



TGF- β Signaling in Control of Cardiovascular Function

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Genetic studies in animals and humans indicate that gene mutations that functionally perturb transforming growth factor β (TGF- β) signaling are linked to specific hereditary vascular syndromes, including Osler–Rendu–Weber disease or hereditary hemorrhagic telangiectasia and Marfan syndrome. Disturbed TGF- β signaling can also cause nonhereditary disorders like atherosclerosis and cardiac fibrosis. Accordingly, cell culture studies using endothelial cells or smooth muscle cells (SMCs), cultured alone or together in two- or three-dimensional cell culture assays, on plastic or embedded in matrix, have shown that TGF- β has a pivotal effect on endothelial and SMC proliferation, differentiation, migration, tube formation, and sprouting. Moreover, TGF- β can stimulate endothelial-to-mesenchymal transition, a process shown to be of key importance in heart valve cushion formation and in various pathological vascular processes. Here, we discuss the roles of TGF- β in vasculogenesis, angiogenesis, and lymphangiogenesis and the deregulation of TGF- β signaling in cardiovascular diseases.

Transforming growth factor β 1 (TGF- β 1) is the prototype of a large family of structurally related, secreted dimeric proteins that have pleiotropic effects and play important roles in cell-to-cell signaling. Other members of this family include the closely related TGF- β 2 and - β 3 and more distantly related proteins like activins and inhibins, nodal proteins, and bone morphogenetic proteins (BMPs) (Hinck et al. 2016; Morikawa et al. 2016). TGF- β s regulate a large variety of cellular processes in many different cell types. Their effects are context-dependent, including the induction of proliferation, apoptosis, migration, adhesion, extracellular matrix (ECM) protein production, and cytoskeletal organization (Massagué 2012; Mori-

kawa et al. 2016). Consequently, many TGF- β family cytokines play essential roles in embryonic development, stem cells, and cell fate determination and in adult tissue homeostasis and repair (Moustakas and Heldin 2009; Wu and Hill 2009; Itoh et al. 2014). Perturbations in the actions of TGF- β can lead to pathological conditions, including cardiovascular diseases, fibrotic disorders, and cancer (Harradine and Akhurst 2006; Ikushima and Miyazono 2010; Dooley and ten Dijke 2012; Pardali and ten Dijke 2012; Morikawa et al. 2016). Therapeutic intervention to normalize perturbed TGF- β signaling is an emerging area of intense research (Hawinkels and ten Dijke 2011; Akhurst and Hata 2012; Chang 2016).

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Misregulated TGF- β signaling in humans causes vascular pathologies and cardiovascular disease such as arteriovenous malformations (AVMs), aneurysms, atherosclerosis, cardiac fibrosis, vascular remodeling of the retina (retinopathy), and valvular heart disease. Additionally, TGF- β signaling contributes to endothelial tumors like hemangiomas (Pardali et al. 2010; Akhurst and Hata 2012). The importance of the TGF- β signaling pathways in the spatial and temporal regulation of heart and blood vessel morphogenesis, as well as cardiovascular homeostasis, is evident when analyzing the phenotypes of mice deficient in components of the TGF- β signaling cascade (Goumans and Mummery 2000; Goumans et al. 2009). The multifunctional and context-dependent activities of TGF- β and its interactions with nonvascular cells (e.g., immune cells) complicate the interpretation of its *in vivo* roles in cardiovascular biology. In this review, we only focus on TGF- β as the role of BMP in angiogenesis is discussed elsewhere (Goumans et al. 2017). First, we discuss vascular development and TGF- β signaling, followed by the mechanisms that are at the basis of TGF- β 's control of vascular function, its effects on endothelial cells (ECs), smooth muscle cells (SMCs), and pericytes, and how a misbalance in TGF- β signaling leads to vascular dysfunction.

BLOOD AND LYMPHATIC VASCULAR NETWORK FORMATION

The Vascular System

The heart, blood, and blood vessels make up the vascular system, which supplies oxygen and nutrients to all cells of the body and removes waste products (Potente et al. 2011). This is achieved by pumping blood through a highly branched vascular network of specialized blood vessels (i.e., arteries, capillaries, and veins). Blood vessels are lined with a single layer of ECs, and stabilized by a basal lamina and a layer of connective tissue containing SMCs or pericytes. The amount of connective tissue and number of smooth muscle cells or pericytes present in the vessel wall depends on the diameter of the vessel and its function.

This vascular network is constructed using two highly coordinated and sequential processes, vasculogenesis and angiogenesis. During vasculogenesis (Fig. 1), mesoderm will first differentiate into proliferating EC precursors known as angioblasts. These angioblasts will differentiate into ECs that align, fuse, and gradually acquire a lumen (Ferguson et al. 2005). Vasculogenesis ends with the formation of a honeycomb-like primary vascular plexus. During angiogenesis (Fig. 1), the primary capillary plexus is remodeled into a stable hierarchical branched network, containing arteries, capillaries, and veins. Vascular endothelial growth factor (VEGF) signaling is a key pathway involved in vascular remodeling, as VEGF loosens cell–cell contacts within the newly formed capillaries and causes local degradation of the ECM. Activated ECs start to proliferate in response to VEGF or basic fibroblast growth factor (bFGF or FGF-2), and form a new sprout (Fig. 1). Loosening of cell–cell contacts can also result in fusion of capillaries to form arteries and veins.

The final step in vascular development is initiated by the blood circulation. In this phase, nonfunctional sprouts that were unable to fuse to patent passages are pruned. The remaining vessels are shaped and remodeled by the blood flow to suit local tissue needs (Adams and Alitalo 2007; Carmeliet and Jain 2011). VEGF is also steering vascular remodeling and pruning. Once this process is finished, TGF- β and platelet-derived growth factor (PDGF)-BB are secreted by the endothelium and stabilize the mature vascular network (Fig. 1). TGF- β stimulates the production and stabilization of the ECM and, together with PDGF-BB, attracts and regulates the differentiation of pericytes and SMCs, which surround and enclose the primitive vascular tube (Bergers and Song 2005). Finally, TGF- β promotes arterial-venous specification necessary for the establishment and enlargement of arteries and veins (Fish and Wythe 2015).

Although vasculogenesis occurs mainly during embryonic development, angiogenesis is responsible for the formation of new blood vessels after birth. Angiogenesis will result in vascularization of growing tissues and during healing, and in the formation of capillaries,

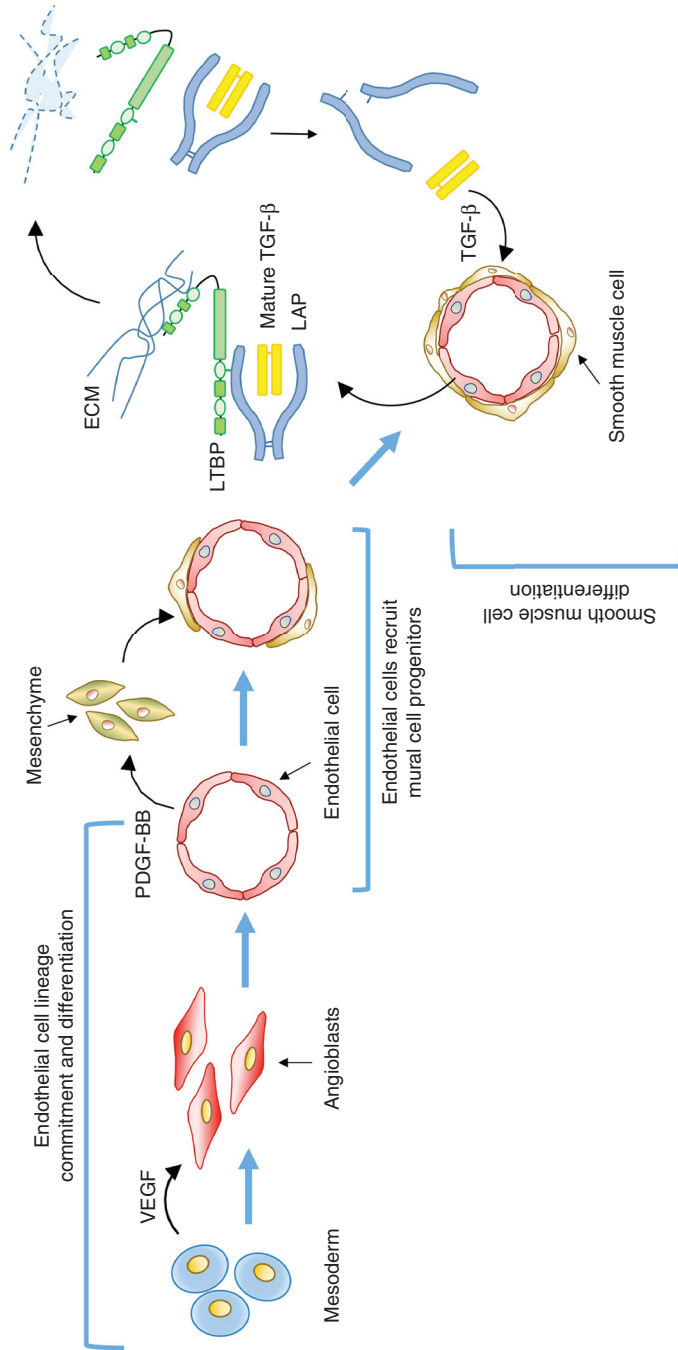


Figure 1. Process of vasculogenesis. Vasculogenesis starts with the differentiation and proliferation of mesodermal cells into angioblasts followed by their differentiation into endothelial cells (ECs) in response to vascular endothelial growth factor (VEGF). These ECs fuse and form a lumen in a honeycomb structure. Platelet-derived growth factor (PDGF) secreted by ECs induces recruitment of mesenchymal cells that will differentiate into smooth muscle cells (SMCs) or pericytes. When SMCs or pericytes adhere to ECs, the ECs start to produce TGF- β . TGF- β is produced as a propeptidopeptide, which is proteolytically processed, and forms a small latent TGF- β complex consisting of the mature TGF- β dimer associated with two latency-associated peptides (LAPs). The small latent TGF- β complex binds to latent transforming growth factor β binding protein (LTBP), and this complex is secreted as the large latent TGF- β complex. LTBP binds to extracellular matrix (ECM) proteins such as fibronectin. Activation of TGF- β by, for example, proteolytic release from LAP will induce growth arrest and terminal differentiation of SMCs.

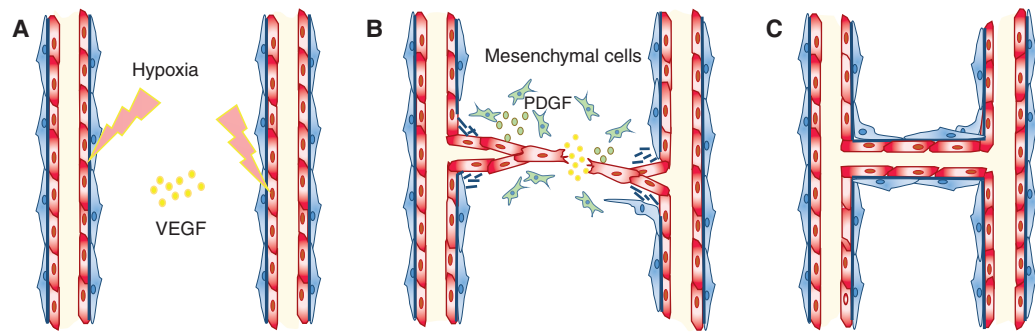


Figure 2. Process of angiogenesis. (A) Angiogenesis begins when endothelial cells are activated, for example, by hypoxia. These cells proliferate and the tip cells migrate into the perivascular space toward a vascular endothelial growth factor (VEGF) gradient (B). During the resolution phase, Endothelial cells stop dividing and vascular sprouts fuse, rebuild their extracellular matrix, and attract pericytes and smooth muscle cells in a platelet-derived growth factor (PDGF)-dependent manner. (C) These latter cells will stabilize the newly formed sprout.

the smallest blood vessels in our body (Fig. 2). The formation of new blood vessels is a tightly regulated process, governed by a balance between proangiogenic factors such as VEGF and bFGF and antiangiogenic factors such as angiostatin 1 and thrombospondin (Adams and Alitalo 2007; Carmeliet and Jain 2011; Herbert and Stainier 2011). TGF- β 's effect on blood vessel formation is context- and concentration-dependent and can be either pro- or antiangiogenic (Pardali et al. 2010). Hypoxia induces the secretion of VEGF, which in turn activates the endothelium and induces the formation of tip cells and stalk cells by binding to vascular endothelial growth factor receptor 2 (VEGFR2 or Flk-1). Conversely, the presence of VEGFR1 on the EC surface inhibits angiogenesis, partly by functioning as a VEGF ligand trap.

