Chapter 13
TGF-β and Cardiovascular Disorders

Laurens A. van Meeteren, Marie-José Goumans, and Peter ten Dijke

Abstract Transforming growth factor β (TGF-β) is a secreted pleiotropic cytokine that is involved in a wide range of biological processes and has essential roles in development and tissue homeostasis. TGF-β elicits cellular effects by activating serine/threonine kinase receptors located at the plasma membrane and intracellular Smad effector proteins. TGF-β is an important (cardio)vascular regulator as shown by many in vitro studies on cultured endothelial and mural cells, in vivo studies in animal models, and genetic studies in which mutations in human TGF-β signaling components have been causally linked to cardiovascular diseases. Here we review recent progress in our understanding of the (dys)function of TGF-β in the cardiovascular system.

Keywords Angiogenesis • Cardiovascular • Endothelium • TGF-β

13.1 Introduction

Transforming growth factor β (TGF-β) is one of the most studied members of a large family of structurally related secreted pleiotropic cell to cell signaling molecules. Different members of the family include the activins and bone morphogenetic proteins (BMPs). Many of these cytokines have essential roles in numerous processes during development, but also in maintenance of tissue homeostasis and tissue repair in the adult (Massagué 1998). Not surprisingly,

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when their action is perturbed, they have an important role in pathological conditions, like vascular diseases, cancer, and fibrosis (Blobe et al. 2000; ten Dijke and Arthur 2007).

The ligands of the TGF-β family mediate their effects by binding specific single transmembrane receptors at the cell membrane (Feng and Derynck 2005). Upon ligand-induced heteromeric complex formation, the type I receptor is phosphorylated by the type II receptor. There are seven different type I receptors (ALK1 to ALK7) (also known as activin receptor-like kinases (ALK)) and five type II receptors (activin receptor type IIA (ActRIIA), activin receptor type IIB (ActRIIB), BMP type II receptor (BMPRII), TGF-β type II receptor (TGF-βRII), and AMH type II receptor (AMHRII)). Different ligands can bind different combinations of type I and type II receptor thereby creating specificity of signaling. TGF-β signals mostly via ALK5 and TGF-βRII, activins via ALK4 with ActRIIA or ActRIIB, and BMPs signal via ALK1, 2, 3, and 6 together with BMPRII, ActRIIA, or ActRIIB. For regulation of endothelial function by TGF-β, ALK1 and ALK5 signaling are most important (van Meeteren and ten Dijke 2012).

After binding of the ligand the type I receptor phosphorylates specific transcription factors, receptor regulated (R)-Smads. Upon activation by type I receptors, R-Smads form heteromeric complexes with the common mediator (Co)-Smad (Smad4) and these heteromeric complexes accumulate into the nucleus, where they regulate the transcription of specific target genes (Moustakas and Heldin 2009).

Inhibitory Smads (I-Smads) inhibit the activation of R-Smads by competing for type I receptor interaction and by recruiting phosphatases and ubiquitin ligases to the activated receptor complex leading to dephosphorylation or proteosomal degradation of the receptor complex (Itoh and ten Dijke 2007). R-Smads can be divided into two groups based on the type I receptor that is activating them. The first group consists of Smad1, 5, and 8 and these are activated by ALK1, 2, 3, and 6. The second group contains Smad2 and 3 and is activated by ALK4, 5, and 7. In addition to Smad signaling, TGF-β and BMP signaling can result in activation of pathways where Smads are not directly involved. Non-Smad pathways include various branches of MAP kinase pathways, Rho-like GTPase signaling pathways, and PI3K/AKT pathways (Moustakas and Heldin 2005; Zhang 2009). Non-Smad signaling pathway have been found to modulate Smad signaling and vice versa (Moustakas and Heldin 2005) (Figs. 13.1).

Co-receptors are receptors that do not signal by themselves since they lack intracellular enzymatic motifs such as kinase domains (Kirkbride et al. 2005). For TGF-β signaling co-receptors endoglin and betaglycan (also called TGF-β receptor III) have been identified (Cheifetz et al. 1992; Wang et al. 1991). Both receptors are structurally related and have a small intracellular tail, a single transmembrane domain, and a large extracellular domain. Endoglin is highly expressed in proliferating endothelial cells, hence its name endoglin.

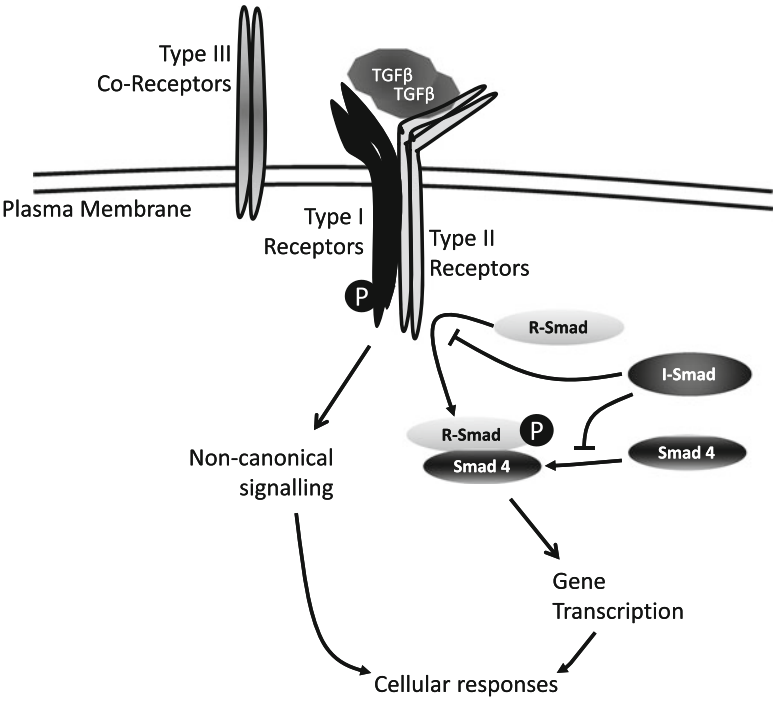

Fig. 13.1 Basic signaling unit of the TGF-β system. TGF-β family ligands such as TGF-β and BMPs bind a complex of type I receptors, type II receptors, and possibly a type III co-receptor leading to activation of R-Smads. R-Smads then form a complex with Smad4 leading to specific gene-transcription. I-Smads inhibit the activation of R-Smads and complex formation of Smad4 with R-Smads. In addition, the receptor complex can also activate non-Smad pathways

13.2 TGF-β Signaling in Vascular Biology

13.2.1 Regulating Endothelial Function by TGF-β

Genetic studies in mice and humans revealed the importance of components of the TGF-β signaling pathway in vascular morphogenesis and angiogenesis. Deletion of components of the TGF-β pathway (ligands, receptors, and intracellular Smad mediators) in mice leads to embryonic lethality due to vascular abnormalities (see below) (van Meeteren et al. 2011).

