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Review

Controlling angiogenesis by two unique TGF-β type I receptor signaling pathways

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Summary. Genetic studies in mice and humans have revealed a pivotal function for transforming growth factor-beta (TGF-β) in vascular development and maintenance of vascular homeostasis. Mice deficient for various TGF-β signaling components develop an embryonic lethality due to vascular defects. In patients, mutations in TGF-β receptors have been linked to vascular dysplasia like Hereditary Hemorrhagic Telangiectasia (HHT) and pulmonary arterial hypertension (PAH). Besides indirect effects by regulating the expression of angiogenic regulators, TGF-β also has potent direct effects on endothelial cell growth and migration, and we have proposed that TGF-β regulates the activation state of the endothelium via two opposing type I receptor/Smad pathways, activin receptor-like kinase (ALK)1 and ALK5. TGF-β is also critical for the differentiation of mural precursors into pericytes and smooth muscle cells. Furthermore, defective paracrine TGF-β signaling between endothelial and neighboring mural cells may be responsible for a leaky vessel phenotype that is characteristic of HHT. In this review, we discuss our current understanding of the TGF-β signaling pathway and its regulation of endothelial and vascular smooth muscle cell function.

Key words: Angiogenesis, BMP, Hereditary hemorrhagic telangiectasia, Preeclampsia, Pulmonary arterial hypertension

Introduction

Angiogenesis is a complex, highly regulated process and involves multiple players. *De novo* blood vessels are formed in early development, during a process called vasculogenesis. A mass of proliferating mesodermal cells forms an inner core of hematopoietic precursor cells and an outer layer of vascular cells, termed blood island. These blood island migrate, fuse and organize into a primary capillary plexus (Carmeliet, 2005). Branching, growth and remodeling of preexisting vessels is called angiogenesis. Angiogenesis occurs in embryonic and postnatal development, and can be reactivated during adulthood as well. All new capillaries begin as thin-walled endothelial-lined structures. During the angiogenic progression, some remain as capillaries covered by pericytes, and others develop into large vessels supported by a smooth muscle cell layer to form a stable vessel wall. Angiogenesis is tightly regulated, and unbalanced angiogenesis contributes to multiple pathological conditions, such as tumor growth, proliferative retinopathies and age related muscular degeneration (Carmeliet, 2005).

Transforming growth factor-beta (TGF-β) is the prototypic member of a family, which also includes bone morphogenetic proteins (BMPs) and activins. All 33 family members in man are multifunctional secreted regulators of cell behavior. Depending on the cell type and extracellular surrounding they mediate different, and sometimes even opposite effects, on the same cells. TGF-β family members signal via transmembrane type I and type II serine/threonine kinase receptors and intracellular Smad transcriptional effector proteins (Fig. 1) (Heldin et al., 1997). The essential role of TGF-β in regulation of angiogenesis first became apparent by genetic studies in mice (Goumans and Mummery, 2000; Goumans et al., 2009). Inactivation of several components of the TGF-β signaling pathway results in embryonic lethality due to abnormal yolk sac vasculogenesis and/or angiogenesis in the embryo proper. In addition, TGF-β is essential for vascular homeostasis in the adult (ten Dijke and Arthur, 2007), as well as for the maintenance of anti-inflammatory characteristics of endothelial cells. Deregulation of TGF-β signaling is linked to multiple vascular pathologies, such as preeclampsia (Venkatesha et al., 2006), pulmonary arterial hypertension (PAH) (Davies and

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Morrell, 2008; Machado et al., 2009), hereditary hemorrhagic telangiectasia (HHT) (Govani and Shovlin, 2009), and tumor angiogenesis (Pardali and ten Dijke, 2009). TGF-β1 inhibits the production of pro-inflammatory regulators by endothelial cells, such as E-Selectin and IL-8 (Gamble et al., 1993; Smith et al., 1996). In addition, systemic inhibition of TGF-β-related ligands with sEng results in an increase of leukocyte adhesion to the endothelium, increased leukocyte rolling and increase leukocyte infiltration into tissues (Walshe et al., 2009).

In this review we will highlight recent findings on the role of TGF-β family members, TGF-β type I receptors, i.e. activin receptor-like kinase (ALK)1 and ALK5, and endoglin in the vasculature and vascular associated diseases.

TGF-β signaling in endothelial cells

The TGF-β incorporates a large superfamily of ligands and receptors (for review see (ten Dijke and Hill, 2004; Moustakas and Heldin, 2009; Wu and Hill, 2009)). The TGF-β ligands comprise more than 33 different family members, such as TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins and nodal. TGF-β receptors are heterodimers and possess Ser/Thr kinase activity (Fig. 1). Type I receptors can be functionally divided based on their ability to activate Smads; ALK4/5/7 activate Smad2/3 and ALK1/2/3/6 induce phosphorylation of Smad1/5. Type II receptors, including TGF-β type II receptor (TβRII), BMP type II receptor (BMPRII) and activin type II receptor (ActRII), posses constitutive kinase activity, which allows them to phosphorylate and activate type I receptors upon ligand binding and transmit signals to the nucleus via phosphorylation and activation of Smad transcription factors. In addition, the TGF-β co-receptors (type III receptors), endoglin and betaglycan, can regulate ligand-receptor interactions.