Angiogenesis is a multistep process (Fig. 2). The first step is a vascular activation phase when VEGF increases vascular permeability and basement membrane degradation, allowing ECs to proliferate and migrate into the extracellular space where they form new capillary sprouts. The second step is a resolution phase when the ECs cease proliferation and migration, reconstitute the basement membrane, and promote vessel maturation (Adams and Alitalo 2007; Carmeliet and Jain 2011). During vessel maturation, ECs secrete TGF- β to recruit mesenchymal cells to the perivascular space, where they differentiate into pericytes and SMCs, and

form a tight, protective coat around the newly formed vessel (Armulik et al. 2011).

The Lymphatic System

The lymphatic system is formed by lymphatic capillaries and collecting lymphatic vessels. The lymphatic vasculature drains the interstitial fluid that leaks out of blood capillaries and returns it to the circulation to maintain interstitial fluid pressure. The lymphatic system is also important for the immune response by transporting white blood cells and antigen-presenting cells (Alitalo and Carmeliet 2002). Like blood vessels, lymphatic vessels are built from ECs and SMCs or pericytes (Karpanen and Alitalo 2008).

Lymphatic vessels originate from blood vessels (Fig. 3). Within the developing embryo, a subset of cardinal vein ECs starts to express lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), followed by expression of prospero homeobox transcription factor (Prox)-1. Prox-1 is a binary transcriptional switch and induces the expression of VEGFR3. Prox-1 is required for lymphangiogenesis because *Prox1*^{-/-} mouse embryos do not form lymphatic endothelial cells (LECs). Prox-1 activates the expression of LEC markers and represses the expression of blood endothelial cell (BEC) markers in mature ECs (Johnson et al. 2008). The Prox-1-expressing BECs start to migrate from the cardinal vein toward VEGF-C-express-

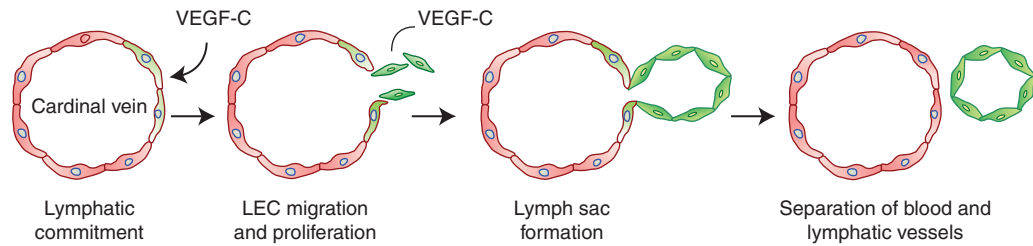


Figure 3. Process of lymphangiogenesis. Lymph vessels originate from blood vessels when and where some of the cardinal vein endothelial cells start to express lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and prospero homeobox transcription factor (Prox)-1 (green cells). The Prox-1-expressing lymphatic endothelial cells (LECs) migrate out of the vessel, proliferate, and form the lymphatic sac, the first lymphatic structure.



ing mesenchymal cells (Karkkainen et al. 2004) and form the first lymphatic structures called the primary sac. Some LECs differentiate into lymphatic valve-forming cells, dedifferentiate into ECs with BEC-like features, or transdifferentiate into fibroblast-like cells (Yang and Oliver 2014a,b). For example, when challenged with blood flow or shear stress, LECs can change into BEC-like cells and incorporate into the blood vessel wall (Chen et al. 2012a). Postnatal lymphangiogenesis is also stimulated by LYVE-1-expressing macrophages; although macrophages may differentiate into LECs (Lee et al. 2010; Ran and Montgomery 2012), they mainly stimulate proliferation of existing LECs by inducing the secretion of growth factors, including VEGF-C or bFGF (Adams and Alitalo 2007).

TGF- β FAMILY SIGNALING

TGF- β is synthesized as an inactive precursor protein (ten Dijke and Arthur 2007; Shi et al. 2011). The TGF- β precursor contains an amino-terminal signal peptide to direct it to the endoplasmic reticulum. After removal of the signal peptide, the remaining propeptide is cleaved by the endoprotease furin (Dubois et al. 1995) to generate a short carboxy-terminal protein, which corresponds to mature TGF- β , and a large amino-terminal propeptide called the latency-associated peptide (LAP). LAP and TGF- β remain noncovalently bound to form the small latent complex (Annes et al. 2003; Robertson et al. 2015; Robertson and Rifkin 2016). TGF- β is secreted from cells as a large latent

complex consisting of a mature dimeric TGF- β molecule, LAP, and a latent transforming growth factor β binding protein (LTBP) (see Fig. 1) (Hyytiäinen et al. 2004; Shi et al. 2011). There are four LTBPs, of which LTBP-1, -3, and -4 can form covalent complexes with the small latent TGF- β complex. LTBPs can target the latent TGF- β complex to the ECM by interacting with fibrillin and other ECM components, preferentially fibronectin, through covalent transglutaminase-induced crosslinks (Hyytiäinen et al. 2004; Robertson et al. 2015; Robertson and Rifkin 2016). Targeting of the latent TGF- β complex to the ECM is important for effective TGF- β bioavailability and activation (Robertson et al. 2015; Robertson and Rifkin 2016).

To exert its effects on cells, TGF- β needs to be released from its latent complex and activated before it can bind to the signaling receptors (see Fig. 1) (Robertson and Rifkin 2016). How TGF- β is liberated from the large latent complex and activated is context- and cell type-dependent. The large latent complex is released from the microfibrils by replacement of LTBP with fibrillin-1 fragments (Chaudhry et al. 2007) or from the ECM by proteolytic cleavage by proteases like plasmin and thrombin (Annes et al. 2003; Robertson et al. 2015). Release or shedding of the large latent TGF- β complex from the ECM may also reduce the generation of active TGF- β , because TGF- β can also be activated through ECM-bound mechanisms (see below) using, for example, the $\alpha_v\beta_6$ integrin complex interacting with the ECM-bound large latent TGF- β complex (Annes et al. 2004).

Activation of latent TGF- β complexes in vitro can be achieved through mechanisms that cause protein denaturation like exposure to high temperature, extreme pH, or ionizing radiation. Although radiation treatment can induce tissue fibrosis in several organs, binding of thrombospondin-1 to LAP (Crawford et al. 1998) and binding of the integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ to the RGD (arginine-glycine-aspartate) sequence in LAP (Sheppard 2005) are important mechanisms for in vivo activation (Shi et al. 2011). Thrombospondin activates latent TGF- β by disrupting the noncovalent interaction between LAP and the active TGF- β dimer (Schultz-Cherry et al. 1995; Ribeiro et al. 1999). Integrin-mediated activation of TGF- β involves binding of $\alpha_v\beta_6$ and $\alpha_v\beta_8$ to the RGD sequence in LAP (Sheppard 2005), which results in deformation of the latent complex by physical force generation in a LTBP-1-dependent manner, and release of active TGF- β (Annes et al. 2004).

The importance of ligand activation for TGF- β to exert its cellular responses becomes evident with the phenotypes of mice deficient for thrombospondin-1 due to *Thbs1* inactivation (Crawford et al. 1998), $\alpha_v\beta_6$ (Munger et al. 1999; Aluwihare et al. 2009), or $\alpha_v\beta_8$ (Zhu et al. 2002; Aluwihare et al. 2009). These phenotypes phenocopy those of *Tgfb1*- and *Tgfb3*-deficient mice, whereas mice deficient in LTBP-4 are not able to activate latent TGF- β (Koli et al. 2004).

Active, dimeric TGF- β exerts its effects on cells by binding to a heteromeric receptor complex, composed of structurally related types I and II receptors, and various coreceptors (Fig. 4) (Heldin and Moustakas 2016). The TGF- β types I and II receptors (T β RI and T β RII) are single transmembrane-spanning proteins with an extracellular, cysteine-rich, ligand-binding domain and an intracellular dual specificity kinase domain, often referred to as serine-threonine kinase domain. There are seven type I

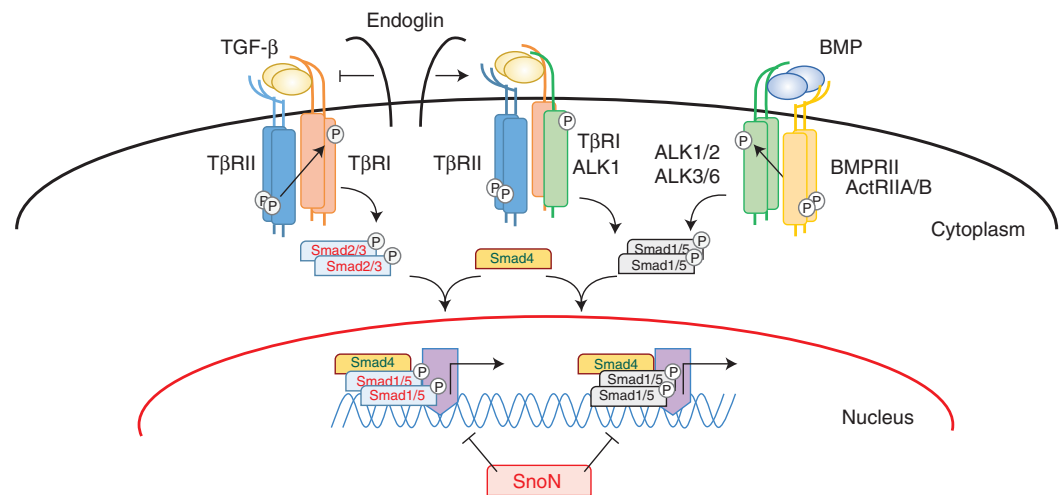


Figure 4. TGF- β signaling pathways. TGF- β s signal by binding to a specific, heteromeric complex that includes types I and II kinase receptors (T β RI/ALK-5 and T β RII, respectively). In most cells, TGF- β binds to a complex of T β RII and T β RI/ALK-5 complex, but in endothelial cells, they can also bind to a complex of T β RII and ALK-1. The coreceptor endoglin inhibits TGF- β binding to the T β RII-ALK-5 complex and promotes TGF- β binding to the T β RII-ALK-1 complex, thus activating ALK-1 signaling. In a similar manner, bone morphogenetic proteins (BMPs) also bind to complexes of two types of receptors—that is, the type I receptors ALK-1, -2, -3, or -6, and the type II receptors BMPRII, ActRIIA, or ActRIIB. Intracellular signaling through Smad activation can be divided into two pathways. In one pathway, ALK-5 phosphorylates, and thus activates, Smad2 and Smad3, and in the other one, ALK-1, -2, -3, and -6 activate Smad1, Smad5, and Smad8. These receptor-activated Smads form heteromeric complexes with the common mediator Smad, Smad4. These complexes translocate into the nucleus, where they act as transcription factor complexes and regulate the expression of specific target genes.

receptors (i.e., ALK-1 through ALK-7), of which ALK-4 and -5 correspond to ActRIB and T β RI, respectively, and five type II receptors (i.e., T β RII, BMPRII, ActRIIA, ActRIIB, and Müllerian inhibiting substance RII). TGF- β binds to a heterotetrameric receptor complex that consists of a T β RII dimer and two type I receptors (Ehrlich et al., 2011). After TGF- β binds to T β RII, T β RI/ALK-5 is phosphorylated by the T β RII kinase on specific serine and threonine residues in the so-called glycine-serine enriched (GS)-domain (Wrana et al., 1994; Hata and Chen, 2016; Heldin and Moustakas, 2016). Upon activation, T β RI initiates intracellular signaling by phosphorylating downstream effectors. Among these, Smad transcription factors play a pivotal role in relaying the signals from the plasma membrane to the nucleus (Heldin et al. 1997; Feng and Derynck 2005; Hill 2016). T β RI phosphorylates Smad2 and Smad3 at their carboxyl termini. Phosphorylated Smad2 and Smad3 form a complex with Smad4 and translocate to the nucleus to activate or repress target gene transcription (Massagué et al. 2005; Ross and Hill 2008; Hill 2016). Activins also induce phosphorylation of Smad2 and Smad3 after binding to a complex of activin receptor IIA or IIB (ActRIIA or ActRIIB) and ALK-4. BMPs bind to heteromeric complexes of either BMP type II receptor (BMPRII), ActRIIA, or ActRIIB with ALK-1, -2, -3, or -6, which then phosphorylate Smad1, Smad5, and/or Smad8 (Heldin et al. 1997; Feng and Derynck 2005; Katagiri and Watabe 2016). Although BMP-9 and -10 bind to ALK-1 and -2 with high affinity (Brown et al. 2005; David et al. 2007), TGF- β binds to a receptor complex of a T β RII dimer and ALK-5 and -1 in cultured ECs (Goumans et al. 2003a). Besides the canonical Smad pathway, TGF- β family members can also activate non-Smad signaling pathways (Derynck and Zhang 2003; Zhang 2016), including TGF- β activated kinase 1 (TAK1)-mediated activation of p38 and c-Jun amino-terminal kinase (JNK) mitogen-activated protein (MAP) kinases (Yamaguchi et al. 1995).