To regulate the activation state of endothelial cells TGF-β can differentially activate two type I receptors, ALK1 and ALK5 (Goumans et al. 2002; Oh et al. 2000). ALK5 is expressed in most tissues, but ALK1 expression is restricted to endothelial cells. TGF-β-induced ALK5 signaling activates Smad2 and Smad3 phosphorylation resulting in inhibition of endothelial cell proliferation, migration, and organization

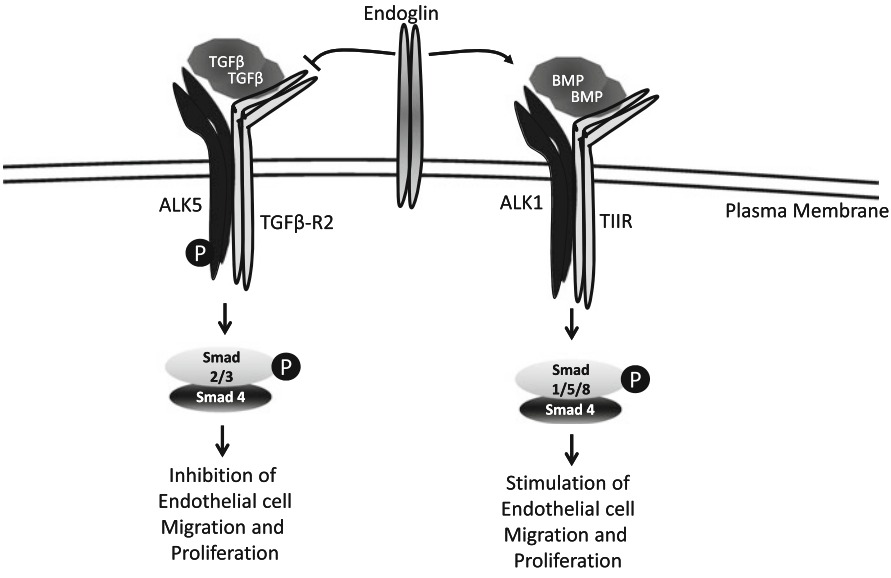

Fig. 13.2 TGF-β signaling in endothelial cells. BMP binding to ALK1 (in complex with a type II receptor and endoglin) activates Smad1, 5, and 8 leading to stimulation of endothelial cell migration and proliferation. ALK1 is antagonized by ALK5 that is activated by TGF-β leading to Smad2 and 3 activation and endothelial cell quiescence

(Goumans et al. 2003) (Fig. 13.2). The ALK5 kinase inhibitor SB-431542 enabled proliferation and sheet formation of embryonic stem cell-derived endothelial cells. SB-431542 upregulated the expression of claudin-5, an endothelial-specific component of tight junctions, suggesting a role of ALK5 signaling in regulating vascular permeability (Watabe et al. 2003). In addition, VEGF and inhibitors of TGF-β type I receptor kinase synergistically promoted blood vessel formation by inducing β 5-integrin expression (Liu et al. 2009). In contradiction to these findings, in vivo, SB-431542 has been reported to induce enhanced delivery of nanoparticles to the tumor tissue extravascular space due to increased vessel leakiness (Kano et al. 2007). Indeed, ALK5 has been reported before to be important for TGF-β-induced permeability and cytoskeletal remodeling of endothelial cells (Birukova et al. 2005). Interestingly a major component of endothelial cell–cell junctions, VE-cadherin, was found to interact with ALK5, ALK1, endoglin, and TGF-βRII, which possibly reveals the link between permeability and TGF-β signaling (Rudini et al. 2008). In conclusion, ALK5 signaling results in keeping endothelial cells in a quiescent state.

TGF-β-induced ALK1 signaling activates Smad1 and Smad5 leading to endothelial cell proliferation, migration, and organization (Goumans et al. 2003). A central intracellular effector of ALK1 is Id1; its upregulation was shown to be required for TGF-β/ALK1-induced endothelial cell migration and tube formation (Valdimarsdottir et al. 2002) and inhibition of ALK1 was shown to inhibit endothelial cell sprouting (van Meeteren et al. 2012). However, inhibitory effects of ALK1

signaling on endothelial cells have also been reported (David et al. 2007; Lamouille et al. 2002; Mallet et al. 2006). The effect of ALK1 is likely dependent on cellular context.

Although ALK1 and ALK5 have opposing effects on endothelial cells, they do interact with each other physically. ALK5-deficient endothelial cells are not only defective in ALK5 signaling but also show impaired ALK1 responses; ALK5 was found to be essential for recruitment of ALK1 into a TGF-β receptor complex, and the kinase activity of ALK5 is essential for full ALK1 activation (Goumans et al. 2003). In the presence of fibronectin ALK1 activity is not dependent on ALK5 activity since in the presence of fibronectin an ALK5 inhibitor has no effect on ALK1-mediated Smad1/5/8 phosphorylation (Tian et al. 2012). On the other hand, ALK1 can directly antagonize ALK5 signaling at the level of Smads (Goumans et al. 2002; Oh et al. 2000). Taken together, the cross talk between ALK1 and ALK5 signaling provides endothelial cells with a sophisticated mechanism to fine-tune endothelial function.

Endothelial cells can express both the TGF-β co-receptors endoglin and betaglycan. Endoglin positive but betaglycan negative endothelial cells are responsive to TGF-β-1 and -3, but not -2 (Cheifetz et al. 1990). In endothelial cells that express both co-receptors endoglin can form a complex with betaglycan and the TGF-β receptor complex simultaneously (Wong et al. 2000).

