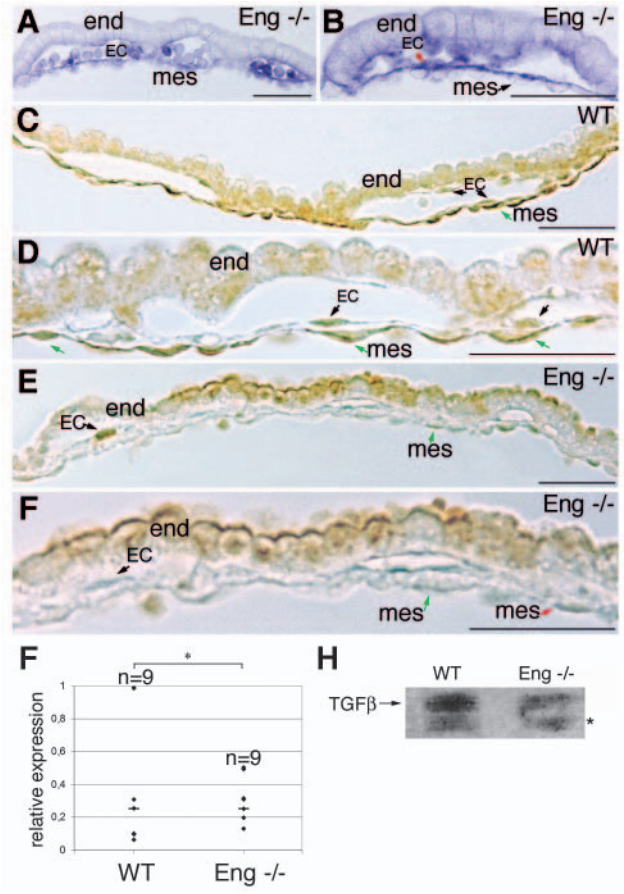

Fig. 6. TGFβ1 mRNA and protein expression in wild-type and *Eng* mutant yolk sacs. Expression of *Tgfb1* mRNA was examined by whole-mount in situ hybridization in wild type (not shown) and (A) *Eng* mutant yolk sacs, and detected specifically in endothelial cells. (B) Higher magnification of A, showing *Tgfb1*-positive ECs (red arrow). TGFβ1 protein was detected by immunohistochemistry following whole-mount staining and sectioning of yolk sacs from (C) wild-type and (D) *Eng* mutant embryos. In the wild-type yolk sac, TGFβ1 was present in the endoderm as well as in both endothelial and mesothelial cell layers (C,D). (E,F) Higher magnification of the yolk sac layers. Positive endothelial cells are indicated by black arrows and positive mesothelial cells are indicated by green arrows. (E) In the *Eng* null yolk sac, TGFβ1 was present in the endoderm but was almost absent in the endothelial and mesothelial cell layers. A TGFβ1-positive endothelial cell is indicated by black arrow and a TGFβ1-positive mesothelial cell is indicated by a green arrow. (F) Higher magnification of the yolk sac. TGFβ1-negative endothelial cell is indicated by black arrow, TGFβ1-negative mesothelial cell is indicated by green arrow and TGFβ1-positive mesothelial cell is indicated by red arrow. (G) Real-time RT-PCR analysis of *Tgfb1* mRNA expression in wild-type and *Eng* mutant yolk sacs. Expression of *Tgfb1* mRNA compared with β-actin (loading control) in wild-type and *Eng* mutant yolk sacs. The number of yolk sacs analysed is shown (n). Samples were corrected for loading difference using β-actin primers. Contamination with genomic DNA was not evident in samples without RT (not shown). The Wilcoxon Test was used for statistical analysis of significance; medians are shown as a black stripe (*P>0.5). (H) The decreased expression of TGFβ1 in $Eng^{-/-}$ yolk sacs was confirmed by Western blot analysis. *Unspecific band. Scale bars: 0.5 mm. Abbreviations: EC, endothelial cell layer; end, endoderm; mes, mesothelial cell layer.