The inhibitory Smads, Smad6 and Smad7, repress TGF- β family signaling. They compete

with R-Smads for phosphorylation or promote degradation of the R-Smads and receptors by recruiting E3 ubiquitin ligases like Smurf1 and Smurf2 (Itoh and ten Dijke 2007; de Boeck and ten Dijke 2012; Miyazawa and Miyazono 2016). Smads regulate gene transcription together with coactivators (e.g., p300 or CBP) and corepressors like c-Ski or SnoN (Massagué et al. 2005; Ross and Hill 2008; Hill 2016). SnoN represses the Smad-mediated transcriptional activity by disrupting the functional heteromeric complexes of Smad2 and/or Smad3 with Smad4 (Stroschein et al. 1999). Upon TGF- β stimulation, SnoN is rapidly degraded in a Smad3-dependent manner. Already after 2 h, the expression of SnoN is markedly increased and, in a negative-feedback loop, terminates TGF- β signaling (Stroschein et al. 1999).

In the TGF- β pathway, endoglin and betaglycan, also known as the T β RIII receptors, have been characterized as accessory receptors. They do not display intrinsic enzymatic activity but, instead, regulate the access of TGF- β ligands to the signaling receptors (Fig. 4) (ten Dijke et al. 2008; Bernabéu et al. 2009; Bilandzic and Stenvers 2011). These two coreceptors possess TGF- β binding sites in their extracellular domains, and their intracellular domains are rich in serine and threonine residues of which specific residues are phosphorylated by T β RI and T β RII (Koleva et al. 2006). Betaglycan must be present at the cell surface for TGF- β 2 to bind to T β RII (Lin et al. 1995). Endoglin and betaglycan have also been reported to enhance BMP signaling of defined BMP family members (David et al. 2007; Kirkbride et al. 2008).

Endoglin exists in two variants arising by alternative splicing, a long form and a short form (L- and S-endoglin, respectively). L-endoglin has a cytoplasmic domain of 47 residues and is the predominant isoform. S-endoglin contains a cytoplasmic tail of only 14 amino acids (Bellón et al. 1993; Pérez-Gómez et al. 2005). Both L- and S-endoglin are able to bind ligand (Bellón et al. 1993), but they differ in their phosphorylation levels (Lastres et al. 1994) and their capacity to regulate certain TGF- β -dependent responses (Lastres et al. 1996).



TGF- β SIGNALING IN ECs

TGF- β has pleiotropic effects on ECs. TGF- β inhibits or promotes angiogenesis depending on several factors like the origin of the EC, ligand concentrations, serum components, the type of exogenously provided ECM, treatment duration, cellular density, and combination of TGF- β receptors expressed at the cell surface. ECs express the more widely distributed T β RI/ALK-5 at their cell surface, but also express ALK-1 to which TGF- β can bind with low affinity. Furthermore, the ALK-5 and -1 pathways have different effects on the expression and signaling of VEGF (Liu et al. 2009; Shao et al. 2009), which together with differential binding of TGF- β to these two type I receptors, may explain the biphasic effect of TGF- β on neovascularization, being either angiogenic, thus stimulating new vessel formation, or antiangiogenic, that is, inhibiting EC proliferation and migration and stimulating maturation of the vessel (Oh et al. 2000; Goumans et al. 2002).

The antiangiogenic effect of TGF- β is mainly mediated by TGF- β signaling through T β RII and ALK-5 (Goumans et al. 2003a). Overexpression of a constitutively active ALK-5 kinase inhibits the proliferation and migration of ECs and induces the expression of plasminogen activator inhibitor 1 (PAI-1) (Goumans et al. 2002). Conversely, inhibition of the ALK-5 kinase with the small molecule SB-431542 stimulates EC proliferation and sheet formation of mouse embryonic ECs in culture (Watabe et al. 2003) and blood vessel formation in an ex vivo fetal mouse metatarsal assay (Liu et al. 2009).

The antiangiogenic properties of this signaling pathway are confirmed in vivo (Table 1). Mice carrying null mutations in the genes encoding TGF- β 1 (Dickson et al. 1995; Bonyadi et al. 1997), T β RII (Oshima et al. 1996), or T β RI/ALK-5 (Larsson et al. 2001) and mice expressing a dominant-negative T β RII mutant in the extraembryonic mesoderm of the yolk sac (Goumans et al. 1999) all die at around embryonic day (E)10.5 because of impaired remodeling of the yolk sac vascular plexus, which prevents the formation of a robust network of vessels. Furthermore, these embryos show re-

duced ECM production, which results in loosely attached layers in the vessel wall. Interestingly, endothelial-specific deletion of T β RII and T β RI recapitulates the phenotype of *Tgfbr2*^{-/-} and *Tgfbr1*^{-/-} mice, respectively, suggesting that the primary cause of the vascular phenotype is a loss of endothelial TGF- β signaling and, consequently, an impairment in SMC recruitment and differentiation (Carvalho et al. 2007). These studies, both in cell culture and in vivo, show that TGF- β signaling through T β RII and ALK-5 keeps the ECs in a quiescent state and is required for vascular network maturation.

The angiogenic capacity of TGF- β results from TGF- β binding to the complex of T β RII with ALK-1 and endoglin, which stimulates EC proliferation, migration, and tube formation (Goumans et al. 2003b). Adenoviral expression of constitutively active ALK-1 stimulates the proliferation of ECs and the formation of endothelial tube-like structures (Goumans et al. 2002; Wu et al. 2006). ALK-1 is also present in EC caveolae, in which it interacts with caveolin-1. This results in enhanced ALK-1 signaling, whereas TGF- β signaling through ALK-5 and Smad2 and Smad3 activation is inhibited (Santibanez et al. 2008). In a mouse model for pancreatic tumors, heterozygosity of the gene encoding for ALK-1 results in a reduction of vascular density within the tumor (Cunha et al. 2010). These cell culture and in vivo findings suggest that ALK-1 signaling is proangiogenic and maintains ECs in an activated state.

Endothelial-specific deletion of *Acvr11/Alk1* in mice shows that ALK-1 is crucial for EC proliferation and the establishment of an arterial identity during development, whereas ALK-1 controls vessel stabilization and integrity during adult life (Tual-Chalot et al. 2014). Expression of a constitutively active ALK-1 in ECs inhibits cell proliferation, migration, and adhesion, indicative of the maturation phase of angiogenesis (Lamouille et al. 2002). Furthermore, silencing ALK-1 expression in cultured ECs results in enhanced vascular sprouting and loss of ephrinB2 (Kim et al. 2012). ALK-1-deficient ECs show enhanced migration and sprout formation in

Table 1. Genetic defects in the cardiovascular (CV) system

Protein (gene name)	Age of death	CV phenotype, animal model	Human genetic CV disorder	References
TGF- β 1 (<i>TGFB1</i>)	E8.5–adult	Owing to gene modifier, ranging from embryonic lethal with (yolk sac) vascular defects to postnatal lethality, caused by autoimmune disease	No CV malformations	Letterio et al. 1994; Goumans and Mummery 2000; Tang et al. 2005
TGF- β 2 (<i>TGFB2</i>)	P0	Defect in cardiac septation and valve remodeling	Loeys–Dietz syndrome type 4; syndromic TAA disorder; Kawasaki disease	Sanford et al. 1997; Azhar et al. 2011; Shimizu et al. 2011; Lindsay et al. 2012; Kruihof et al. 2013; Leutermann et al. 2014
TGF- β 3 (<i>TGFB3</i>)	P0	Defect in pulmonary development and cleft palate. No CV disease	Unknown	Kaartinen et al. 1995; Proetzel et al. 1995
BMP-9 (<i>BMP9</i>)	Viable	Defects in lymphatic drainage, retina vascularization, and valve maturation	Vascular anomaly similar to HHT	Ricard et al. 2012; Levet et al. 2013; Wooderchak-Donahue et al. 2013
T β RII (<i>TGFB2</i>)	E10.5	Abnormal development of yolk sac vasculature and capillary vessel formation in the embryo	Loeys–Dietz syndrome types 1 and 2; MFS	Oshima et al. 1996; Loeys et al. 2005, 2006; Stheneur et al. 2008
BMPRII (<i>BMP2</i>)	E6.5–E9.5	Defect in mesoderm induction; heterogenic: pulmonary hypertension	PAH; HHT	Beppu et al. 2000, 2004; Lane et al. 2000; Soubrier et al. 2013
T β RI/ ALK-5 (<i>TGFB1</i>)	E10.5	Abnormal angiogenesis, impaired EC migration, and fibronectin production	Loeys–Dietz syndrome type 1	Larsson et al. 2001; Loeys et al. 2005, 2006
ALK-1 (<i>ACVRL1</i>)	E11.5	Abnormal angiogenesis, impaired differentiation, and SMC recruitment	HHT-2; PAH	Johnson et al. 1995; Oh et al. 2000; Urness et al. 2000; Fujiwara et al. 2008; Soubrier et al. 2013
Endoglin (<i>ENG</i>)	E10.5–11.5	Angiogenesis defect, arrested remodeling and SMC development, cardiac defects; heterogenic: HHT	HHT-1	McAllister et al. 1994; Bourdeau et al. 1999; Li et al. 1999; Arthur et al. 2000
T β RIII/ betaglycan (<i>TGFB3</i>)	E16.5–P0	Defect in compaction of ventricular wall and cardiac septation	Unknown	Stenvers et al. 2003
Smad1 (<i>SMAD1</i>)	E9.5	No allantois formed; disorganized vessels	Unknown	Lechleider et al. 2001
Smad3 (<i>SMAD3</i>)	Viable	Accelerated wound healing; aortic aneurysm	Aneurysm-osteoarthritis syndrome	Ashcroft et al. 1999; van de Laar et al. 2011; van der Linde et al. 2012; Tan et al. 2013; Wischmeijer et al. 2013

Continued

Table 1. *Continued*

Protein (gene name)	Age of death	CV phenotype, animal model	Human genetic CV disorder	References
Smad4 (<i>SMAD4</i>)	<E7.5	Gastrulation defect, defect in visceral endoderm differentiation	Myhre syndrome, HHT	Sirard et al. 1998; Yang et al. 1998; Piccolo et al. 2014
Smad5 (<i>SMAD5</i>)	E9.5–10.5	Defect in angiogenesis, ectopic vascularization	No CV syndrome	Chang et al. 1999, 2000; Yang et al. 1999
Smad6 (<i>SMAD6</i>)		Cardiovascular abnormalities, defect in endocardial cushion formation	BAV	Galvin et al. 2000
Fibrillin-1 (<i>FBN1</i>)	P0	Aortic aneurysm and rupture, impaired pulmonary function.	MFS1	Carta et al. 2006
Fibrillin-2 (<i>FBN2</i>)	Viable	No CV defects	No CV syndrome	Arteaga-Solis et al. 2011
Fibrillin-1/2 (<i>FBN1/2</i>)	E14.5–16.5	Defects in fiber formation of aortic wall, aortic aneurysms	No CV syndrome	Carta et al. 2006
LTBP-1 (<i>LTBP1</i>)	P0	Persistent truncus arteriosus and aortic arch defects	No CV syndrome	Todorovic et al. 2007
LTBP-3 (<i>LTBP3</i>)		Thoracic aneurism	No CV syndrome	Zilberberg et al. 2015
LTBP-4 (<i>LTBP4</i>)	P9-14	Pulmonary emphysema and cardiomyopathy	ARCL1C	Bultmann-Mellin et al. 2015
LTBP-4S	Adult	Cardiomyopathy	ARCL1C	Sterner-Kock et al. 2002
Thrombospondin-1 (<i>THBS1</i>)	4 wk	Cerebral hemorrhages	No CV syndrome	Crawford et al. 1998

TAA, Thoracic aortic aneurysm; HHT, hereditary hemorrhagic telangiectasia; PAH, pulmonary arterial hypertension; EC, endothelial cell; SMCs, smooth muscle cells; BAV, bicuspid aortic valve; MFS, Marfan syndrome; ARCL1C, autosomal-recessive cutis laxa type IC.

response to angiogenic stimuli in cell culture (Choi et al. 2013). Finally, mouse embryos deficient for ALK-1 expression display dilated large vessels and delayed SMC differentiation and migration. *Alk-1*^{-/-} embryos show excessive fusion of capillary networks and the major arteries and veins, thus giving rise to AVMs (Oh et al. 2000; Urness et al. 2000; Park et al. 2008), suggesting a role for ALK-1 in vessel maturation. The contradictory findings on the role of ALK-1 in TGF- β -stimulated angiogenesis suggest a delicate balance between the pro- and antiangiogenic effects of TGF- β . This balance must be unraveled before we can fully understand how TGF- β signaling affects angiogenesis during development and disease.