Endoglin is predominantly expressed in highly proliferating endothelial cells, but is also expressed in hematopoietic cells, syncytiotrophoblasts of term placenta, stromal cells, smooth muscle cells, and mesenchymal cells (Bot et al. 2009; Dağdeviren et al. 1998). Ectopic expression of endoglin inhibits TGF-β-induced growth inhibition of endothelial cells, monocytes and myoblasts (Lastres 1996; Lebrin et al. 2004), and extracellular matrix deposition (Obreo et al. 2004). Endoglin plays an important role in balancing ALK1 and ALK5 signaling pathways. Endoglin overexpression potentiates TGF-β/ALK1 signaling and inhibits TGF-β/ALK5 signaling, whereas endoglin knockdown resulted in impaired TGF-β/ALK1 signaling responses (Blanco et al. 2005; Lebrin et al. 2004). Endothelial cells isolated from endoglin heterozygote or homozygote embryos displayed significantly reduced proliferation and migration, increased collagen production, and decreased nitric oxide (NO) synthase expression and VEGF secretion (Jerkic et al. 2006). Hypoxia upregulates endoglin levels and this upregulation was shown to protect endothelial cells from hypoxia-induced apoptosis (Li et al. 2003).

A general feature of co-receptors is that they can be cleaved from the cell membrane with the consequence that they exist as soluble forms. Indeed, also betaglycan and endoglin can be shedded by membrane type matrix metalloprotease-1 (MT-MMP) from the plasma membrane giving rise to a soluble betaglycan and soluble endoglin (sol-endoglin) (Hawinkels et al. 2010; Velasco-Loyden et al. 2004). Interestingly, sol-endoglin levels in plasma are a prognostic marker for several pathological conditions, including pregnant women suffering from preeclampsia (Levine et al. 2006; Liu et al. 2012). Furthermore, several labs have reported increased levels of sol-endoglin in serum from cancer patients as a marker of poor prognosis (Li et al. 2000a).

13.2.2 Regulating Mural Cell Function by TGF-β

Both endothelial cells and their supporting cells, such as vascular smooth muscle cells (VSMCs) and pericytes, are needed for proper endothelial function. Tight regulation and close coordination between endothelial cells and VSMCs and pericytes are needed to form a mature vascular network (Armulik et al. 2005, 2011). VSMCs express type I and type II receptors for TGF-β family members (Bobik 2006), and TGF-β is a potent stimulator of VSMC differentiation (Owens 1995). VSMCs form gap junctions with endothelial cells and upon receiving signals from VSMCs, endothelial cells line up and recruit more VSMCs. TGF-β is an important regulator of the interaction between endothelial cells and the supporting cells. Endothelial cells produce latent TGF-β that upon endothelial–VSMC interaction can be activated leading to VSMC differentiation and function (Ding et al. 2004). Knockout mice of several TGF-β signaling players reveal the significance of TGF-β signaling in VSMC–endothelial cell interaction. ALK1- and endoglin knockout mice show defective remodeling of the primary capillary plexus of the yolk sac and failure in SMC development (Arthur et al. 2000; Goumans and Mummery 2000; Oh et al. 2000). TGF-βRII, Smad5, Smad1, and TGF-β1 knockout mice show defects in vasculature structure or blood vessel organization indicative of a defect in endothelial cell lining and impaired VSMC development (ten Dijke and Arthur 2007).

13.2.3 Lessons from Knockout Mice

TGF-β ligands receptors and downstream signaling components are involved in various aspects of vascular biology, in physiology as well as pathophysiology and in development as well as the adult stage (ten Dijke et al. 2008).

Knockout studies in mice offered the first indication that TGF-β signaling plays an essential role in vascular function and development. We will focus mainly on the animal models with disrupted TGF-β signaling components that exhibit defects in angiogenesis and vasculogenesis. For a summary of these knockouts, see Table 13.1.

The first reports on TGF-β knockout mice showed that TGF-β was only necessary for postnatal survival and not for embryonic development (Kulkarni et al. 1993; Shull et al. 1992). However, subsequently it was reported that on the genetic background used for the first studies placental and lactational transfer of maternal TGF-β1 from heterozygous mothers to knockout embryos and pups could have rescued their embryonic lethality (Kallapur et al. 1999; Letterio et al. 1994). In a mixed 129×NIH/Ola×C57BL/6 background, 50 % of the mutant embryos die around embryonic day 10.5 (E10.5) due to inadequate yolk sac development (Bonyadi et al. 1997; Dickson et al. 1995) as a result of failure in both vasculogenesis and hematopoiesis. A defect in terminal differentiation of the endothelial cells in the yolk sac affecting endothelial tube formation and/or its integrity resulting in insufficient capillary tube formation and weak vessels was observed. However, there was no

Table 13.1 List of TGF-β signaling component knockout mice that show vascular defects

Gene	Phenotype knockout mice	References
Ligands		
TGF-β1	Embryonic lethal (E10.5) with vascular defects such as inadequate yolk sac development, defective vasculogenesis, and hematopoiesis	Dickson et al. (1995), Kulkarni et al. (1993), Letterio et al. (1994), Shull et al. (1992)
Receptors		
ALK1	Embryonic lethal (E11.5) due to severe vascular abnormalities such as large shunts between arteries and veins. Also defects in differentiation and recruitment of VSMCs	Oh et al. (2000), Urness et al. (2000)
ALK5	Embryonic lethal (E11.5), severe defects in vascular development of the yolk sac and placenta, and absence of circulating red blood cells	Larsson et al. (2001)
Co-receptors		
Endoglin	Embryonic lethal (E10.5) due to angiogenesis defects and defects in VSMC differentiation	Arthur et al. (2000), Bourdeau et al. (1999), Carvalho et al. (2004), Li (1999)
Betaglycan	Embryonic lethal (E13.5) with defects in coronary vessels and increased apoptosis in the liver	Compton et al. (2007), Stenvers et al. (2003)
Smads		
Smad4	Endothelial-specific knockout is embryonic lethal (E10.5) due to cardiovascular defects and defects in VSMC differentiation and recruitment	Lan et al. (2007)
Smad5	Embryonic lethal (E10.5) due to angiogenesis defects in the yolk sac and enlarged blood vessels that failed to recruit VSMCs	Yang et al. (1999)

obvious defect in vascular development within the TGF-β1-deficient embryos at E9.5. TGF-β2 and TGF-β3 knockout mice die perinatal with no obvious vascular defects (Kaartinen et al. 1995; Sanford et al. 1997).