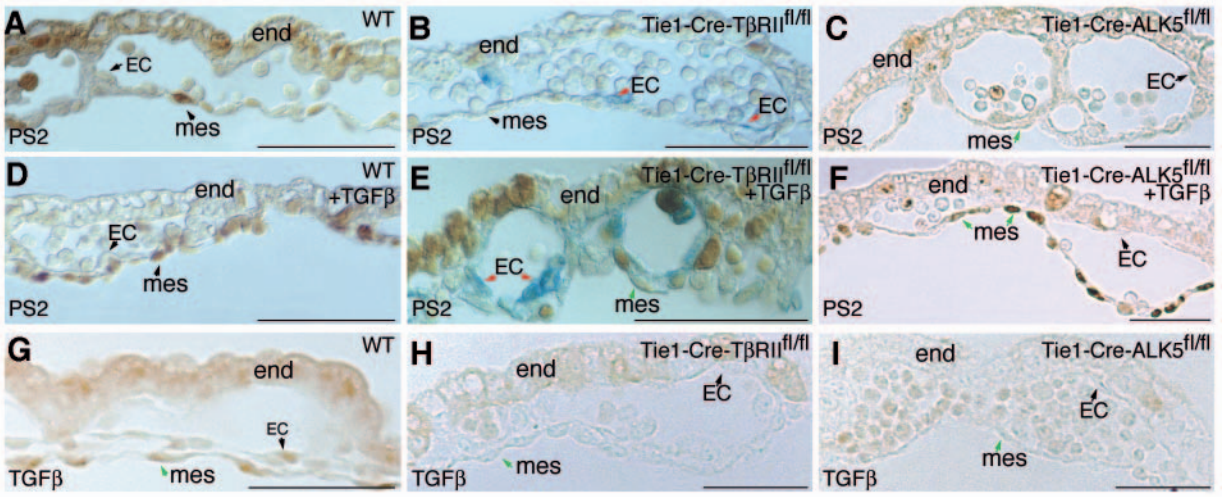

Fig. 7. Smad2 phosphorylation and TGFβ1 protein expression in *tie-1*-Cre/*TβRII*$^{fl/fl}$ and *tie-1*-Cre/*ALK5*$^{fl/fl}$ double transgenic mutant yolk sacs. PSmad2 was analysed by immunohistochemistry in sectioned whole mounts of both untreated (A-C) and TGFβ1-treated (D-F) yolk sacs. Phosphorylation of Smad2 was observed in wild-type yolk sacs (A,D). In the yolk sacs of *tie-1*-Cre/*TβRII*$^{fl/fl}$ (B) and *tie-1*-Cre/*ALK5*$^{fl/fl}$ (C) mice, PSmad2 was not present in the mesothelial cell layer (green arrow) but phosphorylation of Smad2 was restored after TGFβ1 treatment (E,F, green arrows) for 1 hour. To determine the expression of functional Cre in vivo, *lacZ* expression was analysed in double transgenic yolk sacs. β-Gal staining was observed in the endothelial cells of *tie-1*-Cre/*TβRII*$^{fl/fl}$ (B and E, red arrows) and *tie-1*-Cre/*ALK5*$^{fl/fl}$ (not shown). TGFβ1 protein expression was analysed by immunohistochemistry on whole-mount staining of yolk sacs from (G) wild-type, (H) *tie-1*-Cre/*TβRII*$^{fl/fl}$ mutant embryos and (I) *tie-1*-Cre/*ALK5*$^{fl/fl}$ mutant embryos. In wild-type yolk sacs, normal expression of TGFβ1 was observed in the endothelial (black arrow) and mesothelial (green arrow) cell layers, while in both *tie-1*-Cre/*TβRII*$^{fl/fl}$ and *tie-1*-Cre/*ALK5*$^{fl/fl}$ mutant yolk sacs, TGFβ1 was no longer detected in the mesothelial cell layer (green arrows). Scale bars: 0.5 mm. Abbreviations: EC, endothelial cell layer; end, endoderm; mes, mesothelial cell layer.

been deleted in the adjacent ECs. These results demonstrated that the mesothelial layer indeed interacts closely with the endothelial layer and more importantly demonstrated that disabling any one of several receptors affecting TGFβ signalling in ECs has a knock-on effect on TGFβ responses in the adjacent mesothelium.