Endoglin is highly expressed in ECs and plays an important role in regulating the balance between the pro- and antiangiogenic responses of TGF- β . Endoglin enhances TGF- β signaling through ALK-1 and Smad1 and Smad5 activation and inhibits TGF- β signaling through ALK-5 and Smad2 and Smad3 phosphorylation (Lebrin et al. 2004; Pomeranec et al. 2015). Endoglin-deficient mice die around E11.5, and both the yolk sac and the embryonic vasculature are affected (Table 1) (Li et al. 1999; Arthur et al. 2000; Bourdeau et al. 2000b), suggesting a proangiogenic role of endoglin. *Eng*^{-/-} mouse embryos develop enlarged and weak vessels and at E9.5 lack SMCs around the major vessels in the embryo (Li et al. 1999). This defect in SMC de-

velopment precedes the defect in endothelial remodeling. However, defective TGF- β signaling in ECs reduces the endogenous levels of TGF- β 1 protein and impairs the recruitment and differentiation of the SMC layer (Carvalho et al. 2004). Furthermore, *Eng*^{-/-} mouse embryonic stem cells can differentiate into ECs, whereas endoglin deficiency impairs VEGF-induced angiogenesis (Liu et al. 2014). Finally, endoglin contributes to both shear-induced collateral artery growth and ischemia-induced angiogenesis in a mouse hind limb ischemia model, suggesting a role for endoglin in stimulating both angiogenesis and arteriogenesis (Seghers et al. 2012).

SnoN inhibits decapentaplegic (DPP)- and activin-induced vein formation in the *Drosophila* wing model (Ramel et al. 2007). Introducing a mutant form of SnoN, which is unable to bind Smads and abolishes the ability of SnoN to repress TGF- β signaling, in the original mouse *Skil* locus that encodes for SnoN results in embryonic lethality because of cardiovascular defects. Although vasculogenesis does occur, angiogenesis is impaired in both the yolk sac and the embryo proper (Pan et al. 2009; Zhu et al. 2013). Furthermore, the embryos expressing the mutant SnoN develop AVMs, a typical feature of *Alk1*^{-/-} embryos, and show reduced expression of the arterial marker ephrinB2. ECs isolated from these SnoN mutant embryos show an increased expression of PAI-1, which is encoded by a T β RI/ALK-5-induced Smad3 target gene and represses EC migration (Zhu et al. 2013). The SnoN mutation reduces the expression of the inhibitor of differentiation 1 (Id1), encoded by an ALK-1-induced Smad1 and/or Smad5 target gene, and reduces the ability of ALK-1 to form complexes with Smad1 and Smad5 after TGF- β or BMP-9 stimulation. In contrast, no difference in ALK-5-induced Smad2 and Smad3 complex formation is observed between wild-type and SnoN mutant cells (Zhu et al. 2013). These results suggest that SnoN prevents functional Smad interaction at targeted promoters.

Similar to the biphasic effects reported for TGF- β (Pepper et al. 1993; Goumans et al. 2002), BMP-9 also regulates EC proliferation and differentiation in a context-dependent manner.

BMP-9 inhibits EC proliferation at high concentrations (David et al. 2007; Scharpfenecker et al. 2007) but stimulates proliferation of multiple EC types at low concentrations (Suzuki et al. 2010). The underlying mechanism for this differential response is not clear. One explanation might be the presence of BMP-10, because both BMP-9 and -10 are expressed at significant levels in human and mouse sera (Chen et al. 2013) and are found to be critical for postnatal retinal vascular remodeling (Ricard et al. 2012). Although BMP-9 is dispensable during embryonic and neonatal vascular development (Chen et al. 2013), early postnatal lymphatic development is disrupted on genetic deletion, antibody suppression, or when an ALK-1-Fc ligand trap is used to inhibit both BMP-9 and -10. This defect in development of the lymphatic microvessels and maturation of the lymphatic plexus is similar to that observed as a result of silencing ALK-1 expression in mice (Niessen et al. 2010). Analyses of retinal vascular differentiation showed that endothelial-specific silencing of either *Eng* or *Alk1* causes a delay in vascular remodeling of the retinal capillary plexus (Mahmoud et al. 2010; Tual-Chalot et al. 2014). Treating mice with a ligand trap for BMP-9 and -10 (ALK-1-Fc) or for BMP-10 (anti-BMP-10 antibody) in a *Bmp9*-deficient background revealed that BMP-10 can substitute for BMP-9 in retinal angiogenesis. This apparent substitution may be explained partly by enhanced Notch signaling, and partly by decreased expression of the pro-angiogenic factor apelin, which activates the G-protein coupled receptor apelin receptor (APLNR, alias APJ) (Ricard et al. 2012).

Lymphatic Endothelium

TGF- β signaling has a dual effect on LECs. TGF- β prevents differentiation of LECs by inhibiting the expression of Prox-1 and LYVE-1 in cultured LECs (Oka et al. 2008). In a mouse model of pancreatic ductal adenocarcinoma, inhibition of TGF- β signaling using a T β RI kinase inhibitor results in enhanced lymphangiogenesis (James et al. 2013). LEC-specific deletion of *Tgfb2* or *Tgfb1* in vivo using a Prox-1-specific Cre-driver (*Prox1-Cre*^{ERT2}; *Tgfb2*^{f/f} and *Prox1-*

Cre^{ERT2}; Tgfb¹^{f/f}) results in severe reduction of lymphatic vessel sprouting and remodeling and inhibits LEC proliferation (James et al. 2013). Stimulation of LECs with BMP-9 reduces the number of LECs (Yoshimatsu et al. 2013). Furthermore, systemic intraperitoneal administration by injection of the BMP-9 ligand trap ALK-1-Fc into neonatal mice at P1, P3, and P5 disturbs postnatal lymphatic vessel development in the retina, tail, and skin (Niessen et al. 2010). *Alk1*- or *Bmp9*-deficient mice develop enlarged lymphatic vessels (Yoshimatsu et al. 2013), again highlighting the importance of this signal transduction pathway for lymphatic vessel maturation and lymphatic valve formation.

BMP-9 signaling through ALK-1 was also shown to convert ECs from a LEC phenotype to a BEC phenotype (Yoshimatsu et al. 2013). The loss of LEC-specific markers like Prox-1 coincides with the activation of several genes known to be involved in lymphatic valve formation, such as *Foxc2* and *Nrp1* (encoding neuropilin-1), and maturation of the lymphatic vessels (Levet et al. 2013). *Bmp9* inactivation results in enlarged lymphatic-collecting vessels as a result of an increase in number of LECs in the lymphatic wall (Levet et al. 2013). Finally, BMP-9 prevents tumor lymphangiogenesis and inhibits lymphangiogenesis in a chronic aseptic peritonitis mouse model (Yoshimatsu et al. 2013). This result shows that TGF- β family signaling is not essential for lymphatic system function under physiological conditions. However, it remains to be elucidated how both TGF- β signaling through ALK-5 and BMP-9 signaling through ALK-1 inhibit LEC proliferation, decrease Prox-1 expression, and reprogram LECs to become BECs.

Mural Cells and Interplay with ECs

In addition to its effects on ECs, TGF- β also has potent, context-dependent effects on pericytes and SMCs. TGF- β stimulates SMC proliferation by inducing the expression of growth factors, including PDGF; however, at high TGF- β concentrations, this response is inhibited (Battegay et al. 1990). Increased levels of Smad3 can switch TGF- β from an inhibitor of SMC prolifer-

ation to an activator of SMC proliferation. TGF- β -induced growth inhibition, causing a G₀/G₁ arrest, requires the activity of p38 MAP kinase (Seay et al. 2005). Intimal hyperplasia, caused by SMC proliferation, is induced by activation of p38 MAP kinase, the extracellular signal-regulated kinase (Erk) MAP kinases, and PI3K (phosphatidylinositol-3 kinase)-Akt (Suwanabol et al. 2012) signaling. Furthermore, increased VEGF expression enhances intimal hyperplasia because VEGF functions as an autocrine inhibitor of SMC apoptosis (Shi et al. 2014). When SMCs are deprived of serum, TGF- β can both inhibit and promote apoptosis. Again, the effect of TGF- β appears to depend on the cell physiological context, including cell-ECM interactions (Hishikawa et al. 1999; Pollman et al. 1999). Additionally, TGF- β was found to inhibit SMC migration by inducing N-cadherin expression, which mediates RhoA activation and modulates the actin cytoskeleton (Nuesse et al. 2011).

TGF- β stimulates SMC differentiation, which is apparent by the induction of α -SMA (α -smooth muscle actin, gene name *ACTA2*), calponin1, and SMC1a (structural maintenance of chromosomes 1A) expression. The effects of TGF- β on the expression of SMC-specific genes require phosphorylation of Smad2 or Smad3 (Chen et al. 2003; Hu et al. 2003; Wang et al. 2012) but also depend on CARG-binding transcription factors such as serum response factor (SRF) and myocardin (Owens et al. 2004). SRF and myocardin physically interact with Smad3 and synergistically transactivate the promoters of *TAGLN* (i.e., the gene encoding SM22 α) and *ACTA2* in a Smad-binding element (SBE)-dependent but CARG box-independent manner (Qiu et al. 2003, 2005). TGF- β can induce pluripotent embryonic stem cells to differentiate into SMCs (Sinha et al. 2004). TGF- β -induced Smad2, Smad3, and p38 MAP kinase activation are required for SMC differentiation (Chen and Lechleider 2004; Deaton et al. 2005) in a RhoA-dependent manner (Chen et al. 2006). Inhibition of RhoA blocks TGF- β -induced SMC contractility (Chen et al. 2006). The ability of TGF- β to induce stem cells to differentiate into SMCs is, in part, mediated by the induced ex-



pression of the Notch ligand, Jagged (Kurpinski et al. 2010). The Notch pathway and the TGF- β pathway also cooperate in promoting mesenchymal stem cell differentiation. The canonical Notch effector, C-promoter binding factor-1 (CBF1), interacts with phosphorylated Smad2 and Smad3 to activate SMC promoters (Darland and D'Amore 2001; Tang et al. 2012).

Interactions between ECs and surrounding mural cells are essential for vascular development (Fig. 1). In elegant, three-dimensional, coculture studies, it was shown that latent TGF- β deposited in the ECM is activated when ECs interact with multipotent 10T1/2 mesenchymal cells. Activated TGF- β then stimulates the differentiation of 10T1/2 cells into SMCs or pericytes, and stabilizes the endothelial, capillary-like structures (Ding et al. 2004). Together with the induction of the expression of the serine protease furin that cleaves the TGF- β precursor, shear stress on ECs stimulates the formation of active TGF- β , which enhances TGF- β signaling and, consequently, the effects of TGF- β on ECs and SMCs (Negishi et al. 2001). Shear stress also enhances TGF- β signaling by inducing the expression of TGF- β 1 and - β 3 in ECs (Egorova et al. 2011; Walshe et al. 2013).

TGF- β signaling controls the differentiation and function of SMCs. Deletion of *Smad4* in SMCs, or inhibition of TGF- β signaling using specific pharmacological inhibitors or small-interfering RNAs against Smad2 or Smad3, decreases SMC proliferation, migration, and expression of SMC-specific gene markers and contractile proteins like α -SMA, SM22 α , calponin, and myosin heavy chain 11 (MYH11, also known as SMMHC), whereas inhibition of BMP signaling only affects cell migration (Mao et al. 2012). Targeted *Smad4* inactivation in ECs shows that Smad4 is required for cerebral vascular integrity. By cooperating with Notch, Smad4 mediates activation of N-cadherin expression, and contributes to stabilizing the interactions of ECs with pericytes (Li et al. 2011).

Endothelial-to-Mesenchymal Transition

ECs lining the inside of blood vessels and lymphatic vessels can undergo a transition into mes-

enchymal cells, a process known as endothelial-to-mesenchymal transition (EndMT) (Goumans et al. 2008; Kovacic et al. 2012; Medici and Kalluri 2012). In three-dimensional and in vivo models, ECs undergo EndMT when stimulated with TGF- β (Fig. 5). During EndMT, the ECs lose cell–cell contact, delaminate from the endothelium, and invade and migrate into the underlying tissue, thereby displaying a mesenchymal phenotype. EndMT is characterized by the loss of EC markers, like platelet/endothelial cell adhesion molecule-1 (PECAM1, also known as CD31) and vascular endothelial cadherin (VE-cadherin), and the expression of mesenchymal cell markers, including α -SMA, vimentin, procollagen I, and fibroblast-specific protein 1 (FSP1, also known as S100A4). Thus, EndMT may contribute to the generation of fibrotic myofibroblasts in vivo. In addition, the concomitant loss of functional ECs may also lead to capillary rarefaction (a reduction in capillary density), thus causing tissue ischemia, a potent driver of the fibrotic process. EndMT was first described during embryonic cushion formation (Fig. 6); in that scenario, cells detach from the endocardial layer, acquire a mesenchymal phenotype, and migrate into the cardiac jelly where they ultimately form the cardiac valves (Markwald et al. 1977; Kruithof et al. 2012). EndMT has been implicated in a wide variety of organ pathologies, including cardiac fibrosis, kidney fibrosis, pulmonary fibrosis, and cancer (Kalluri and Weinberg 2009).