Acvrl1 (encoding for mouse ALK1) homozygous knockout embryos die at midgestation (around E11.5) due to severe vascular abnormalities such as large shunts between arteries and veins and hyperdilation of large vessels. Furthermore, defects in differentiation and recruitment of supporting cells such as VSMCs were observed (Oh et al. 2000; Urness et al. 2000). Interestingly, a zebrafish with a mutation in ALK1 suffers from an abnormal circulation pattern in which blood flows through a limited number of dilated cranial vessels and failure to perfuse the trunk and tail (Roman et al. 2002).

Mice deficient for ALK5 die also at midgestation, exhibiting severe defects in vascular development of the yolk sac and placenta, and an absence of circulating red blood cells. Endothelial cells from ALK5-deficient embryos show enhanced cell proliferation, improper migratory behavior, and impaired fibronectin production in vitro, defects that are associated with the vascular defects seen in vivo (Larsson et al. 2001).

When *Alk5* was knocked out in only ALK1 expressing endothelial cells, no vascular phenotype was observed. This would suggest that ALK5 plays no important roles in endothelial cells (Park et al. 2008) in line with earlier reports from the same lab that found no detectable expression of ALK5 in endothelial cells (Seki et al. 2006). However, it is in conflict with earlier studies that showed that conditional *Alk5* crossed with the endothelial-specific *tie-1*-cre mice did show a lethal vascular phenotype (Carvalho et al. 2004). This discrepancy is most likely due to different expression pattern between the *tie-1*-cre and the *alk1*-cre. *Tie-1*-cre is supposedly expressed earlier in endothelial cells, and therefore it could mean that ALK5 plays an essential role in the earliest stages of mouse vascular development. Indeed, in the *alk1*-cre mice cre is only expressed at a stage when the *tie-1*-cre ALK5 embryos have already died.

Knockout mice for many other ALKs are also embryonic lethal but at stages before vascular development. For example knockout mice for *Alk2*, *Alk3*, and *Alk4* die around E7.5 (Goumans and Mummery 2000). Therefore it cannot be excluded that also these have roles in the development of the vascular system. By crossing conditional knockout mice for these receptors with an endothelial-specific inducer these roles could be revealed in the future.

Mice deficient for the ALK1 co-receptor endoglin were described by three independent groups. All endoglin knockout mice showed early embryonic lethality at E10.5–11.5 days caused by angiogenesis defects of the yolk sac and abnormal heart development (Arthur et al. 2000; Bourdeau et al. 1999; Li 1999). The formation of the primitive vascular plexus in the yolk sac occurred normally but the successive branching and remodeling associated with angiogenesis fails to proceed. Subsequent analysis of the endoglin knockout mice showed that endoglin is required for downstream TGF-β signaling from the endothelial cell to the adjacent smooth muscle cell to promote smooth muscle cell differentiation (Carvalho et al. 2004). Mice deficient for the co-receptor betaglycan die around E13.5 due to defects in the formation of the coronary vessels and increased apoptosis in the liver (Compton et al. 2007; Stenvers et al. 2003).

Smad1, *2*, and *4* deficient mice all die before vascular development starts due to defects and abnormalities in germ layer formation. In addition, the allantois in Smad1 knockouts fails to connect with the placenta (Huang et al. 2002).

Endothelial-specific *Smad4* knockouts die at E10.5 due to cardiovascular defects, including attenuated vessels sprouting and remodeling, collapsed dorsal aortas, enlarged hearts with reduced trabeculae, and failed endocardial cushion formation. Furthermore, vessels that formed showed impaired development of VSMCs and association between endothelial and VSMCs. Noticeably, *Smad4*-deficient endothelial cells from these mice demonstrated an intrinsic defect in tube formation in vitro (Lan et al. 2007).

Smad5 knockout mouse embryos die between days 10.5 and 11.5 due to defects in angiogenesis. The mutant yolk sacs lacked normal vasculature and had irregularly distributed blood cells, although they contained hematopoietic precursors capable of erythroid differentiation. In addition, *Smad5* mutant embryos had enlarged blood vessels surrounded by decreased numbers of VSMCs, suffered massive apoptosis of mesenchymal cells (Yang et al. 1999).

13.2.4 Endothelial-to-Mesenchymal Transition

Recent evidence has demonstrated that endothelial cells can have a remarkable plasticity. By a process called endothelial-to-mesenchymal transition (EndMT) endothelial cells convert to a more mesenchymal cell type that can give rise to cells such as fibroblasts, but also bone cells. EndMT is essential during embryonic development and tissue regeneration (van Meeteren and ten Dijke 2012). Interestingly, it also plays a role in pathological conditions like fibrosis of organs such as the heart and kidney (Goumans et al. 2008). In addition, EndMT contributes to the generation of cancer-associated fibroblasts that are known to influence the tumor-microenvironment favorable for the tumor cells. EndMT is a form of the more widely known and studied epithelial-to-mesenchymal transition (EMT) (Thiery et al. 2009).

Many endothelial cells can be induced to undergo EndMT when stimulated with TGF-β or Notch ligands (Frid et al. 2002; Ishisaki et al. 2003; Noseda et al. 2004; Timmerman et al. 2004; Zeisberg et al. 2007a, b), albeit that embryonic cells are more plastic than adult cells. The molecular mechanism behind TGF-β-induced EndMT has been found to involve the Snail family of transcription repressors. In mouse embryonic stem cell derived endothelial cells TGF-β stimulation induced EndMT and increased the expression of Snail. This upregulation of Snail by TGF-β was shown to be dependent on the activation of Smad, MEK, PI3K, and p38 MAPK by TGF-β (Medici et al. 2010). Subsequent knockdown of Snail blocked the TGF-β-induced EndMT (Kokudo et al. 2008). Although overexpression of Snail was sufficient to induce EMT (Cano et al. 2000), for EndMT Snail expression alone is insufficient. The inhibitor of Snail, GSK-3β, needs to be inhibited by phosphorylation by kinases such as AKT to induce EndMT (Medici et al. 2010).