Multiple studies demonstrated the opposite function of TGF-β in endothelial cells. The effect of TGF-β on endothelial cells is concentration-dependent and can be enhanced or inhibited in the presence of other regulators (Pepper, 1997). At low concentration TGF-β possesses pro-angiogenic activities, while at higher concentration it inhibits angiogenesis. In addition, the biological outcome from TGF-β signaling is dependent on the presence or absence of other cell specific mediators. For

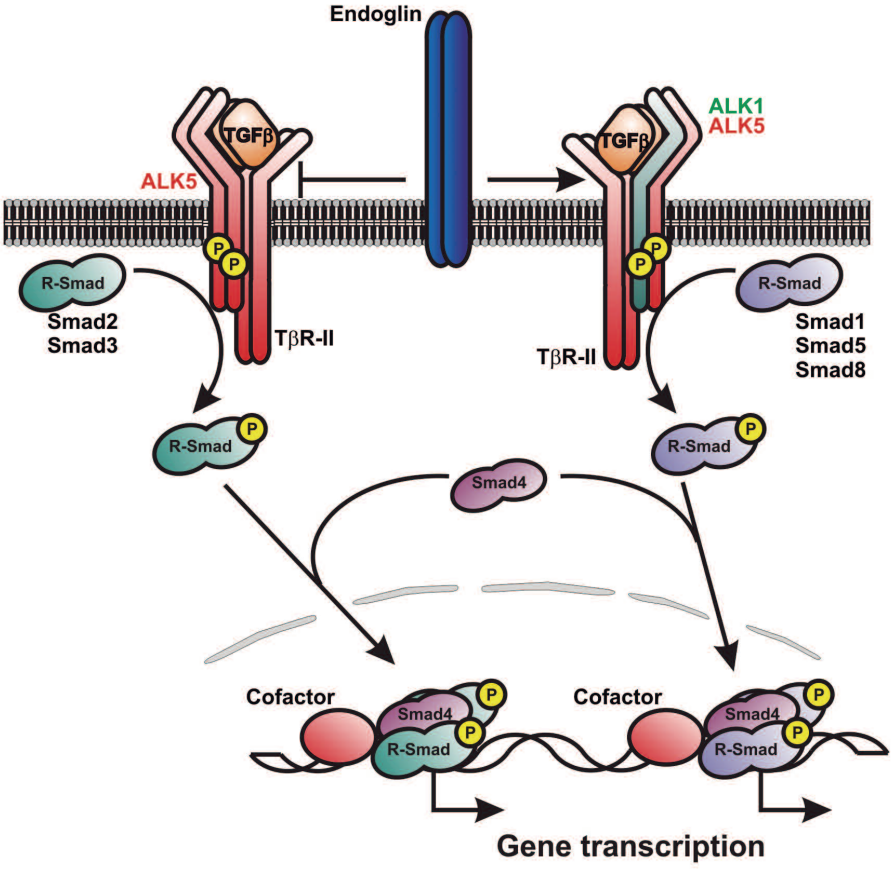

Fig. 1. TGF-β signals via distinct TGF-β receptor complexes. TGF-β binds to heteromeric complex consisting of TβRII/ALK5 and TβRII/ALK1/ALK5 in endothelial cells. Actvation of ALK5 will induce Smad2/3 phosphorylation and ALK1 activation will mediate Smad1/5/8 phosphorylation. Activated R-Smads form heteromeric complexes with Smad4, which accumulate in the nucleus, where these complexes, together with other co-factors, participate in gene regulation. Endoglin is a co-receptor that negatively regulates TGF-β/ALK5 signaling, and positively regulates TGF-β /ALK1 signaling. Not depicted here is that ALK1 together with BMPRII and ActRII can bind BMP9.

example, a recent study provided evidence regarding the importance of VE-Cadherin in the anti-angiogenic function of TGF-β (Rudini et al., 2008). VE-Cadherin deficient endothelial cells displayed a loss of TGF-β mediated inhibitory effects on cell proliferation and migration. Moreover, inhibition of homotypic VE-Cadherin interaction with blocking antibodies (BV9) resulted in less TGF-β/ALK5 induced Smad3 phosphorylation.

This biphasic activity of TGF-β can also be explained by activation of different receptors on endothelial cells. Endothelial cells express a certain subset of type I receptors, mainly ALK1 and ALK5, and TGF-β was shown to bind to both receptors (Fig. 1). Interestingly, in endothelial cells TGF-β has been shown to induce phosphorylation of both TGF-dependent Smad2/3, as well as BMP-dependent Smad1/5 (Goumans et al., 2002). Knockdown experiments, together with overexpression of constitutive active forms of ALK5 and ALK1, confirmed that TGF-β-induced Smad2/Smad3 phosphorylation was ALK5-dependent, whereas Smad1/Smad5 phosphorylation was ALK1-dependent. Interestingly, functional studies identified opposite roles of these receptors in endothelial cells (Goumans et al., 2002). TGF-β signaling via ALK5 promoted a quiescent endothelial cell phenotype and resulted in inhibition of endothelial cell proliferation, migration and induction of plasminogen activator inhibitor (PAI)-1 expression. In addition, the ALK5 kinase inhibitor SB-431542 increases the expression of the tight junction component Claudin-5 in mouse embryonic stem cell-derived ECs, suggesting a role for ALK5 signaling in regulating vascular permeability (Watabe et al., 2003). In contrast, TGF-β signaling via ALK1 activated endothelial cells and resulted in higher proliferation, migration, induction of the expression of inhibitor of differentiation-1 (Id1) and increased vascular endothelial growth factor (VEGF) expression (Oh et al., 2000; Shao et al., 2009). As a result, TGF-β regulates both pro- and anti- angiogenic endothelial cell function, and the balance between ALK5 and ALK1 receptors determines the net result upon stimulation with TGF-β.