Abundant examples show that TGF- β family signaling is a major molecular mediator in the pathological activation of EndMT (Goumans et al. 2008; Kovacic et al. 2012). In human disease, EndMT is observed during pathological processes in the heart that involve fibrosis of ischemic areas (Zeisberg et al. 2007). EndMT is also implicated in the pathology of fibrodysplasia ossificans progressiva (FOP), a rare genetic disorder, in which acute inflammation induces heterotopic ossification of soft tissues and the formation of ectopic skeletal structures (Medici and Olsen 2012). The fibroblast growth factor (FGF)-induced microRNA (miR) let-7 (miR-let-7) can suppress TGF- β signaling by reducing the stability of T β RI mRNA, thereby

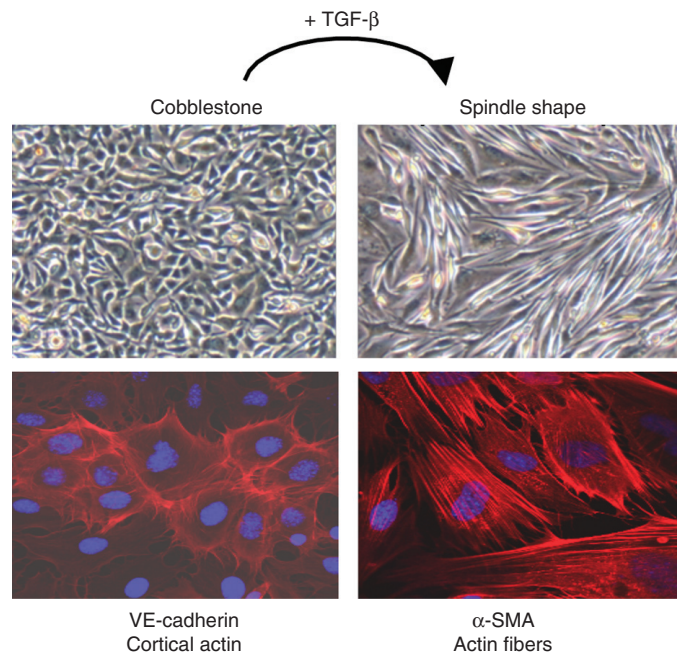


Figure 5. Endothelial-to-mesenchymal transition. When stimulated with TGF- β , endothelial cells undergo EndMT. They lose their cobble-stone morphology (upper panels, bright field), reduce the expression of endothelial cell markers such as platelet/endothelial cell adhesion molecule-1 (PECAM1), and start to express mesenchymal markers such as α -smooth muscle actin (α -SMA). Cortical actin (in red) is reorganized into stress fibers. The nucleus is stained with DAPI in blue.

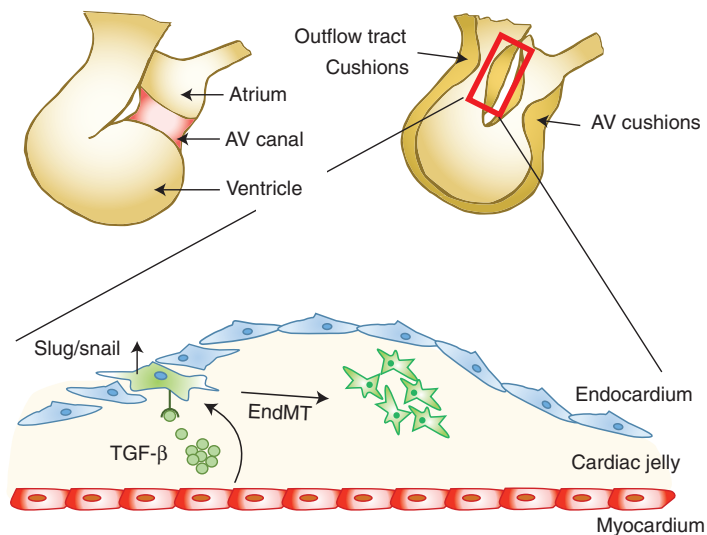


Figure 6. Cardiac cushion formation. The developmental formation of cardiac cushions involves endothelial-to-mesenchymal transition (EndMT). The endothelial cells lose their cobblestone morphology, adopt a mesenchymal phenotype, and invade the cardiac jelly. AV, Arteriovenous.

preventing the induction of EndMT (Chen et al. 2012b). TGF- β inhibition also promotes the generation and maintenance of phenotypically stable, embryonic stem cell–derived ECs (James et al. 2010). Although inhibition of TGF- β signaling, by repressing *Smad3*, contributes to the ability of Akt to maintain stable embryonic stem cell–derived ECs (Israely et al. 2014), TGF- β rapidly induces endothelial expression of miR-21. miR-21 facilitates EndMT through a PTEN/Akt-dependent pathway, as shown by a reduction in PECAM1 and endothelial nitric oxide synthase (eNOS) (Kumarswamy et al. 2012). miR-21 expression is also induced in cardiac ECs during pressure overload–induced cardiac remodeling and fibrosis, a process well known to involve TGF- β signaling (Zeisberg et al. 2007). In animals treated with transaortic constriction to produce cardiac pressure overload, a fraction of the ECs coexpresses endothelial and fibroblast markers, which is strongly inhibited by an antagomir against miR-21 (Kumarswamy et al. 2012). Finally, TGF- β induces miR-155 expression in ECs. miR-155 stimulates EndMT by repressing the Rho kinase activity (Bijkerk et al. 2012).

EndMT also occurs in the vasculature in the brain of patients with cerebral cavernous malformations (Maddaluno et al. 2013). Patients with this genetic disorder have enlarged, irregular blood vessels, which often give rise to cerebral hemorrhage. Endothelial-specific deletion of cerebral cavernous malformation protein 1 (CCM1; also known as KRTI1), one of three CCM-encoding genes, recapitulates the disease phenotype. In this context, EndMT is apparent by the disorganized VE-cadherin staining, and increased expression of the mesenchymal markers, N-cadherin, Slug, Id1, and α -SMA results from increased TGF- β and/or BMP signaling. Moreover, in mice with endothelial-specific *Ccm1*-inactivation, inhibition of the TGF- β pathway, by treating the animals with LY-364947 and SB-431542, reduces vascular malformations and prevents vascular leakage (Maddaluno et al. 2013). The development of cerebral cavernous malformations in the endothelial-specific *Ccm1*-deficient mouse is also reduced by silencing *Klf4* expression. Apparently, KLF4

is required in TGF- β - or BMP-induced EndMT of CCM1-deficient ECs (Cuttano et al. 2015).

EndMT has also been observed in vascular injury. Lineage tracing in a vein graft model revealed that endothelial-derived mesenchymal cells comprise the majority of the neointima (Cooley et al. 2014). Biomechanical injury to the luminal ECs initiates a TGF- β signaling cascade, which results in maladaptive EndMT. ECs transdifferentiate into proliferating smooth muscle-like cells within the neointima in a TGF- β -Smad2/Smad3-Slug-dependent manner (Cooley et al. 2014). Aberrant neointima formation can undermine the longevity of coronary bypasses and contribute to the pathogenesis of cardiovascular disease by narrowing the vessel diameter. Patients that undergo percutaneous angioplasty often show massive neointimal formations. Angioplasty usually involves inflating a balloon to open the artery and allow for blood flow. Stents or scaffolds may be placed at the site of the blockage to hold the artery open. To prevent neointima formation, a drug-eluting stent may be used, eluting a compound that will inhibit EndMT or SMC proliferation. For example, inhibiting TGF- β signaling by placing gene silencer pyrrole-imidazole polyamide eluting stents, which target the activator protein (AP)-1 binding site in the *TGFBI* promoter (Yao et al. 2009), or anti-endothelin antibody-eluting stents (Cui et al. 2014), may reduce or prevent pathological neointimal hyperplasia.

ECs that line the blood vessels can sense changes in hemodynamics exerted by blood flow regulation. Shear stress induces the expression of the Krüppel-like transcription factors KLF2 (Dekker et al. 2006) and KLF4, which results in quiescent ECs. Conversely, oscillating flow will not induce expression of these factors; instead, an inflammatory profile is activated in these ECs. Like an antenna, the endothelial primary cilium, a specialized, membrane-covered, rod-like protrusion, is capable of sensing, transducing, and amplifying information about the direction, viscosity, and velocity of the flow (Egorova et al. 2012; Praetorius 2015). ECs are devoid of cilia in areas of high shear stress and are ciliated in areas of oscillating flow. High shear stress and nonciliated ECs coincide with

areas of EndMT, and these ECs release growth factors, including TGF- β 1 and - β 3 (Egorova et al. 2011). Thus, the absence of a cilium facilitates TGF- β -dependent EndMT in embryonic ECs. Moreover, EndMT was also found to depend on the expression of KLF4 (Egorova et al. 2011; Cuttano et al. 2015). Shear-induced KLF2 expression induces the expression of Smad7, which subsequently inactivates TGF- β -induced Smad signaling in human umbilical vein ECs (Boon et al. 2007). Shear-induced changes in mechanical forces at the blood–endothelial interface can also lead to morphological changes of the EC monolayer and rearrangement of the endothelial cytoskeleton at the onset of atherogenesis; these changes depend on activation of TGF- β , KLF2, and EndMT (Boon et al. 2007; Walshe et al. 2013).

Transdifferentiation of LECs into fibroblast cells is also stimulated by TGF- β . bFGF is required for maintaining the differentiated LEC identity by inhibiting TGF- β -induced EndMT. bFGF depletion synergizes with TGF- β to induce EndMT and results in increased α -SMA expression in LECs (Ichise et al. 2014). TGF- β -induced EndMT also depends on Notch signaling. Although BECs can be immortalized and maintained with an endothelial morphology, immortalized LECs lose their EC markers and express α -SMA (Ichise et al. 2014). TGF- β signaling might be required for lymphatic regeneration and function in adulthood; however, endothelial-specific deletion of *Smad2* does not impair developmental lymphangiogenesis (Itoh et al. 2012).

TGF- β SIGNALING IN VALVE DEVELOPMENT AND DISEASE

Cardiac valve formation is essential for proper function of the four-chambered heart. Septal malformations, such as prolapses in bicuspid valves and mitral valves, are the most common congenital heart defects, and affect 1%–2% of the population (Michelena et al. 2015). During heart development, when the cardiac chambers appear, valves form in the atrioventricular canal, which separates the atria and ventricles, and in the outflow tract, which connects the ventricles

to the aortic sac and ensure proper blood flow (Kruithof et al. 2012). The endocardial cushions are the primordial valves and, during the initial stages of cardiac valve formation, endocardial cells overlying the cushions undergo EndMT. They lose cell–cell contact, differentiate into mesenchymal cells, and migrate into the cardiac jelly, which is the ECM between the endocardium and myocardium and is rich in collagen, proteoglycans, and hyaluronan (Fig. 6).

TGF- β signaling represents one of three recognized signaling pathways that play key roles in cushion formation, in which it promotes EndMT of endocardial cells and their invasion as mesenchymal cells into the cardiac cushions (Garside et al. 2013). The developmental expression patterns suggest that TGF- β 1 and - β 2 promote EndMT (Akhurst et al. 1990; Molin et al. 2003). At E8, TGF- β 1 is expressed in the endocardium and, when EndMT takes place, TGF- β 1 expression becomes restricted to the atrioventricular canal endocardial cells. TGF- β 1 expression remains restricted to valve endocardial cells until shortly after birth. TGF- β 2 is expressed in atrioventricular myocardial and endocardial cells that flank the cushions (Akhurst et al. 1990; Molin et al. 2003). In contrast, TGF- β 3 is expressed in the cushion mesenchyme after EndMT, and its expression increases as valve development proceeds after birth, suggesting that it may play a role in postnatal valve remodeling and function (Camenisch et al. 2002).

In several mouse studies, genes for TGF- β signaling components were inactivated, either in all tissues or in specific tissues, to understand their function during valve formation. Because TGF- β 1 can cross the placental membranes, only *Tgfb1*-deficient embryos born from *Tgfb1*^{-/-} mothers are thought to not be developmentally exposed to TGF- β 1. These embryos show severe heart defects, including disorganized atrioventricular valves (Letterio et al. 1994). Embryos deficient in TGF- β 2 expression show numerous defects that indicate that TGF- β 2 is critical for cardiac valve and septal development. *Tgfb2*^{-/-} embryos frequently show incomplete ventricular septation of the heart and, at E14.5, have enlarged, hypercellular cardiac cushions (Sanford et al. 1997; Bartram et al.