Notch can, as TGF-β, induce EndMT in endothelial cells in vitro. In this Notch-induced EndMT the Snail family member Slug has been shown to be essential (Leong et al. 2007). Snail and Slug are known to repress the expression of VE-cadherin (Lopez et al. 2009). Since VE-cadherin is essential for endothelial cell–cell junctions, this obviously could provide a link to a mechanism by which EndMT occurs. A different factor involved in TGF-β-induced EndMT was shown to be plasminogen activator inhibitor-1 (PAI-1). Although elevated levels of PAI-1 are implicated in tissue fibrosis (Ghosh and Vaughan 2012), lack of PAI-1 in the heart is associated with the development of cardiac fibrosis in aged mice (Ghosh et al. 2010). It was shown that in the PAI-deficient endothelial cells of these mice both Smad and non-Smad TGF-β signaling is spontaneously activated. This spontaneous activation leads to EndMT and subsequently the fibrosis observed in these animals. In addition, it was recently shown that c-Abelson tyrosine kinase (c-Abl) and Protein Kinase C (PKC)-δ are crucial for TGF-β-induced EndMT, and therefore that imatinib mesylate and rottlerin (inhibitors of c-Abl and PKC-δ, respectively) might be effective therapeutic agents for fibroproliferative pathologies in which EndMT plays a role (Li and Jimenez 2011).

It has also been reported that pathways other than TGF-β can lead to EndMT. In myocardial infarction (MI) for example, canonical (β-catenin-dependent) Wnt signaling is induced 4 days after experimental MI in the subepicardial endothelial cells

and perivascular cells. Coincidently with canonical Wnt activation EndMT was also triggered after the infarction. In addition, canonical Wnt signaling induced mesenchymal characteristics in cultured endothelial cells, suggesting a direct role of canonical Wnt signaling in EndMT (Aisagbonhi et al. 2011).

13.3 TGF-β in Vascular Pathologies

Given the importance of TGF-β signaling for blood vessels, it is not surprising that TGF-β signaling is involved in several pathological conditions concerning the endothelium (Fig. 13.3).

13.3.1 Hereditary Hemorrhagic Telangiectasia

Hereditary hemorrhagic telangiectasia (HHT), or Osler–Rendu–Weber syndrome, is an autosomal dominant vascular dysplasia characterized by the development of mucocutaneous telangiectasias and arteriovenous malformations in the lungs, brain, liver, and gastrointestinal tract (Abdalla and Letarte 2006). Many HHT patients suffer from recurrent nosebleeds, which severely affect their quality of life and are clinically difficult to treat. Mutations in three different genes have been shown to be responsible for HHT. Interestingly these are *ENG* (encoding endoglin), *ACVRL1* (encoding ALK1), and *SMAD4*, and the proteins encoded by these genes are all important players in TGF-β signaling in endothelial cells. Mutations in *ENG* cause

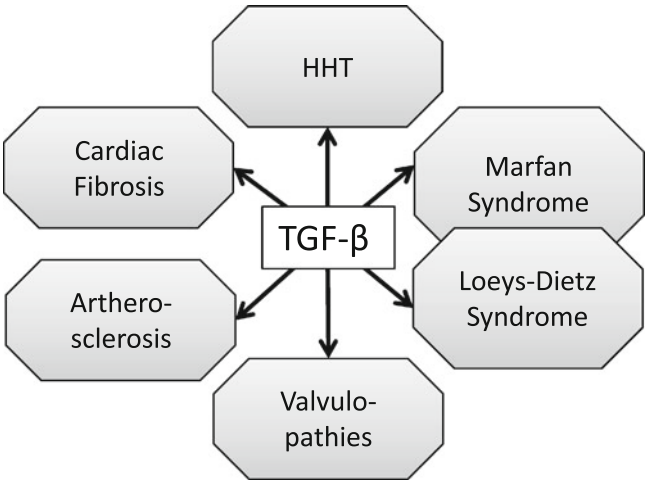

Fig. 13.3 TGF-β plays a central role in vascular pathologies like Marfan and Loeys–Dietz syndrome, hereditary hemorrhagic telangiectasia (HHT), atherosclerosis, cardiac fibrosis, and congenital heart valve defects

HHT type 1 (HHT-1) (McAllister et al. 1994); *ACVRL1* mutations cause HHT-2 (Johnson et al. 1996); and mutations in *SMAD4* cause a syndrome consisting of both juvenile polyposis and a HHT phenotype (Gallione et al. 2006). Endoglin heterozygous knockout mice have dilated and fragile blood vessels which resemble clinical manifestations of HHT-1 patients (Arthur et al. 2000; Bourdeau et al. 2001). Endothelial-specific ALK1 knockout mice suffer from vascular malformations mimicking all pathologic features of HHT-2 (Park et al. 2009). Several clinical trials in HHT patients are ongoing with anti-angiogenesis agents, including bevacizumab, a neutralizing antibody against VEGF and thalidomide to inhibit bleedings and other vascular malformations associated with HHT. Recently, it was reported that thalidomide treatment of a small group of HHT patients reduced the severity and frequency of nosebleeds (epistaxis) in the majority of the patients. In addition, in heterozygous endoglin knockout mice thalidomide treatment stimulated mural cell coverage and thus rescued vessel wall defects. Thalidomide treatment increased platelet-derived growth factor-B (PDGF-B) expression in endothelial cells and stimulated mural cell activation. The effects of thalidomide treatment were partially reversed by pharmacological or genetic interference with PDGF signaling from endothelial cells to pericytes (Lebrin et al. 2010).