In order to determine whether this was again the result of reduced TGFβ availability, we examined TGFβ1 protein levels in both *tie-1*-Cre-*TβRII* and *tie-1*-Cre-*ALK5* double transgenic knockout embryos. As in the *Eng* mutant mice, TGFβ was undetectable in most mesothelial cells of yolk sacs lacking either TβRII (Fig. 7H) or ALK5 in the ECs (Fig. 7I) and was clearly present wild-type yolk sac (Fig. 7G). The results confirmed that an indirect effect in the mesothelium is a common feature of disruption of TGFβ signalling in ECs by one of several means and is not specific for endoglin.

As mRNA levels of TGFβ1 are not affected in the *Eng* knockout mice yet TGFβ1 protein (probably taken up in an active form) is no longer detected in mesothelial cells, TGFβ production is most likely to have been affected at the post-transcriptional level. Either the amounts of latent protein are reduced or activation of the latent TGFβ is altered. We were, however, unable to determine whether this was the case directly as TGFβ levels in protein extracts or short-term conditioned medium from yolk sacs were below the detection limit of available bioassay or ELISAs.

Discussion

The earliest vascular structures in the mouse embryo are the blood islands that arise in the mesodermal layer of the yolk sac around E8.0. At E9.5, blood islands are surrounded by endothelial cells and supported by smooth muscle cells. Normal embryonic development is crucially dependent on the proper functioning of the yolk sac. Mice lacking functional *Tgfb1, Alk5* or *Tgfbr2* genes show defects in yolk sac vasculogenesis and hematopoiesis and die around day 10 of gestation (Dickson et al., 1995; Oshima et al., 1996). The endothelial expression of endoglin is essential for angiogenesis and heart development, as well as normal development of vascular smooth muscle (Arthur et al., 2000). It has been shown that the extra-embryonic mesodermal layer expresses the receptors ALK5, TβRII and ALK1, whereas none of them was detected in the visceral endoderm (Goumans et al., 1999).

In the present study we analysed yolk sacs from endoglin deficient mice and from mice lacking TβRII or ALK5 specifically in endothelial cells. The data showed that loss of an intact TGFβ signalling pathway in endothelial cells has a strong impact on Smad2 phosphorylation in the adjacent mesothelial layer. Rescue by TGFβ1 suggested a TGFβ-mediated interaction between the endothelial layer and the mesothelial layer of the yolk sac.

During blood vessel assembly, endothelial cells recruit mesenchymal progenitors and induce their differentiation into smooth muscle cells or pericytes. In the *Eng* mutant, yolk sac expression of α-smooth muscle actin and caldesmon are clearly affected, indicating that the differentiation of mesothelial cells into smooth muscle cells is impaired, as has also been also shown by Li et al. (Li et al., 1999). Importantly, however, we found that reduced α-sma expression in mesothelial cells could be rescued by treatment with TGFβ1. Microarray analysis in ECs, infected with constitutively active (ca)ALK5 showed that caldesmon was one of the genes upregulated by the TGFβ signalling pathway (Ota et al., 2002). A number of other transcription factors are involved in VSMC development. Mice lacking *Fli1* (Hart et al., 2000), *Mef2c* (Lin et al., 1998), *Smad5* (Yang et al., 1999), *Hand2* (Yamagishi et al., 2000), *Hand1* (Morikawa and Cserjesi, 2004) and *Cx43* (Hirschi et al., 2003) also show reduced smooth muscle development around the vessel. It is not known whether TGFβ processing or availability is affected in these mutants although in *Cx43* mutant mice, it was shown that the mechanism by which gap junctions mediate endothelial-induced mural differentiation involves the activation of TGFβ (Hirschi et al., 2003). Nevertheless, disruption of TGFβ signalling maybe a common feature of some mice mutants with defective yolk sac vasculogenesis.