2001), suggesting that TGF- β 2 is involved in the initiation and termination of EndMT (Sanford et al. 1997; Azhar et al. 2009, 2011). The valves in *Tgfb2*^{-/-} mice at E18.5 display defective remodeling and differentiation (Azhar et al. 2011), which might indicate additional roles for TGF- β 2. *Tgfb3*^{-/-} mice do not have valve defects (Azhar et al. 2003); however, TGF- β 3 has been implicated in epithelial-to-mesenchymal transition in the developing palate and lung (Karttinen et al. 1995), as well as during chicken valvulogenesis (Boyer et al. 1999), and complementary activities of TGF- β 1 may have masked a function for TGF- β 3 in EndMT. Endothelial-specific inactivation of *Tgfb1* using Cre-mediated recombination driven by the *Tie2* promoter leads to formation of severely hypoplastic atrioventricular cushions at E10.5. The lack of mesenchymal cells migrating into the cushions is consistent with a crucial role for TGF- β signaling in EndMT progression (Sridurongrit et al. 2008). Endothelial-specific inactivation of *Tgfb2* results in embryonic death at E11.5, but EndMT occurs normally in the atrioventricular cushions. Although mesenchymal cells are detected in the inferior cardiac cushion, their proliferation is impaired (Jiao et al. 2006). Inducible, Cre-mediated inactivation of *Tgfb2* in VE-cadherin-expressing ECs at E11.5 results in a ventricular septal defect because of failure of cushion fusion (Robson et al. 2010).

LTBP-1 also stimulates cardiac EndMT. *Ltbp1*^{-/-} mice show hypoplastic cushions and enlarged valves as a result of attenuated EMT (Todorovic et al. 2011). Furthermore, an LTBP-1 antibody inhibits EndMT of mouse atrioventricular explants in three-dimensional collagen culture assays (Nakajima et al. 1997). LTBP-1 is made as a short (LTBP-1S) and long (LTBP-1L) isoform, and LTBP-1L binds to the ECM more efficiently than LTBP-1S (Olofsson et al. 1995). Lack of either LTBP-1L (Todorovic et al. 2007) or both LTBP-1 isoforms (Horiguchi et al. 2015) during embryonic development leads to hypoplastic endocardial cushions as a result of delayed EndMT in early valve development and hyperplastic mitral and tricuspid valves because of prolonged EndMT in late valvulogenesis. The thickening of the valves is accompanied by re-

duced TGF- β activity in the valvular interstitial cells (VICs) (Todorovic et al. 2007), revealing an important *in vivo* role for LTBP-1 as an extracellular regulator of TGF- β activity.

Targeted inactivation of either the *Eng* gene, encoding the TGF- β coreceptor endoglin, or *Tgfb3*, encoding the coreceptor betaglycan, results in embryonic death caused by cardiovascular defects. *In vivo* lineage tracing shows that endoglin is required for efficient EndMT and cushion development (Nomura-Kitabayashi et al. 2009). Endoglin-deficient embryos show hypocellular cardiac cushions, consistent with a role for endoglin in EndMT (Li et al. 1999; Arthur et al. 2000; Bourdeau et al. 2000a). Haplotype analysis revealed a relationship between bicuspid aortic valves, pathological fusion, EndMT, and endoglin expression (Wooten et al. 2010). Betaglycan is also expressed in the endocardium during cushion formation (Stenvers et al. 2003), and *Tgfb3*^{-/-} mice die just before birth because of a thin myocardial wall and poorly developed septa (Compton et al. 2007).

The leaflets of the aortic valve are highly specialized structures, consisting mostly of VICs and ECM (Hinton and Yutzy 2011). VICs maintain valve tissue homeostasis by regulating ECM biosynthesis and transdifferentiating into osteoblast-like cells (Taylor et al. 2003; Wang et al. 2014c). Because TGF- β is a potent inducer of ECM deposition and remodeling, it is tempting to speculate that a causal relationship exists between aberrant TGF- β activation, dysfunctional flow, and changes in valve function. Indeed, in syndromes like Marfan syndrome (MFS) (Judge et al. 2011) and Williams syndrome (Hinton et al. 2010), atrioventricular valves frequently undergo pathological changes associated with impaired TGF- β signaling, VIC activation, increased proliferation, and abnormal reorganization and production of ECM and proteoglycans. These changes may induce mitral valve leaflet thickening and elongation, which can result in a prolapsed valve. However, perturbed TGF- β signaling is associated with syndromic and nonsyndromic valvulopathies. Myxomatous mitral valve disease is associated with impaired TGF- β signaling, VIC activation, proliferation, leaflet thickening, fibrosis, matrix

remodeling, and leaflet prolapse. Activation of TGF- β signaling is seen as a major contributor to this pathology (Hagler et al. 2013; Rizzo et al. 2015).

The involvement of TGF- β and T β RI signaling in heart valve integrity may complicate the development of cancer treatments, based on inhibition of the T β RI kinase activity with a small molecule. Indeed, inhibition of T β RI function results in heart valve lesions, including hemorrhage, inflammation, degeneration, and interstitial cell activation and proliferation (Ander-ton et al. 2011). TGF- β signaling may be involved in the continued remodeling of the adult valve (Walker et al. 2004) as a result of ongoing mechanical stress caused by valve opening and closing during each heart cycle; inhibiting T β RI activity may therefore disturb this process. However, the T β RI kinase inhibitor LY2157299 does not show cardiac toxicity and thus represents an interesting candidate to treat patients with cancer (Kovacs et al. 2015). In a mouse model of MFS and Loeys–Dietz syndrome (LDS) (Habashi et al. 2006; Gallo et al. 2014), losartan, an angiotensin-II type 1 (AT1) receptor blocker, inhibits TGF- β -induced Smad2 and Erk phosphorylation and reduces the EndMT-driven fibrotic response. Losartan prevents mitral valve lengthening and thickening, which are typically caused by changes in hemodynamics and/or cytokine production after myocardial infarction (MI), and does so by inhibiting EndMT in cardiac mitral valve ECs. In cultured mitral valve ECs, losartan prevents EndMT by inhibiting TGF- β -induced Erk MAP kinase pathway activation (Wylie-Sears et al. 2014).

Aortic valve stenosis is characterized by valve fibrosis and calcification. This aortic valve narrowing causes high shear stress across the valves, which activates latent TGF- β that is released by platelets. In turn, TGF- β release induces EndMT (Mahler et al. 2013), which causes further narrowing of the valves. Although valvular interstitial and ECs show activation of Smad2 and Smad3, as well as Erk MAP kinase signaling (Wang et al. 2014b), leukocytes only show Smad2 and Smad3 activation. In mice, the stenotic phenotype is reverted when floxed

Tgfb1 is excised in platelets by Cre recombinase expressed from the promoter of the *Pf4* gene (Wang et al. 2014b), suggesting that platelet-derived TGF- β 1 contributes to aortic valve stenosis.

TGF- β SIGNALING IN HEREDITARY VASCULAR DISEASES

Hereditary Hemorrhagic Telangiectasia

The TGF- β and BMP coreceptor endoglin and the type I receptor ALK-1 are highly expressed by ECs. The involvement of ALK-1 and endoglin in TGF- β -regulated angiogenic responses is evident from the inherited vascular dysplasia in humans, named hereditary hemorrhagic telangiectasia (HHT) or Osler–Rendu–Weber syndrome (García de Vinuesa et al. 2016; Morrell et al. 2016; Goumans et al. 2017). HHT is an autosomal-dominant disorder that affects approximately 1 in 5000 people and arises from mutations in the genes that encode endoglin (*ENG*), ALK-1 (*ACVRL1*), or Smad4 (*SMAD4*). HHT is characterized by many telangiectasias (i.e., red spots that emerge from vascular abnormalities in connections between arteries and veins). In small arteries and veins, these spots are associated with loss of capillary beds. Larger arteries and veins develop AVMs (Fig. 7), which are predominantly found in the lungs, liver, gastrointestinal (GI) tract, and brain (Abdalla and Letarte 2006). These fragile vessels are prone to severe bleeding. HHT typically manifests as recurrent nosebleeds (epistaxis), which are difficult to treat and severely affect quality of life. In more severe cases, HHT may result in ischemic injury and stroke, which require major clinical attention.

HHT is divided into four subtypes, HHT type 1 (HHT-1) to HHT-4. These subtypes are caused, respectively, by mutations in *ENG* encoding endoglin (McAllister et al. 1994), *ACVRL1* encoding ALK-1 (Johnson et al. 1995), chromosome 5 (Cole et al. 2005), and chromosome 7 (Bayrak-Toydemir et al. 2006). *SMAD4* mutations are linked to a combined syndrome with characteristics of juvenile polyposis and HHT (Gallione et al. 2006, 2010). Ad-

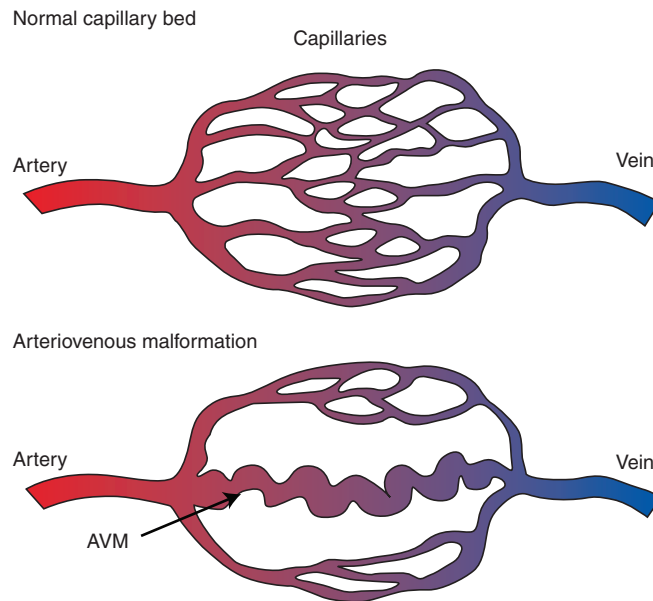


Figure 7. Arteriovenous malformations (AVMs) in hereditary hemorrhagic telangiectasia (HHT). One hallmark of HHT is the development of an AVM. In the AVM, the capillary bed is lost, and the artery drains directly into the vein via a tortuous, weak vessel, which is prone to rupture.

ditionally, *BMP9* mutations are linked to a vascular anomaly that phenotypically overlaps with HHT (Wooderchak-Donahue et al. 2013).

To date, the genetic underpinnings of HHT and the clinical phenotype points to roles of ALK-1 and endoglin in vascular development and vascular integrity. Nevertheless, the specific cellular and molecular mechanisms remain to be clearly understood. Ongoing clinical trials are evaluating antiangiogenesis agents in patients with HHT. These agents include bevacizumab, a neutralizing antibody against VEGF, which inhibits HHT-associated pathologies (Epperla and Hocking 2015), and thalidomide, which inhibits bleeding and other vascular malformations associated with HHT (Lebrin et al. 2010), although not all patients benefit from this treatment (Hosman et al. 2015).

Endothelial-specific *Alk1* inactivation in mice results in vascular malformations, including systemic enlargement of arteriovenous connections, GI bleeding, and all the pathological features of HHT-2 (Park et al. 2008). Although these mice lack ALK-1, AVMs do not develop, suggesting the requirement of a second hit.

VEGF-stimulated angiogenesis triggers the development of AVMs in endothelial-specific *Alk1* mice, both in the GI tract (Park et al. 2008) and in the brain (Chen et al. 2014). Furthermore, angiogenic stimulation after wounding and increased expression of VEGF gives rise to AVMs. Conversely, VEGF blockade prevents AVM formation and normalizes established arteriovenous shunts in ALK-1-deficient mice (Han et al. 2014).

Although treatment with anti-VEGF antibodies is beneficial in patients with HHT-1, different mechanisms are at the basis of HHT-1 and -2. Mice heterozygous for *Eng* show dilated, fragile blood vessels, which resemble the clinical symptoms observed in HHT-1 (Arthur et al. 2000; Bourdeau et al. 2000b, 2001; Sorensen et al. 2003). In addition, *Eng* heterozygous mice show dysregulated microvascular density in the lungs, associated with increased thrombospondin-1 expression. In contrast, mice heterozygous for *Alk1* inactivation, thus mimicking HHT-2, show secondary right ventricular hypertrophy, associated with increased expression of angiopoietin-2 (Ardelean et al. 2014). Anti-

VEGF treatment normalizes the pulmonary microvascularization anomaly, and attenuates the right ventricular hypertrophy in this mouse model (Ardelean et al. 2014; Ardelean and Lertarte 2015).