13.3.2 Marfan Syndrome and Loeys Dietz Syndrome

Marfan syndrome (MFS) is a genetic disorder that has been linked to mutations in the *FBN1* gene, which encodes for the extracellular matrix glycoprotein Fibrillin-1 (Dietz et al. 1991). This protein forms an important component of elastic fibers in the aorta, ligaments, eye, and other tissues. Inactivating *FBN1* mutations in MFS affect cardiovascular, pulmonary, ocular, and skeletal tissues, among other tissues (Ramirez and Dietz 2007). Most dangerous are the aortic aneurysms, which carry the risk of sudden rupture and death. Initially it was thought that MFS was caused by structural defects in elastic fibers. However, fibrillin-1 not only serves an important role in providing structural integrity of connective tissues, but also acts as reservoir for growth factors such as TGF-β (ten Dijke and Arthur 2007). The latter function appears to be prominently associated with the vascular pathologies of MFS (Bolar et al. 2012). Fibrillin-1 contains multiple latent TGF-β binding motifs, and can associate with LTBP-1 and control the availability of active extracellular TGF-β (Chaudhry et al. 2007). Insufficient or nonfunctional fibrillin-1 as result of a *FBN1* mutation may lead to the release of bioactive TGF-β, and thereby trigger elevated TGF-β/Smad2 signaling (Habashi et al. 2006; Neptune et al. 2003).

A disorder closely related to MFS is Loeys Dietz syndrome (LDS). The latter has been linked to mutations in *ALK5*, *TGFBR2*, and *TGF-β2* (Lindsay et al. 2012; Loeys et al. 2005). LSD overlaps clinically to a large extent with MFS, but LSD has also unique properties, including widely spaced eyes. Some of the TGF-β receptor mutations in LSD were found to be inactivating mutations (Horbelt et al. 2010; Loeys et al. 2006), which paradoxically leads to overactive TGF-β/Smad2 signaling.

Both MFS and LDS demonstrate an upregulation of TGF-β/Smad2 signaling. Consistent with the notion of overactive TGF-β signaling, neutralizing antibodies for TGF-β in animal models for MFS prevented the formation of fragmented elastin fibers and aortic aneurysms (Neptune et al. 2003). Importantly, losartan, an angiotensin inhibitor that inhibits TGF-β signaling through not well-understood mechanisms, also demonstrated a protective effect against aortic aneurysms (Habashi et al. 2006). Losartan has recently moved into phase III clinical trial in patients with MFS. Recently, the non-Smad signaling pathways, i.e., extracellular regulated kinase (ERK) and Jun N-terminal kinase (JNK) MAP kinases, were found to be elevated in MFS and to contribute to aortic aneurysms development (Holm et al. 2011). Selective inhibition of ERK or JNK in a MFS mouse model was found to inhibit aortic growth. Thus, inhibition of ERK and JNK may be beneficial for treatment of MFS.

While aortic aneurysms are one of the complications in MFS and LDS, familial thoracic aortic aneurysms and dissections can also result from mutations in these genes, resulting in loss of signaling through the TGF-β type I and II receptor, in the absence of all features of MFS and LDS (Milewicz et al. 1996; Pannu et al. 2005; Tran-Fadulu et al. 2009). Furthermore, mutations in *SMAD3* have been linked to the aneurysms osteoarthritis syndrome, a form of TAAD with tortuosity of the arterial tree and early onset of osteoarthritis (van de Laar et al. 2012; Regalado et al. 2011). In addition, the Shprintzen–Goldberg syndrome (SGS) that has considerable phenotypic overlap with MFS and LDS, including aortic aneurysm, was found to be linked with mutations in the proto-oncogene SKI, a known repressor of TGF-β activity (Doyle et al. 2012). Whether alterations in these TGF-β signaling components are inhibiting or enhancing TGF-β signaling is still a matter of debate.

13.3.3 TGF-β Signaling and Valvulopathies

TGF-β is crucial for valve development and homeostasis. The development of the valve starts with the formation of the cardiac cushion by EndMT of the endocardial cells. This occurs in the atrioventricular canal, which separates the atria and ventricles, and the outflow tract, which connects the ventricles with the aortic sac of the developing heart (Fig. 13.4) (Kruithof et al. 2012). Endocardial cushions are the primordia of the septa and valves and become mature structures by mesenchymal cell proliferation, remodeling, and valve elongation, resulting in thin leaflets with highly organized extracellular matrix. Valve maturation continues after birth before reaching the adult configuration with the mechanical properties to withstand the continuous changing of the hemodynamic environment during the heart cycle (Kruithof et al. 2012).

TGF-β is one of the key regulators of EndMT and an increased expression of TGF-β1 can be seen in the endocardial cells overlying the cushions, while TGF-β2 is expressed in the myocardium and endocardium flanking the cushions during initiation of cushion development (Akhurst et al. 1990; Molin et al. 2003). The

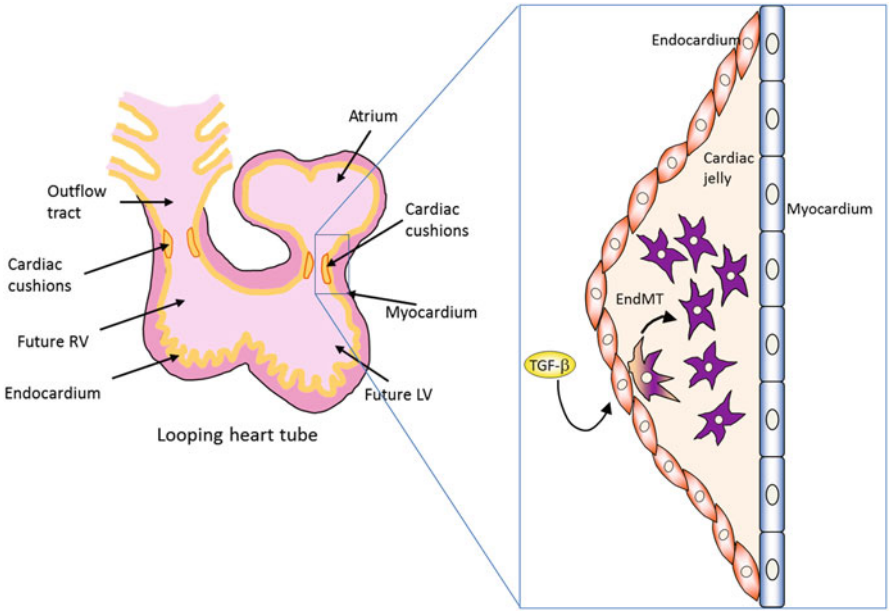

Fig. 13.4 Cardiac valve development. During embryogenesis the cardiac valves develop by the TGF-β-dependent mechanism of EndMT of the endocardial cells