Interestingly, ALK1 and ALK5 receptors not only mediate opposite responses in endothelial cells, but they also cross talk with each other. ALK5 is not only necessary for the recruitment of ALK1 into a TGF-β receptor complex, but kinase activity of ALK5 is also necessary for maximal ALK1 activation (Goumans et al., 2003). Furthermore, ALK1 can directly antagonize ALK5/Smad2/3 signaling on the level of the SMADs (Goumans et al., 2003; Lebrin et al., 2004). This cross-talk between ALK1 and ALK5 signaling provides a delicate TGF-β switch to fine tune endothelial cell function (Fig. 1).

Recently, BMP-9 and -10 were identified as novel ligands of ALK1 (Brown et al., 2005; David et al., 2007; Scharpfenecker et al., 2007). Interestingly, high doses of BMP-9 inhibited endothelial cell proliferation and migration *in vitro* (David et al., 2007; Scharpfenecker et al., 2007). In addition, BMP-9 inhibited VEGF-induced angiogenic sprout formation in a mouse metatarsal assay *ex vivo* (Scharpfenecker et al., 2007), inhibited bFGF-induced angiogenesis in a murine sponge assay and blocked angiogenesis in the chick CAM assay *in vivo* (David et al., 2008). Interestingly, high doses of BMP-9 could also block the pro-angiogenic TGF-β-mediated effects in endothelial cells and was proposed as a vascular quiescence factor (Shao et al., 2009). However, the BMP-9-induced gene expression pattern was similar to the pattern obtained upon expression of constitutively active ALK1 (caALK1) (Lux et al., 2006; David et al., 2007; Upton et al., 2009), and resulted in upregulation of genes associated with an activated (Id1, endoglin) and proinflammatory (IL-8, E-Selectin) endothelial cell phenotype (Lux et al., 2006; David et al., 2007; Scharpfenecker et al., 2007; Upton et al., 2009). Recently, BMP-9 was reported to promote the growth of mouse embryonic-stem-cell-derived endothelial cells *in vitro* (Suzuki et al., 2010). In addition, BMP-9 enhanced angiogenesis in the allantois, in matrigel plug assays and in pancreatic carcinoma xenografts in mice. Furthermore, BMP-9 in combination with TGF-β potentiated VEGF induced proliferation of endothelial cells in vitro and VEGF/bFGF induced angiogenesis in vivo (Cunha et al., 2010). Thus, BMP-9 signaling in endothelial cells might exhibit similar biphasic activity to TGF-β, with the functional outcome depending on multiple factors, including ligand concentration and cellular context.

The biphasic effect of TGF-β is further fine-tuned via the TGF-β co-receptor endoglin. Endoglin is upregulated on proliferating endothelial cells and required for efficient signaling of TGF-β via ALK1 (Arthur et al., 2000; Lebrin et al., 2004). Moreover, overexpression of endoglin potentiates BMP-9 response in NIH-3T3 cells co-transfected with ALK1 (David et al., 2007) and, *vice versa,* stimulation with BMP-9 increases endoglin expression in bovine aortic endothelial cells (BAECs) (Scharpfenecker et al., 2007). While necessary for TGF-β/ALK1 signalling, endoglin indirectly inhibits TGF-β/ALK5 signaling (Lebrin et al., 2004; Bernabeu et al., 2007). Interestingly, ectopic expression of endoglin inhibits TGF-β-induced growth inhibition not only in endothelial cells, but also in monocytes and myoblasts (Lastres et al., 1996; Scherner et al., 2007), and protects endothelial cells from TGF-β induced apoptosis and extracellular matrix deposition (Obreo et al., 2004). In addition, hypoxia induces expression of endoglin, ALK1 and phospho-Smad1/5 *in vitro* and *in vivo* (Tian et al., 2010). Moreover, soluble endoglin sEng inhibits endothelial cell sprouting, and patients with colorectal cancer display reduced levels of sEng compared to healthy volunteers (Hawinkels et al., 2010). In summary, endoglin contributes to the activation phase of angiogenesis and is an important regulator for the fine tuning of ALK1/ALK5 signaling in

endothelial cells (Fig. 1).