The low levels of α-sma in endoglin knockout, *tie-1*-Cre-*TβRII* and *tie-1*-Cre-*ALK5* embryos suggests VSMC differentiation is defective in mice with disrupted TGFβ signalling in ECs. Several studies have demonstrated that TGFβ is activated upon endothelial-induced mesenchymal cell differentiation and that it upregulates α-smooth muscle actin expression (Assoian and Sporn, 1986; Majack, 1987; Merwin et al., 1991; Morisaki et al., 1991). Chen and Lechleider also showed that activation of Smad pathway is necessary for smooth muscle cell differentiation from neural crest cells (Chen and Lechleider, 2004). We examined both TGFβ1 mRNA and protein expression and found the TGFβ1 protein levels are indeed affected, demonstrating that ECs are the crucial source of TGFβ in VSMC recruitment to blood vessels. Furthermore, the importance of endothelium-derived TGFβ in VSMC recruitment suggested that TGFβ acts mainly through a paracrine route. Bourdeau et al. (Bourdeau et al., 2001) have shown that the 129/Ola mouse strain has lower levels of plasma TGFβ1 compared with C57Bl/6 and is more susceptible to HHT. Plasma TGFβ was further reduced in *Eng* heterozygous mice and as ECs are the major source of circulating TGFβ, this reduction would be compatible with our present findings. However, these results have not been reproduced by others and determination of levels of circulating TGFβ has been notoriously difficult and subject to artefacts. Whether TGFβ receptor knockout mice exhibit lower levels of TGFβ1 remains to be determined.

Autocrine regulation of TGFβ1 expression has been described previously in a number of different normal and transformed cells (Van Obberghen-Schilling et al., 1988). This autoregulation might explain why affecting TGFβ signalling pathway also affects TGFβ1 protein. The lack of endoglin, TβRII or ALK5 in ECs could thus lead to reduced activation of TGFβ1 in all cases via an autocrine mechanism.

The composition and organization of vascular extracellular matrix is also responsible for the mechanical properties of the vessel wall, forming complex networks of structural proteins. Fibronectin and collagen are major components of the ECM and promote cell adhesion and spreading, cell migration and cytoskeletal organization (Hynes, 1991). It has been shown that TGFβ upregulates expression of ECM components, such as fibronectin and collagen, via both Smad-dependent (Verrecchia et al., 2001) and Smad-independent pathways (Hocevar et al., 1999; Runyan et al., 2004). Reduced levels of TGFβ protein may affect fibronectin deposition between the endoderm and

mesoderm layers of the yolk sac as has already been described in chimaeric mice generated from ES cells expressing dnTβRII (Goumans et al., 1999) and in more general terms affect ECM deposition. However, in the yolk sac of *Eng* mutant mice, we were unable to detect any differences in fibronectin or collagen expression, as has also been reported for the embryo proper (Arthur et al., 2000).

Our studies indicate a general mechanism through which defects in components of TGFβ signalling pathway in ECs affect yolk sac vasculogenesis and would explain why the phenotypes of many of these mutant mice are so similar and also similar to the *Tgfb1* mutant mice itself. All may be mediated by altered TGFβ protein levels. Deletion/mutation of other genes that give rise to a vascular phenotype in the yolk sac (Carvalho et al., 2002) may act up or downstream of TGFβ signalling. One example includes tissue factor (*Tf*; *F3* – Mouse Genome Informatics) gene, a procoagulant receptor, also involved in vascular integrity by affecting the maturation of the muscular wall around endothelial cells (Carmeliet and Collen, 1998) and stimulated by TGFβ1 (Ranganathan et al., 1991). Another is latent TGFβ-binding protein 1, which seems to play an important role in the targeting and release of TGFβ1 in response to arterial injury (Sinha et al., 2002).

Compared with vascular endothelial cells in normal tissues, a stronger staining for endoglin was detected in vascular endothelial cells in tissues undergoing active angiogenesis, such as in regenerating and inflamed tissues or tumours (Bodey et al., 1998a; Bodey et al., 1998b; Miller et al., 1999; Seon et al., 1997; Wang et al., 1994). Increased endoglin expression was also observed in ECs of microvessels from pathological skin lesions and in established atherosclerotic lesions (Burrows et al., 1995).

In individuals with HHT, maximum achievable levels of endoglin expression would be significantly lower than in normal subjects. Should similar mechanisms operate as described here for the *endoglin* mutant mice, then the ECs in individuals with HHT may make less TGFβ protein available than their normal counterparts. As a consequence, less TGFβ1 may be present in the circulation as ECs are the major source of circulating TGFβ1, but more importantly, less TGFβ1 may be available locally to induce differentiation of pericytes or smooth muscle cells and recruit them to the neovasculature leaving the vessels weak and susceptible to further damage and haemorrhage, typical of individuals with HHT.

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