Impaired mural coverage of the vasculature is one cause for fragile vessels in mice heterozygous for *Eng*. Interestingly, treatment of these mice with thalidomide results in increased expression of PDGF-B, which will attract mural cells and stimulate their differentiation into pericytes and SMCs, resulting in vessel coverage and rescue of vessel wall defects (Lebrin et al. 2010). In humans, thalidomide treatment of a small group of patients with HHT-1 reduced the severity and frequency of nosebleeds and attenuated bleeding GI angiodysplasia (Alam et al. 2011; Franchini et al. 2013). However, thalidomide is thrombogenic (Penaloza et al. 2011), and might stimulate the development of peripheral neuropathy. Therefore, safer thalidomide homologs are needed for treating patients with HHT.

Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by progressive remodeling of the pulmonary vasculature because of abnormal proliferation of pulmonary endothelial and SMCs. The resulting increase in pulmonary vascular resistance will ultimately lead to the development of right heart failure (Morrell et al. 2016). The hereditary form of this disease is mostly linked to mutations in the coding region of BMPRII, although mutations in *ENG*, *ACVRL1/ALK1*, and *BMP9* have also been reported. The role of BMPRII in arterial hypertension is shown by the increased susceptibility of mice heterozygous for *Bmpr2* to experimentally induced pulmonary hypertension (Beppu et al. 2004; Song et al. 2005, 2008). Cell culture studies have shown that pulmonary ECs and SMCs from PAH patients have reduced levels of Smad1 and Smad5 activation. TGF- β 1 increases the proliferation of PAH SMCs compared with control SMCs, and elevated Smad2 activation is found in plexiform lesions of PAH patients (Gore et al. 2014). There-

fore, both reduced BMP signaling, as well as elevated TGF- β signaling, contribute to the pathogenesis of PAH.

New treatment modalities are now focusing on either activating BMP signaling or inhibiting the TGF- β pathway. High-throughput screening revealed that the immunosuppressive drug tacrolimus (also known as FK506) stimulates BMP signaling by forming a complex with FKBP12. Treating human pulmonary microvascular ECs with a low dose of tacrolimus releases FKBP12 from the BMP type I receptor and increases the level of Smad1 and Smad5 activation (Spiekerkoetter et al. 2013). Tacrolimus was shown to prevent the development of PAH in endothelial deficient *Bmpr2*^{-/-} mice, as well as in rats treated with monocrotalin to injure pulmonary ECs. A few PAH patients have been successfully treated with a low dose of tacrolimus (TransformPAH trial; Spiekerkoetter et al. 2015) and have been free from hospitalization ever since. Enhancing BMP signaling by administration of BMP-9 to mice carrying one allele encoding a human BMPRII mutant that has been associated with PAH prevents spontaneous development of PAH (Long et al. 2015). Additionally, treating rats with experimentally induced PAH using BMP-9 also reverses their pulmonary hypertension. These elegant studies show that restoring BMP signaling provides a potential treatment strategy for PAH. To find treatment options to inhibit TGF- β signaling, prostacyclin was found to reduce the TGF- β -induced Smad3 activation and inhibit proliferation of PAH-mouse pulmonary artery SMCs in culture (Ogo et al. 2013), and in vivo, monocrotalin-treated mice show a reduction in disease progression when treated with prostacyclin.

Aortic Aneurysms and Dissection: Lessons Learned from MFS and LDS

The aortic aneurysm is a cardiovascular disorder with a high mortality rate due to the risk of aortic dissection and sudden rupture. TGF- β stimulates the formation of aortic aneurysm, apparent from elevated levels of nuclear Smad2 in vascular tissue from patients that experienced a thoracic aortic aneurysm (TAA)



(Gomez et al. 2009). Inherited forms of TAA can be autosomal-dominant or autosomal-recessive. Autosomal-dominant TAAs include MFS, which is caused by mutations in the gene encoding the microfibrillar glycoprotein, fibrillin-1 (*FBN1*) (Dietz et al. 1991), and LDS, which is linked to mutations in *TGFB2* (Lindsay et al. 2012), *TGFB3* (Rienhoff et al. 2013), or the *TGFBR1* or *TGFBR2* genes encoding the two TGF- β receptors, T β RI and T β RII (Loeys et al. 2005). Autosomal-recessive TAA is found in cutis laxa, a disease caused by mutations in the genes encoding fibulin-4 (Renard et al. 2010) or LTBP-4 (Callewaert et al. 2013). Furthermore, aortic aneurysms have been associated with mutations in *SMAD3* and *SKI*, which encode a repressor of TGF- β signaling. Mutations in *Smad3* that disturb Smad3 protein function also lead to the aneurysms-osteoarthritis syndrome, a form of TAA with tortuosity of the arterial tree and early onset of osteoarthritis (Regalado et al. 2011; van de Laar et al. 2012). Mutations in the R-Smad binding domain of c-Ski hampers the formation of a Smad3-c-Ski complex, results in enhanced TGF- β signaling, and causes Shprintzen–Goldberg syndrome (SGS) (Carmignac et al. 2012; Doyle et al. 2012; Schepers et al. 2015). Finally, mutations in LTBP-4 and TGF- β 3 have been associated with abdominal aortic aneurysm progression (Thompson et al. 2010).

MFS has served as a paradigm for understanding the pathogenesis of TAAs and aortic dissections. Fibrillin-1 is an important component of the microfibrils in elastic fibers; these fibers provide stiffness and elasticity to dynamic structures like blood vessels. Mutant fibrillin-1, which is defective in microfibril assembly and function, increases the aortic wall susceptibility to dissection and rupture (Marque et al. 2001; Ramirez and Dietz 2007). In addition to its function in connective tissues, fibrillin-1 contains multiple LTBP binding sites with conserved 8-cysteine motifs (Chaudhry et al. 2007), which allows it to sequester TGF- β (ten Dijke and Arthur 2007; Bolar et al. 2012; Doyle et al. 2012). Nonfunctional fibrillin-1, like the one found in MFS, results in release of the latent TGF- β complex from the ECM,

pronounced TGF- β activation, and elevated TGF- β -induced Smad2 activation (Neptune et al. 2003; Habashi et al. 2006). Impaired binding of LTBP-3, when present in the latent TGF- β complex that associates with fibrillin-1, causes enhanced TGF- β signaling in MFS (Zilberberg et al. 2015). MFS mice deficient for *Ltbp3* show improved survival and no aortic aneurysms and rupture.

Neutralizing anti-TGF- β antibodies administered to a mouse model of MFS heterozygous for an *Fbn1* allele encoding fibrillin-1 with the cysteine substitution, Cys1039 \rightarrow Gly (C1039G) (i.e., *Fbn1*^{C1039G/+} mice) inhibit the increased TGF- β signaling associated with the pathology of MFS, apparent by its level of Smad2 activation, and the development of aortic root aneurysm (Neptune et al. 2003). Moreover, aortic growth is inhibited in the MFS mouse, when Erk or JNK MAP kinase activation is inhibited (Holm et al. 2011). Thus, inhibiting either the canonical pathway or the Erk and JNK MAPK pathways, although not TGF- β specific, may be beneficial for treating MFS. Similar protection against aortic aneurysms is also achieved by treating the MFS mouse with losartan (Habashi et al. 2006). Blocking angiotensin-II signaling reduces the expression of TGF- β ligands and receptors (Fukuda et al. 2000); this effect is apparent from the decreased Smad2 phosphorylation (Fukuda et al. 2000). Losartan also attenuates non-Smad signaling through inhibition of the Erk MAP kinase pathway (Habashi et al. 2011; Holm et al. 2011). This beneficial effect of losartan on aortic dilatation is supported by the results of a phase III clinical trial in patients with MFS (Groenink et al. 2013). Finally, doxycycline, an anti-inflammatory agent and matrix metalloproteinase (MMP) inhibitor, also reduces aortic root dilation in MFS mice by inhibiting TGF- β -induced MMP activation and MMP-induced release of TGF- β (Xiong et al. 2008; Lindeman et al. 2009), but not by reducing inflammation (Chung et al. 2008; Franken et al. 2014).

It is still unclear whether excessive or diminished TGF- β signaling is the primary cause for aortic root aneurysms because systemic abrogation of TGF- β signaling by treating mice with a



neutralizing anti-TGF- β 1 antibody promotes angiotensin-II-induced abdominal aortic aneurysm formation (Wang et al. 2010). Furthermore, some *TGFBR2*, *SMAD3*, and *SKI* mutations found in MFS, LDS, or TAA, which cause aneurysmal disorders (Loeys et al. 2006; Horbelt et al. 2010; Gallo et al. 2014), lead to decreased TGF- β -induced Smad2 and Smad3 phosphorylation. Conditional inactivation of *Tgfb2* in postnatal SMCs using *Myh11-Cre^{ERT2}* (Li et al. 2014) or *Acta2-Cre* (Yang et al. 2016) induces aortic aneurysms and dissection as a result of enhanced elastin degradation, and accelerates disease progression in the murine model for MFS. Loss of *Tgfb2* reduces Smad2 phosphorylation in SMCs but increases Erk MAPK phosphorylation. This was unexpected because these pathologies are typically observed in patients with MFS and linked to increased TGF- β signaling. *Smad4* deletion in SMCs using *Acta2-Cre* results in the development of TAA and abdominal aortic aneurysm, and blood vessel rupture between weeks 3 and 10 after birth because of enhanced elastin degradation and enhanced SDF-1 α -stimulated macrophage infiltration (Yang et al. 2016). In contrast to loss of *Tgfb2*, *Smad4*-deficient aortic walls show elevated activation of Smad2, Smad3, and JNK and enhanced TGF- β 3 and CTGF expression. The LDS mouse model, which carries LDS-associated missense mutations in either *Tgfb1* or *Tgfb2*, does recapitulate the vascular phenotype of LDS (Gallo et al. 2014).

The aortic wall in TAA can be recognized by the characteristic degeneration of the medial layer, which is caused by decreased expression of contractile proteins and collagens (Crosasmolist et al. 2015). The loss of contractile SMCs, which leads to greater cellular and ECM stiffness and vessel wall rigidity, is a TGF- β -dependent process and is seen as a major contributor to the pathogenesis of aortic aneurysms. These conditions result in SMC apoptosis and, eventually, vessel dissection. miR-29b levels are increased in the SMCs of the ascending aorta of *Fbn1^{C1039G/+}* MFS mice, and treatment with losartan or TGF- β inhibitors decreases miR-29b expression, thus inhibiting SMC apoptosis and stimulating ECM production (Merk

et al. 2012). Furthermore, delivery of a locked nucleic acid (LNA) that targets and inhibits the activity of miR-29b to MFS mice prevents aneurysm formation and reduces aortic wall apoptosis (Merk et al. 2012).

Familial TAA can also result from loss of T β RI or T β RII signaling, when no other features of MFS or LDS are apparent (Milewicz et al. 1996; Pannu et al. 2005; Tran-Fadulu et al. 2009; Inamoto et al. 2010; Lerner-Ellis et al. 2014). Additionally, increased TGF- β signaling is also observed in TAA aortic walls of patients with mutations in *ACTA2*, encoding α -SMA or *MYH11*, two genes involved in SMC contraction. This result suggests that reduced vascular contractility might weaken the aortic wall and make it susceptible to aneurysm formation (Renard et al. 2013a). Dysregulated TGF- β signaling is also associated with a vulnerable aortic wall in patients with mitral valve disease (Renard et al. 2013b) or bicuspid aortic valves (Padang et al. 2013; Grewal et al. 2014a,b). Reduced TGF- β signaling in the vessel wall of patients with bicuspid aortic valves may be a result of the observed elevated expression of LTPB-3, and increased generation of large latent TGF- β complexes (Paloschi et al. 2015).

TGF- β SIGNALING IN ATHEROSCLEROSIS

Atherosclerosis is a progressive, chronic disease, characterized by plaque formation in the vascular wall. Atherosclerosis causes gradual luminal narrowing or, when a plaque ruptures, an acute thrombotic occlusion (Libby et al. 2011). Stable plaques are primarily composed of collagen and SMCs, and unstable plaques are primarily composed of macrophages and a large lipid core, covered with a thin fibrous cap. Unstable lesions are prone to rupture, which exposes the lipid core to the blood (Bentzon et al. 2014). The lipid core is rich in tissue factor and other strong procoagulants, which promote the propagation of a thrombus. Clinical symptoms caused by a thrombotic event include cerebrovascular accidents, aneurysms, or an MI (Bentzon et al. 2014). Atherosclerosis arises from an injury in the vessel wall, and TGF- β most likely modulates the fibrotic and inflammatory components

of the lesion. TGF- β is produced by vascular cells and inflammatory cells, but there is some controversy in the literature as to the precise function of TGF- β in atherosclerosis (Grainger 2004; Toma and McCaffrey 2012).