TGF-β signaling in mural cells

Mural cells are essential structural components of mature vessels, which stabilize tubes of endothelial cells. Mural cells are called pericytes in smaller capillaries or smooth muscle cells (SMC) in a large artery or vein. Aberrations in mural cell coverage are linked to different vascular diseases. For instance, alterations in pericyte coverage are associated with different vascular pathologies, such as diabetic retinopathy, brain hemorrhages, HHT and tumor angiogenesis (Hirschi and D'Amore, 1996; Bergers and Song, 2005). Vascular SMCs (vSMCs) play a crucial role in the regulation of blood flow. Interestingly, vSMCs are not terminally differentiated and posses certain levels of plasticity. An alteration in switching between a quiescent/contractile phenotype to a de-differentiated synthetic/migratory phenotype is associated with different cardiovascular diseases, such as atherosclerosis, restenosis and hypertension (Owens et al., 2004; Morrell et al., 2009).

Different *in vitro* and i*n vivo* studies demonstrated the important role of TGF-β in induction and maintenance of the differentiated phenotype in mural cells. For instance, TGF-β1 induces differentiation of smooth muscle cells from the mesenchymal cell line 10T1/2, neural crest stem cell line Monc-1 and embryonic stem cells (Hirschi et al., 1998; Chen and Lechleider, 2004; Sinha et al., 2004).

TGF-β mediated vSMC differentiation is regulated by zinc finger transcription factor δEF1 (ZEB-1) (Nishimura et al., 2006). The proposed mechanism involves cooperation between δEF and Smad3 in activation of the α-smooth muscle actin (SMA) promoter. Moreover, overexpression of caALK5 alone or together with δEF or Smad3 potentiates activity of the α-SMA promoter as well. Interestingly, Smad3 deficient mice develop dramatic neointima thickening upon injury of the femoral artery. Moreover, Smad3 deficient mouse aortic smooth muscle cells are less responsive to TGF-β1 induced growth inhibition and display a more migratory phenotype (Kobayashi et al., 2005). Besides differentiation, TGF-β was shown to induce growth inhibition and apoptosis of vSMC, but these effects were Smad2 dependent (Redondo et al., 2005, 2010).

In addition, as observed for endothelial cells, endoglin modulates the TGF-β response in vSMCs during both vessel homostasis and vascular pathologies. Endoglin was shown to be required for the differentiation of SMCs from neural crest stem cells (NCSCs) (Mancini et al., 2007). Endoglin promotes atherosclerotic plaque stabilization via the induction of early growth response-1 (EGR-1) transcription factor, which inhibits smooth muscle cell proliferation (Bot et al., 2009; Santiago et al., 1999).

BMPs play a crucial role in maintenance of the differentiated phenotype in vSMCs. For instance, BMP-2, BMP-4 and BMP-7 promote induction of the SMC contractile markers such as SMA, SM22α and calponin (Dorai et al., 2000; Lagna et al., 2007). Moreover, BMPs inhibit smooth muscle cell proliferation (Dorai et al., 2000; Morrell et al., 2001) and induce mitochondria-mediated apoptosis in pulmonary arterial smooth muscle cells (PASMCs) (Lagna et al., 2006). The anti-proliferative effect of BMP-2 involves Smad-independent inhibition of platelet-derived growth factor (PDGF)-BB induced PASMC proliferation via activation of the peroxisome proliferator-activated receptor (PPAR)γ transcription factor and induction of ApoE (Hansmann et al., 2008). Moreover, TGF-β and BMP4 induce microRNA-21, leading to the repression of programmed cell death 4 (PDCD4), an inhibitor of smooth muscle gene expression, thereby stimulating smooth muscle differentiation (Davis et al., 2008). Also, as a feedback loop PDGF-BB induces microRNA-24, which downregulates Tribbles-like protein-3 (Trb3) and results in reduced BMP and TGF-β signaling (Chan et al., 2010). In summary, both TGF-β and BMPs are crucial mediators of vSMC differentiation and maintenance of the quiescent/contractile phenotype necessary for stabilizing the vascular sprouts.

TGF-β function in the interaction between endothelial cells and mural cells

Recruitment and differentiation of mural cell progenitors to the endothelial tube occurs at a later stage of development when primitive vessels are formed and endothelium derived growth factors play an essential role (reviewed in (Armulik et al., 2005; Gaengel et al., 2009)).

For instance, TGF-β can regulate vSMC recruitment via the induction of monocyte chemoattractant protein-1 (MCP-1) in both endothelial cells and vSMCs (Ma et al., 2007; Zhang et al., 2009). The identified mechanism revealed TGF-β/ALK5 and Smad3/Smad4 dependent upregulation of monocyte chemotactic protein (MCP)-1 expression in endothelial cells (Ma et al., 2007) and TGF-β/Smad3 dependent MCP-1 expression in vSMCs, which can further serve as a chemoatractant for e.g. bone marrow derived cells (Zhang et al., 2009).

Heterotypic cell culture of endothelial cells with pericytes activates the latent form of TGF-β (Antonelli-Orlidge et al., 1989). In addition, endothelial cell-derived activated TGF-β1 induces differentiation of SMCs from the mesenchymal cell line 10T1/2 (Hirschi et al., 1998). Depletion of endogenous TGF-β from the cells with either a TGF-β neutralizing antibody or recombinant soluble TβRII abolishes cord and lumen formation in a 10T1/2–endothelial cell 3D co-culture assay. Moreover, depletion of TGF-β resulted in reduced expression of mural cell markers, such as α-SMA and NG2, in a 10T1/2-endothelial cell co-culture indicating a decrease in mural cell differentiation (Darland and D'Amore, 2001).