requirement for TGF-β in cushion formation was initially shown using chicken explant cultures. When cultured in the presence of antisense oligo's or TGF-β neutralizing antibodies, EndMT, necessary for development of the cushions, was blocked (Boyer et al. 1999; Potts et al. 1991). Interestingly TGF-β1-deficient embryos, born to TGF-β1-null mothers, demonstrate severe cardiac abnormalities, including disorganized valves (Dickson et al. 1995). Furthermore, the valves in TGF-β2-deficient mice at E18.5 displayed defective remodeling and differentiation resulting in thickened valves (Azhar et al. 2011; Bartram et al. 2001; Sanford et al. 1997). The expression of TGF-β3 increases as valve development proceeds into advanced stages of heart development suggesting that TGF-β3 may play an important role in valve structure and function postnatal (Camenisch et al. 2002). Endocardium-specific deletion of ALK5 resulted in severe hypoplastic AV cushions, consistent with a crucial role for TGF-β signaling in EndMT (Sridurongrit et al. 2008). Endoglin is expressed in endocardial cells just before EndMT and endoglin deficiency results in embryonic lethality between E10.5 and E11.5 due to vascular abnormalities and disturbed cardiac development including hypocellular cushions (Arthur et al. 2000; Bourdeau et al. 1999; Li 1999). The TGF-β type III receptor betaglycan is also expressed in the endocardium during cushion formation (Stenvers et al. 2003), and betaglycan mutant mice die at the end of gestation harboring a thin ventricular wall and poorly developed septa (Compton et al. 2007).

Cardiac valve formation is crucial for proper heart function and septal/valve malformations comprise the largest part of congenital heart defects. Perturbation of TGF-β signaling pathway has been associated with both syndromic and non-syndromic congenital heart disease. Mutations in fibrillin-1, found in the genetic disorder marfan (see above), may cause thickening and elongation of the mitral valve leaflet, which can result in prolapse of the valve function (Judge et al. 2011). Mutations in Filamin-A, able to regulate TGF-β signaling, was found in patients with thickened valves (Kyndt et al. 2007). Furthermore, Williams syndrome, which has an elastin haploinsufficiency, shows elongated aortic valves, increased proliferation, and decreased TGF-β signaling (Hinton et al. 2010). Interestingly, a recent study, using a small molecule to inhibit ALK5 kinase activity, showed that indeed this receptor is important for the effect TGF-β has on valve formation. Using two independent chemical inhibitors lesions in the heart valves consisting of hemorrhages, inflammation, degeneration and interstitial cell activation, and proliferation were observed (Anderton et al. 2011). Most likely normal tissue repair is blocked as a consequence of ALK5 inhibition, and the valves may be particularly affected as a result of ongoing mechanical stress at opening and closure during each heart cycle.

13.3.4 TGF-β Signaling in Atherosclerosis

Atherosclerosis is a chronic inflammatory response in the arterial wall and characterized by vascular plaque formation (Libby et al. 2011). Stable plaques contain more collagen and smooth muscle cells, whereas unstable plaques have more macrophages and contain a large lipid core covered with a thin fibrous cap. Unstable plaques are more likely to rupture and cause clinical symptoms, e.g., cerebrovascular accidents or myocardial infarction. In atherosclerotic lesions, the TGF-β signaling components are detectable in endothelial cells, SMCs, myofibroblast, dendritic cells, T cells, and monocyte/macrophages and are rapidly upregulated during vascular injury (Bobik et al. 1999; Bot et al. 2009; Frostegård et al. 1999; Kalinina et al. 2004). There is some controversial information regarding the role of TGF-β in atherogenesis (Grainger 2004; Toma and McCaffrey 2012). Some reports show that increased TGF-β1 expression in human atherosclerotic plaques correlated with advanced atherosclerosis (Herder et al. 2012; Panutsopulos et al. 2005; Wang 1997), and other studies showed an inverse relationship between TGF-β1 levels in serum and the development of atherosclerosis, suggesting an anti-atherogenic effect (Grainger et al. 1995; Hering et al. 2002; Mallat et al. 2001). Furthermore, inhibition of TGF-β activity by various approaches resulted in pro-atherogenic changes in the vessel wall in animal models of atherosclerosis. Also an important role of TGF-β as an immune modulating cytokine in atherosclerosis was reported. Inhibiting TGF-β signaling in Apoe$^{-/-}$ mice by using a recombinant soluble TGF-RII (Lutgens 2002) or a blocking TGF-β1 antibody (Mallat et al. 2001) resulted in accelerated atherosclerosis. Lesions exhibited an unstable phenotype that contained low amounts of fibrosis, an increased amount of inflammatory cells, and even intraplaque hemorrhages.

Cardiac overexpression of TGF-β1, resulting in increased plasma levels of TGF-β, limited plaque growth and induced plaque stabilization (Frutkin et al. 2009). Mice with abrogated TGF-β signaling in T cells ($Apoe^{-/-}$CD4-dnTGF-βRII) (Gojova et al. 2003; Robertson et al. 2003) or DC cells ($Apoe^{-/-}$CD11cDNR) (Lievens et al. 2012) both showed accelerated lesion progression, with plaques containing abundant inflammatory cells paralleled by a decrease in plaque fibrosis.

It might be proposed that TGF-β signaling participates in the development of atherosclerosis, but may more interestingly promote a stable lesion phenotype, suggesting its role in the protection of acute ischemic situations like myocardial infarction. Interestingly, human aortic plaque vascular smooth muscles cells were shown to have enhanced levels of endoglin, supporting a role for endoglin in vascular wall integrity (Bot et al. 2009; Conley et al. 2000). In addition, soluble endoglin levels were found elevated in patients with coronary artery disease and atherosclerosis (Li et al. 2000b), and changes in soluble endoglin plasma levels post-myocardial infarction (MI) have predictive value for acute mortality in this patient group (Cruz-Gonzalez et al. 2008).