Although TGF- β signaling may participate in the development of atherosclerosis, several studies have shown that it promotes a stable lesion phenotype by stimulating SMC differentiation and preventing the switch from contractile to proliferative SMCs (Gomez and Owens 2012). The expression of TGF- β 1 is higher in stable lesions than in unstable lesions, and human atherosclerotic plaques with elevated TGF- β 1 expression correlate with advanced atherosclerosis (Panutsopoulos et al. 2005; Herder et al. 2012). Loss of TGF- β signaling in atherosclerotic human coronary arteries coincides with enhanced FGF signaling and loss of SMC contractile markers (Chen et al. 2016). Other studies show an inverse relationship between serum TGF- β 1 levels and the development of atherosclerosis; that is, low levels of circulating TGF- β associate with unstable plaques (Bobik et al. 1999; Kalinina et al. 2004; Bot et al. 2009). Moreover, inducible overexpression of TGF- β 1 in cardiomyocytes, which increases the levels of TGF- β 1 in the plasma, limits plaque growth and induces plaque stabilization (Frutkin et al. 2009). These results suggest that TGF- β released from the cardiomyocytes is antiatherogenic (Tedgui and Mallat 2001; Hering et al. 2002). Furthermore, in *ApoE*^{-/-} mice, which lack apolipoprotein E and represent an animal model of atherosclerosis, inhibition of TGF- β activity by intraperitoneal administration of recombinant, soluble T β RII (Lutgens et al. 2002) or an anti-TGF- β 1 antibody (Tedgui and Mallat 2001) twice or once weekly, respectively, promotes atherogenic changes in the vessel wall. Specifically, the inflammatory response increases, and the lesions show an unstable phenotype, characterized by loss of SMC markers, low amounts of fibrosis, increased numbers of inflammatory cells, and hemorrhage within the plaque.

Disruption of TGF- β signaling, specifically in T cells (Gojova et al. 2003; Robertson et al. 2003) or dendritic cells (Lievens et al. 2013), accelerates the lesion progression and promotes

the development of an unstable plaque phenotype because of the presence of abundant inflammatory cells and a parallel reduction in plaque fibrosis. Furthermore, increased expression of TGF- β 1 in macrophage reduces the development of atherosclerotic lesions in ApoE-deficient mice and stabilizes existing plaques (Reifenberg et al. 2012). Transplantation of *Smad7*^{-/-} T cells in hypercholesterolemic *Ldlr*^{-/-} mice that lack the low density lipoprotein receptor results in larger, but more stable, atherosclerotic lesions with a large collagen-rich cap (Gistera et al. 2013). Increased Smad3 expression in cardiovascular tissues resulting from gene delivery using an adeno-associated virus vector inhibits atherogenesis and enhances the T_H2 response without stimulating fibrosis (Zhu et al. 2014).

Endoglin may be involved in the as yet unresolved role of TGF- β in atherosclerosis. Human aortic plaque SMCs have elevated levels of endoglin, which supports a role for endoglin in vascular wall integrity (Conley et al. 2000; Bot et al. 2009). In addition, elevated soluble endoglin levels are found in patients with coronary artery disease (Seghers et al. 2012), and changes in soluble endoglin levels in the plasma after an MI have predictive value for acute mortality in this patient group (Cruz-Gonzalez et al. 2008). Elevated expression of both the long and short forms of endoglin was found in atherosclerotic lesions. Interestingly, the long form of endoglin has an athero-protective effect by inducing eNOS expression, and both the short form of endoglin and soluble endoglin promote atherogenesis by inhibiting eNOS expression and TGF- β signaling (Jang and Choi 2014).

Finally, although some features are common to the development of calcified valves and atherosclerosis, current atherosclerosis medications have not prevented progression of calcification. The different responses to treatment may partly be explained by differences in flow; the coronary artery supports laminar blood flow, and valves support pulsatile shear flow on the ventricular side and reciprocal shear flow on the aortic side (Rajamannan et al. 2011; Gould et al. 2013).

TGF- β SIGNALING IN CARDIAC FIBROSIS

Cardiac fibrosis is a hallmark of the heart's pathological remodeling response to mechanical or biochemical stresses, like hypertension, pressure overload, cardiac inflammation, or MI (Hill and Olson 2008; Burchfield et al. 2013). The two types of cardiac fibrosis are reactive interstitial fibrosis, when fibrosis develops without significant cardiomyocyte loss, and reparative or replacement fibrosis, when a fibrotic scar forms in response to MI (Krenning et al. 2010; Kong et al. 2014). Cardiac fibrosis is characterized by the induction of expression of profibrotic growth factors, like TGF- β , and by the formation of cardiac fibroblasts into myofibroblasts. Activated fibroblasts differentiate into myofibroblasts, which increases their ability to produce ECM proteins. This change leads to increased myocardial stiffness and, ultimately, cardiac dysfunction and heart failure (Souders et al. 2012; Sullivan and Black 2013). The fibrotic process is driven primarily by local myocardial increases in TGF- β (Goumans et al. 2008; Creemers and Pinto 2011; Dobaczewski et al. 2011). For example, TGF- β 1 expression increases during the development of cardiac hypertrophy (Ruwhof and van der Laarse 2000), dilated cardiomyopathy (Khan and Sheppard 2006), and aortic stenosis (Beaumont et al. 2014), and after an MI (de Sousa Lopes et al. 2003; Frantz et al. 2008).

Genetic mouse models have provided evidence for the roles of different components in the TGF- β signaling pathway in myocardial stiffening and heart failure development. Cardiomyocyte hypertrophy and interstitial fibrosis are enhanced in the hearts of mice that have elevated circulating TGF- β 1 as a result of TGF- β 1 overexpression in the liver (Rosenkranz et al. 2002). These changes result from reduced expression of interstitial collagenases (Seeland et al. 2002) and are inhibited by treatment with the AT1-receptor inhibitor, telmisartan (Seeland et al. 2009). In *Tgfb1*^{-/-} mice (Brooks and Conrad 2000), or in wild-type mice treated with a neutralizing anti-TGF- β antibody (Okada et al. 2005; Ellmers et al. 2008), reduced TGF- β levels prevent collagen accumulation after pressure overload and attenuate diastolic dysfunction.

Inhibition of TGF- β signaling by overexpressing a soluble T β RII (Okada et al. 2005) or a dominant-negative T β RII (Lucas et al. 2010) decreases cardiac fibrosis and improves cardiac function. However, although it reduces collagen deposition after pressure overload, expression of a dominant-negative T β RII also results in increased left ventricular dilatation and diastolic dysfunction (Okada et al. 2005; Lucas et al. 2010). In contrast, inhibiting the T β R kinase activity by treating mice with an orally active pharmacological ALK-5 inhibitor reduces cardiac fibrosis and improves cardiac output (Engelbrechtsen et al. 2014). Finally, the TGF- β coreceptor, endoglin, is also involved in the development of cardiac fibrosis. Endoglin expression increases during angiotensin-II-induced cardiac fibrosis, and treatment with soluble endoglin reduces cardiac fibrosis and attenuates the development of heart failure after aortic bending (Kapur et al. 2012). Interestingly, overexpression of miR-208a in the left ventricle increases endoglin levels and induces cardiac hypertrophy during pressure overload; these effects can be inhibited by treatment with the angiotensin converting enzyme inhibitor atorvastatin or with an antagomir of miR-208a (Wang et al. 2014a). In addition, *Eng*^{+/-} mice show reduced fibrosis and death rate after right ventricular pressure overload (Kapur et al. 2014).

Both Smad and non-Smad pathways are involved in the pathological remodeling of the heart at the transcriptional level. Smad3 deficiency lowers interstitial fibrosis by reducing the deposition of collagen after left ventricular pressure overload (Divakaran et al. 2009). Smad3-deficient fibroblasts show less myofibroblast transformation and less collagen production in response to TGF- β (Dobaczewski et al. 2010). Moreover, fibrotic scar remodeling after MI is also reduced. In addition, *Smad7*^{-/-} mice show enhanced myocardial fibrosis and remodeling during angiotensin-II-induced cardiac hypertrophy (Wei et al. 2013). Finally, TAK1 is activated by myocardial pressure overload, and activated TAK1 induces cardiac hypertrophy and severe systolic dysfunction (Zhang et al. 2000).

Much effort has been invested into developing new treatment modalities for cardiac fibro-



sis. miRs have received increasing attention, and it has become clear that TGF- β can mediate cardiac fibrosis through the control of miR expression. miR-29b protects against angiotensin-II-induced cardiac fibrosis by inhibiting increases in TGF- β 1 levels (Zhang et al. 2014), and miR-378 inhibits TGF- β 1 expression during cardiac remodeling, which reduces fibrosis (Nagalingam et al. 2014). Additionally, miR-34a regulates fibrosis by inhibiting Smad4 expression (Huang et al. 2014). Finally, miR-15 reduces the expression of T β RI and several other components of the TGF- β pathway, and increased miR-15 expression protects against ventricular pressure-induced cardiac fibrosis (Tijssen et al. 2014). The antifibrotic miRs act by inhibiting TGF- β protein expression, and several antifibrotic drugs, like resveratrol, inhibit fibrosis by interfering with the TGF- β 1-Smad3 pathway (Chen et al. 2015).

CONCLUDING REMARKS

Initial studies on angiogenesis have focused mainly on either tyrosine kinase receptor ligands, like VEGF, angiopoietins, FGF, and PDGF, or angiostatin and endostatin inhibitors, like plasminogen fragments and collagen XVIII fragments. Analyzing the different TGF- β - and TGF- β -receptor-deficient mice showed the importance of TGF- β in yolk sac angiogenesis (Dickson et al. 1995; Oshima et al. 1996; Goumans et al. 1999). However, the possible role of TGF- β has remained neglected by the cardiovascular research community; because many of its functions go beyond vascular biology, its effects are highly context-dependent, and its pathways are thought to be insufficiently defined and complex. Nevertheless, an increasing number of cardiovascular diseases have been linked to mutated or inappropriately expressed TGF- β signaling components (ten Dijke and Arthur 2007). Conditional inactivation of genes expressing TGF- β signaling components in endothelial or mural cells produced informative vascular phenotypes (Jakobsson and van Meeteren 2013). Cell culture studies with three-dimensional (co)culture systems have identified the key interactions between TGF- β and other

angiogenic regulators. Those findings provide a mechanistic basis for the multifunctional behavior of TGF- β . Furthermore, TGF- β was found to play important roles in the identity and fate of (vascular) stem cells (Watabe et al. 2003). As a result, TGF- β has taken center stage in vascular research, along with other angiogenic regulators.

Based on this detailed insight into the molecular and cellular mechanisms underlying the TGF- β effects, agents have been developed that selectively inhibit TGF- β , including neutralizing anti-TGF- β antibodies, receptor ligand traps, and small-molecule kinase inhibitors. Exciting progress has been made toward gaining regulatory approval of these agents for treating cardiovascular diseases caused by perturbed TGF- β signaling. For example, losartan has been tested as a treatment to counteract overactive TGF- β signaling in MFS (Möberg et al. 2012; Groenink et al. 2013), neutralizing anti-TGF- β antibodies may potentially inhibit vein graft stenosis (Cooley et al. 2014), and neutralizing anti-ALK-1 antibodies and ALK-1-Fc ligand traps may inhibit tumor angiogenesis (Cunha et al. 2010, 2015; Hu-Lowe et al. 2011; van Meeteren et al. 2011; Vecchia et al. 2013; Necchi et al. 2014; Hawinkels et al. 2016). Because the TGF- β effects are strongly context dependent and can affect many different cell types, inhibition of any pathological response may be accompanied by on-target side effects (Akhurst and Hata 2012). Potential side effects may become a complication in the clinical advancement of TGF- β signaling modulators; careful patient selection and monitoring during treatment are warranted.

Animal models that recapitulate diseases related to perturbed TGF- β signaling are of key importance for the preclinical testing of new drugs. However, with the advent of induced pluripotent stem (iPS) cell technology, cells from patients with cardiovascular disease can be induced to differentiate into specific vascular cells, allowing for the use of these cells for mechanistic studies and drug screening purposes (Bellin et al. 2012; Orlova et al. 2014; Sanchez Alvarado and Yamanaka 2014). Moreover, recently developed gene editing technology, such

as the CRIPR-Cas9 system (Hsu et al. 2014), can be combined with iPS cells to design rescue experiments in which specific genetic alteration(s) in patient-derived cell lines can be corrected with gene editing. Furthermore, isogenic cell lines from embryonic stem cells can be created with specific genetic changes observed in cardiovascular diseases, and these cells can then be differentiated into specific vascular cell types to examine effects on differentiation and function. These technologies will promote the development of new therapeutic modalities for diseases with perturbed TGF- β signaling.

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