Targeted deletion of several TGF-β signaling components revealed the importance of TGF-β signaling

in vSMC-EC interaction. Endoglin or ALK1-deficient embryos developed a primitive capillary plexus, indicating that vasculogenesis is not affected, whereas vessel maturation is severely disturbed. These embryos displayed loss of pericyte and SMC coverage; the mutant vessels appear dilated and tortuous, with abnormal direct connections between arteries and veins without connecting capillary beds (Arthur et al., 2000; Oh et al., 2000; Urness et al., 2000; Sorensen et al., 2003). Mice lacking TβRII, SMAD5, SMAD1 and TGF-β1 all show defects in vasculature structure or blood vessel organization, indicative of a defect in EC lining and impaired vSMC differentiation (Dickson et al., 1995; Goumans et al., 1999; Yang et al., 1999; Goumans and Mummery, 2000; Oh et al., 2000; Lechleider et al., 2001).

Several lines of evidence indicate possible cooperation between TGF-β and Notch signaling to control smooth muscle cell differentiation. Notch3 is highly expressed in vascular smooth muscle cells, where it regulates smooth muscle cell proliferation and differentiation (Domenga et al., 2004). Jagged-1 is the major Notch3 ligand on endothelial cells and Notch3 expression in smooth muscle cells is induced upon interaction with endothelial cells. Moreover, Notch3/Jagged-1 signaling results in an autoregulatory loop that maintains high levels of Notch3 in smooth muscle cells (Liu et al., 2009a). TGF-β1 promotes human mesenchymal stem cell differentiation towards SMC via upregulation of Jagged-1 and smooth muscle cell markers such as α-SMA, calponin 1 and myocardin (Kurpinski et al., 2010).

TGF-β type I receptors in regulation of angiogenesis and lymphangiogenesis

Taking into account the important role of TGF-β signaling in endothelia/mural cells, it can be considered a good target for anti-angiogenic therapy. Furthermore, endothelial cell restricted expression of ALK1, together with endothelial/mural cell function of ALK5 and signaling co-interplay between them, make these TGF-β type I receptors a good choice for targeting. Several selective inhibitors for TGF-β type I receptors were identified recently (Inman et al., 2002; Sawyer et al., 2003; Bueno et al., 2008; Li et al., 2008). These small molecule inhibitors have been shown to specifically inhibit ALK4/5/7 receptors, but not ALK1/2/3/6. Therefore these inhibitors of TGF-β type I receptors can be used to dissect ALK5/ALK1 receptor function *in vivo*.

Interestingly, a combination of VEGF with a TGF-β type I receptor inhibitor enhanced endothelial cell migration and formation of angiogenic sprouts in endothelial cell spheroids (Liu et al., 2009). This effect was most likely ALK5 dependent, as confirmed by treatment with a TGF-β neutralizing antibody. The effect of enhanced angiogenesis with the combination of VEGF and ALK5 inhibitor resulted from upregulation of α5 integrin subunit expression and could be blocked by siRNA-mediated knockdown of the α5 integrin *in vitro* or by a neutralizing antibody to α5 integrin in the matrigel plug assay *in vivo*. Interestingly, upregulation of α5 integrin subunit was a result of *de novo* protein synthesis, as it could be blocked by cyclohexamide (CHX). Although the exact mechanism of the synergistic upregulation of the α5 integrin subunit is not well understood, these findings provide a new intriguing mechanism of TGF-β signaling in endothelial cells.

Another ALK1 targeting strategy was developed based on usage of recombinant ALK1 extracellular domain fused to human Fc protein, the ALK1 ligand trap. Interestingly, ALK1-Fc specifically inhibited BMP-9 or BMP-10-induced activation of a BMP-specific reporter (Smad1/Smad5), but not of a TGF-β-specific reporter (Smad2/Smad3). Furthermore, ALK1-Fc inhibited VEGF-induced endothelial cell sprouting *in vitro* and angiogenesis in the matrigel plug assay *in vivo* induced by VEGF together with bFGF (Cunha et al., 2010). Strikingly, ablation of ALK1 resulted in decreased angiogenesis and tumor growth in the endocrine pancreatic tumorgenesis model (RIP-Tag2) (Cunha et al., 2010).

Treatment of neonatal mice with ALK1-Fc from day 1 to day 5 (p1,3,5) resulted in dramatic abnormalities in retinal vasculature at p8, such as increased vessel density or failure to remodel vessels of the primitive capillary plexus into mature network (Niessen et al., 2009). Moreover, administration of ALK1-Fc to neonatal mice at p1 and p3 resulted in defective development of lymphatic vessels at day 5. VEGF-R3 together with VEGF-C/D is important for lymphatic cell proliferation and survival. Interestingly, inhibition of ALK1 with ALK1-Fc increased endothelial cell apoptosis upon VEGF-R3-Fc treatment. In addition, inhibition of ALK1 decreased podoplanin expression a marker of mature lymphatic endothelial cells. All these data indicate the importance of ALK1 in lymphatic vessel development and maturation.