13.3.5 TGF-β Signaling in Cardiac Fibrosis

The development of heart failure starts with an acute MI or chronic injury, resulting in reduced ventricular performance and increased wall stress. Pathological deposition of extracellular matrix and myocardial hypertrophy are compensatory changes to reduce wall stress as the ventricle dilates. This will eventually lead to heart failure (Cohn et al. 2000). The process of cardiac fibrosis can be divided into two types: reactive fibrosis in response to inflammatory processes and reparative or replacement fibrosis in cell response to cell death and loss of myocardial tissue (Beltrami et al. 1994; Park et al. 2009; Silver et al. 1990). Cardiac fibroblasts are the interconnected cells that lie within the myocytes and extracellular space, and are the primary source of collagen in the heart (Souders et al. 2009). Activation of these fibroblasts or change of the fibroblast phenotype to myofibroblasts is what drives ECM accumulation and the development of pathological fibrosis. The local increase of TGF-β by various stimuli including inflammation, ischemia, mechanical stress, and vasoactive hormones like angiopoietin II is a key driver of this profibrotic process (Creemers and Pinto 2011; Dobaczewski et al. 2011; Goumans et al. 2008). Expression levels of TGF-β are increased in the left ventricle of both hypertrophic and idiopathic-dilated cardiomyopathy (Li et al. 1997; Martin et al. 2005; Pauschinger et al. 1999; Villarreal and Dillmann 1992), and in the border-zone of the infarcted region following myocardial infarction (Chuva de Sousa Lopes et al. 2004; Frantz et al. 2008) and due to diabetes (Connelly et al. 2009; Westermann et al. 2007).

That indeed TGF-β promoted myocardial hypertrophy and fibrosis was shown by overexpressing TGF-β1 in mice (Rosenkranz et al. 2002). These animals developed significant cardiac hypertrophy accompanied by interstitial fibrosis. In addition,

TGF-β1 heterozygous animals were protected from age-associated cardiac fibrosis and diastolic dysfunction (Brooks and Conrad 2000). Furthermore, treating animals with an anti-TGF-β neutralizing antibody prevented collagen accumulation following pressure overload and attenuated diastolic dysfunction without affecting cardiac hypertrophy (Ellmers et al. 2008; Kuwahara et al. 2002; Okada et al. 2005). Interestingly, mice overexpressing a dominant negative TGF-βRII showed markedly reduced collagen deposition following pressure overload, resulting in increased left ventricular dilatation and diastolic dysfunction (Lucas et al. 2010; Okada et al. 2005). Endoglin is also expressed in cardiac fibroblast and found to be increased during Ang-2-induced cardiac fibrosis. Recently, Kapur and coworkers showed that when treating animals with soluble endoglin 1 day before aortic bending, cardiac fibrosis and the development of heart failure were reduced (Kapur et al. 2012).

The functional effects of TGF-β signaling are likely to be biphasic. TGF-β signaling is necessary to preserve cardiac structure to protect the heart from the increased pressure and uncontrolled matrix degradation, while excessive TGF-β signaling results in enhanced collagen deposition, increased myocardial stiffness, and diastolic dysfunction. That TGF-β may function as a master switch is nicely demonstrated by inhibiting TGF-β signaling post MI. Early TGF-β inhibition within 24 h after occlusion of the coronary artery enhanced the inflammatory response and increased mortality (Frantz et al. 2008; Ikeuchi et al. 2004), while late TGF-β inhibition after the inflammatory phase of infarct healing reduced the number of myofibroblasts and improved cardiac output (Okada et al. 2005).

Both the intracellular Smad signaling pathways as well as non-Smad pathways are thought to play a role in the pathological remodeling of the heart. Smad3 was shown to be involved in myofibroblast transformation and mediated the TGF-β-induced ECM production and tissue inhibitor of matrix metalloprotease (TIMP) upregulation (Dobaczewski et al. 2010). In addition, TGF-β-activated kinase (TAK1) is activated in the pressure overloaded myocardium, and activated TAK expressed in the mouse myocardium induces cardiac hypertrophy and severe systolic dysfunction (Zhang et al. 2000). Finally, an indirect effect of TGF-β on cardiac fibrosis, by inducing, e.g., connective tissue growth factor (CTGF) and endothelin (Leask 2010), cannot be excluded.

13.4 Concluding Remarks

TGF-β plays a pivotal role in controlling cardiovascular homeostasis. Mutations in human TGF-β signaling components such as TGF-β receptors and Smads have been directly linked to cardiovascular disorders. Moreover, genetic studies in mouse models revealed that too little or overactive TGF-β signaling in ECs and SMCs leads to vascular dysfunction. Several small molecular weight compounds and antibodies that modulate TGF-β or BMP signaling are being evaluated in (pre)clinical trials for cardiovascular disorders. Examples are: losartan to inhibit overactive TGF-β signaling in Marfan syndrome (Möberg et al. 2012), Bevacizumab

(a VEGF-A neutralizing antibody) and thalidomide to normalize and stabilize the weak vessel phenotype of HHT patients (Dupuis-Girod et al. 2012; Lebrin et al. 2010), and TGF-β receptor kinase inhibitors to correct the BMP/TGF-β imbalance seen in pulmonary hypertension (Long et al. 2009). However, with TGF-β being such a multifunctional model with effects on many different cell types that are highly context dependent, TGF-β signaling components remain very challenging therapeutic targets. Current treatments that target TGF-β or its receptors are not selective for only the pathological signaling pathways triggered by TGF-β. Careful selection and monitoring of patients are needed to guard for side effects.

There remains to be urgent need to more precisely dissect the mechanisms of TGF-β in vascular cells and identify cell type-specific regulators of TGF-β signaling, thereby enabling strategies to selectively target "bad" responses, while leaving "good" effects of TGF-β intact. The use of conditional knockouts and knockins for TGF-β signaling components in different cells, different tissues, and different times will be very instrumental to achieve this. Moreover, induced pluripotent stem cells (iPSCs) technology may be used to generate cardiovascular patient-derived endothelial and smooth muscle cells (Reed et al. 2012). These cells can be used not only to study pathology, but can also be valuable as tools to identify small molecule drugs that rescue the disease cell phenotype. Advances in all the above research fields will provide new opportunities for treatment of increasing number of vascular pathologies that are associated with dysregulation in TGF-β signaling.

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