Vascular disorders

Hereditary hemorrhagic telangiectasia (HHT)

HHT is a genetic disorder that is characterized by multiple telangiectasia in the skin, oral and nasal mucosa, and gastrointestinal tract, and arteriovenous malformations (AVMs) in the brain, lungs, liver, and gastrointestinal tract (Haitjema et al., 1996; Govani and Shovlin, 2009; Dupuis-Girod et al., 2010) (Fig. 2C,D). Approximately 50-80% of HHT patients suffer from severe and recurrent epistaxis (Haitjema et al., 1996a). HHT is caused by an alteration in the TGF-β signaling pathway (van den Driesche et al., 2003) Autosomal dominant mutations in endoglin, ALK1 and Smad4 are linked to type 1 HHT (HHT1), type 2 HHT (HHT2) and combined juvenile polyposis symdrome (JPS)/HHT respectively (McAllister et al., 1994; Berg et al., 1997;

Gallione et al., 2004).

Mouse models with targeted disruption of the gene for endoglin or Alk1 were generated. Strikingly, heterozygous mice for either gene developed a phenotype with similar characteristics to HHT patients (Bourdeau et al., 1999; Oh et al., 2000; Urness et al., 2000; Sorensen et al., 2003; Srinivasan et al., 2003; Park et al., 2008, 2009; Mahmoud et al., 2010). Endothelial specific deletion of ALK1 or endoglin caused formation of AVMs in adult mice, indicating the vital role of TGF-β signaling in endothelial cells (Park et al., 2009; Mahmoud et al., 2010). Moreover, endothelial deficiency of ALK1 or endoglin resulted in abnormal smooth muscle cell coverage. In addition, active angiogenesis was proposed as a triggering factor for AVM formation. For instance, wounding and active angiogenesis during retina development dramatically increases AMV formation in ALK1 and endoglin knockout mice respectively.

Recently Lebrin et al. reported thalidomide as an effective drug to treat the severe nosebleeds in HHT patients (Lebrin et al., 2010). Thalidomide was originally introduced as a sedative drug and later removed from the market for its teratogenic activity, as a result of a potent anti-angiogenic activity. However thalidomide was re-introduced as a potent anti-hemorrhage and anti-inflammatory drug. Thalidomide is currently used to threat severe intestinal bleedings and used as an anti-anti-angiogenic drug in cancer therapy (Laffitte and Revuz, 2004). The discovered mechanism of thalidomide-mediated normalization of the vasculature in HHT patients involves increased proliferation and recruitment of smooth muscle cells to the vessels. Endothelial cell-derived increase in PDGF-B expression was identified as one of the downstream targets of thalidomide, but thalidomide also had a

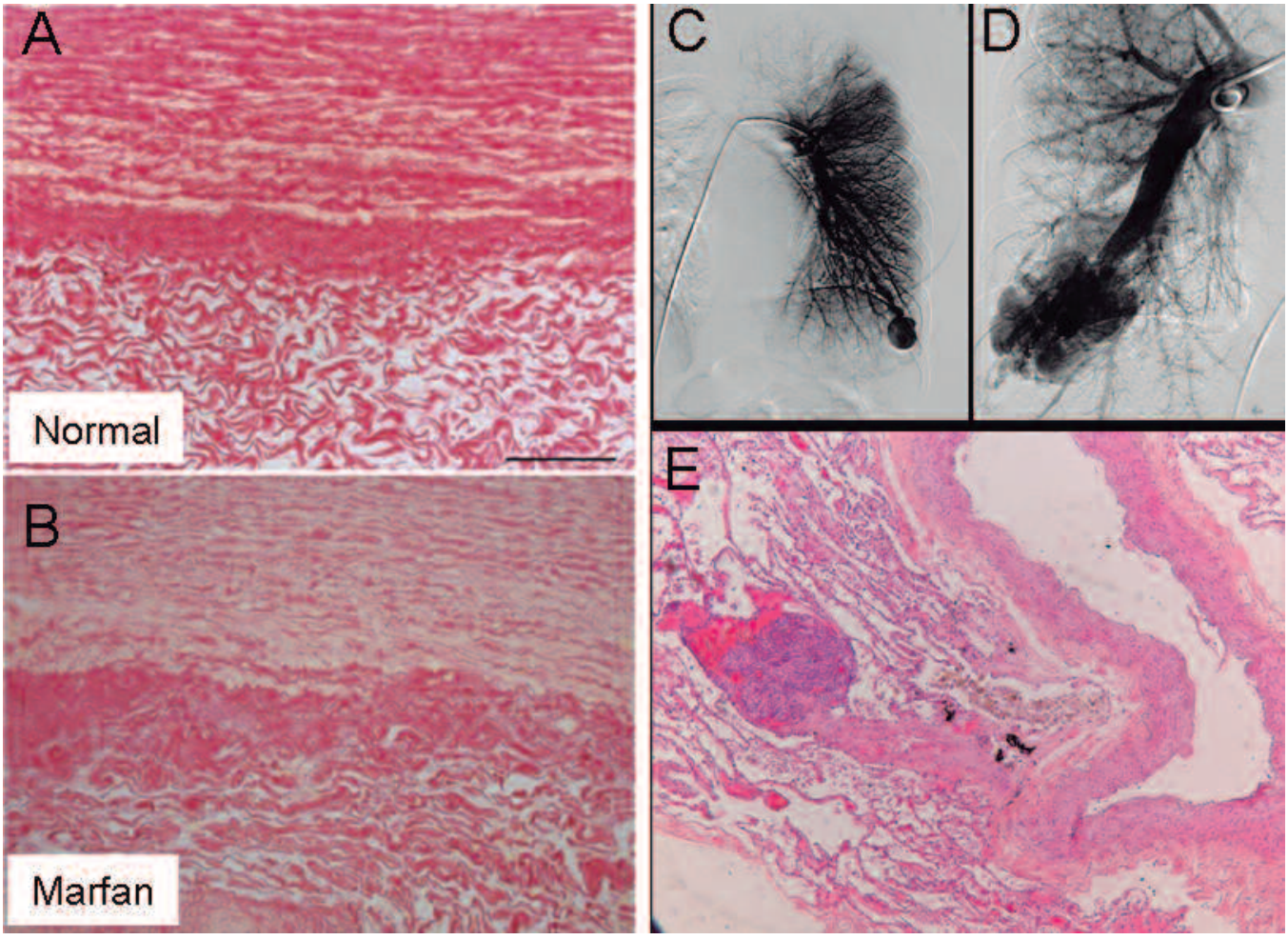

Fig. 2. Vascular disorders with perturbed TGF-β signal transduction. Sirius red staining for collagen deposition in adventitia of thoracic aorta Marfan patient **(B)** and control aorta **(A)** (Lindeman et al., 2010). Fragmented elastin fibres in the aorta are seen in MFS and are associated with release of the large latent TGF-β complex and active TGF-β. Clinical symptoms of HHT and PAH patients include arteriovenous malformations as shown on an angiogram in HHT **(C, D)** and PAH patient with a plexiform lesion in the supernumerary artery **(E)**.

PDGF-B independent effect on mural cell behavior. Therefore, additional studies to understand the mechanism of thalidomide or thalidomide derivatives action on endothelial/mural are required.

Marfan syndromes and Loeys-Dietz syndromes (MFS and LDS)

Marfan syndrome (MFS) is an autosomal dominant disorder of connective tissues caused by mutations in the fibrillin-1 gene on chromosome 15 and involves an increase in interstitial fibrosis in multiple organ systems, including the cardiovascular and pulmonary system (Fig. 2A,B) (Collod-Beroud et al., 2003; Mizuguchi and Matsumoto, 2007). Fibrillin-1 is a 350-kDa glycoprotein with multiple domains, including motifs with homology to latent TGF-β–binding proteins (LTBP). Fibrillin-1 interacts with LTBP and controls TGF-β bioavailability, since TGF-β in a LTBP-fibrillin complex is kept inactive (Neptune et al., 2003) Interestingly, using a mouse model for Marfan deficient for fibrillin-1, it was demonstrated that defective fibrillin-1 diminished sequestration of latent TGF-β in the extracellular matrix, leading to increased TGF-β availability (Neptune et al., 2003). Recently, enhanced levels of circulating TGF-β was also observed in MF patients (Matt et al., 2009). Inhibiting the enhanced TGF-β signaling in fibrillin-1 deficient mice by giving a TGF-β neutralizing antibody the abnormal alveolar septation previously observed was rescued (Neptune et al., 2003). Analyzing the Marfan mouse for aortic root dilatation and skeletal myopathy revealed that the TGF-β neutralizing antibody or the angiotensin II type 1 receptor inhibitor losartan prevented aortic root dilatation, elastic fiber degeneration, Smad2 activation, and also skeletal myopathy was reversed (Cohn et al., 2007; Brooke et al., 2008; Matt et al., 2009).

The important preclinical observation that angiotensin II type I–receptor blockade induces decrease in TGF-β signaling and reduces the pathology in multiple organs, has translated to immediate clinical studies. In a prospective randomized trial, the effects of angiotensin II type I blockade on aortic root growth will be studied in MF patients (Lacro et al., 2007). To date, 426 patients have been enrolled and the results of this study are eagerly awaited.

The Loeys-Dietz syndrome (LDS) is a recently characterized genetic disorder due to mutations in either TβRI or TβRII (Loeys et al., 2005). LDS is an autosomal disorder of the connective tissue. LDS patients present with a variety of features with a phenotypic overlap with MF syndrome and Ehlers-Danlos syndrome, mainly involving the cardiovascular, musculoskeletal and central nervous systems. In particular, LDS patients have arterial tortuosity with widespread vascular aneurysm and dissection, and a high risk of aortic rupture at an early age (Loeys et al., 2006). The molecular mechanism of LDS is still poorly understood, although it is most likely related to TGF-β signaling as reported for MF syndrome (Loeys et al., 2005, 2006). Analyzing biopsies from patients with LDS demonstrated nuclear enrichment of phosphorylated Smad2 and increased expression of collagen and CTGF, indicative of increased TGF-β signaling (Loeys et al., 2006).

Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a severe clinical condition characterized by luminal obliteration of small pulmonary arteries (plexiform lesion) (Fig. 2). Proliferation of myofibroblasts and smooth muscle cells in the arterial wall increases pulmonary vascular resistance and results in elevated pulmonary arterial pressure, ultimately leading to right ventricular failure. Heterozygous mutations in several TGF-β receptor members BMPRII, ALK1 and endoglin have been associated with PAH (Lane et al., 2000; Machado et al., 2001; Atkinson et al., 2002; Harrison et al., 2003, 2005). Interestingly, the pathology of PAH results from deficiency in TGF-β signaling in both endothelial or/and smooth muscle cells. Moreover, recent studies clearly demonstrate the primarily role of endothelial cells in the development of PAH. For instance, TGF-β and BMP-9 induce ALK5 and ALK1 mediated increase in endothelin-1 (ET-1) expression in endothelial cells (Castanares et al., 2007; Star et al., 2010), and up regulation of ET-1 expression has been linked to PAH (Humbert et al., 2004). In addition, a direct effect of misbalanced TGF-β signaling on vSMC proliferation is also one of the possible causes of PAH. PASMCs from patients with primary pulmonary hypertension (PPH) exhibited loss of the BMP induced inhibitory effect on proliferation and increased proliferation upon stimulation with TGF-β1 compared to control PASMCs (Morrell et al., 2001). The identified downstream pathway involves deficiency in BMPRII activation, Smad1 phosphorylation and Id1 gene expression (Yang et al., 2005, 2008). Prostacyclin analogues are currently used for successful treatment of PAH in clinic and a recently identified mechanism involves the induction of Id1 in PASMCs (Yang et al., 2010). Interestingly, administration of iloprost also restores deficiency in Smad1/Smad5 phosphorylation and Id1 levels in BMPRII deficient PASMCs, together with potentiating the inhibitory effect of BMP-4 on PASMC proliferation.

Preeclampsia

Deregulation of TGF-β signaling is linked to the pathogenesis of preeclampsia, a pregnancy complication caused by massive endothelial dysfunction. Preeclampsia affects approximately 3-5% of pregnant women and is a major cause of maternal/prenatal mortality. Clinical symptoms of preeclampsia include proteinuria, hypertension, HELLP (hemolysis, elevated liver enzymes, and low platelets), massive edema or brain edema/eclampsia (Young et al., 2010). An

increased level of circulating soluble VEGF-R1 (sFlt1) precedes the onset of preeclampsia (Maynard et al., 2003; Venkatesha et al., 2006). Interestingly, despite the fact that sFlt1 sequesters major pro-angiogenic factors such as VEGF and PlGF, mouse models demonstrated that the presence of only sFlt1 is not enough to induce all pathologic symptoms of preeclampsia (Maynard et al., 2003). Thus, other factors may contribute to disease pathology. Recently, sEng was identified as an additional soluble factor in preeclampsia (Venkatesha et al., 2006). Administration of sEng to pregnant rats recapitulates all the symptoms of preeclampsia observed in pregnant mothers. The underlying mechanism involves neutralization of active TGF-β1 and TGF-β3. Recently it has been shown that TGF-β1 regulates survival of endothelial cells, and systemic inhibition of TGF-β1 with sEng results in massive retina degeneration (Walshe et al., 2009).

Concluding remarks

The critical importance of TGF-β in keeping vascular morphogenesis in check is illustrated by the embryonic lethality of knock-out mice for specific TGF-β family signaling components caused by defective angiogenesis, and that perturbations in TGF-β signaling are connected to an ever increasing list of human vascular disorders. *In vitro* and *in vivo* studies have demonstrated the powerful context dependent effects of TGF-β on both endothelial cells and pericytes/smooth muscle cells, and the interplay between these two cell types. One important manner to control the activation state of endothelial cells is the differential activation of two opposing TGF-β type I receptors, i.e. ALK1 and ALK5. The co-receptor endoglin inhibits ALK5 but favors ALK1 signaling. Existing recent data have shown that interference with the pro-angiogenic action of ALK1 or endoglin by soluble ALK1-Fc, soluble endoglin or anti-ALK1 or endoglin antibodies interferes with tumor angiogenesis (van Meeteren et al., 2011). Insights into the molecular mechanisms that underlie HHT and Marfan pathology have led to clinical studies with thalidomide and losartan that will normalize the dysfunctional vascular bed (Brooke et al., 2008; Lebrin et al., 2010).

TGF-β was shown to induce the transdifferentiation of endothelial cells into Smooth muscle cell-like cells, termed EndoMT (Goumans et al., 2008). While EndoMT was first discovered to play a role in heart valve formation during embryonic development, this process appears to have an important pathological contribution in fibrosis and cancer progression. Recently, endothelial cells, when cultured under specific conditions, were shown to be able to acquire a stem cell phenotype (Medici et al., 2010). In response to TGF-β2 or BMP-4, endothelial cells converted in an ALK2 and ALK5 dependent manner into multipotent mesenchymal stem cells that could be stimulated to differentiate into chondrocytes, osteoblasts and adipocytes. An interesting area of research will be to examine whether we can exploit this endothelial cell conversion into stem cells for tissue repair.

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