Transforming Growth Factor-ß Signal Transduction in Angiogenesis and Vascular Disorders

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Transforming Growth Factor-β Signal Transduction in Angiogenesis and Vascular Disorders*

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Transforming growth factor (TGF)-β is a multifunctional protein that initiates its diverse cellular responses by binding to and activating specific type I and type II serine/threonine kinase receptors. TGF-β can act as a regulator of proliferation, migration, survival, differentiation, and extracellular matrix synthesis in endothelial cells and vascular smooth muscle cells, as well as in the maintenance of vascular homeostasis. Importantly, genetic studies in humans have revealed the pivotal role of TGF-β as well as its signaling components in angiogenesis. Mutations in two TGF-β receptors (*ie*, the activin receptor-like kinase (ALK) 1 and the accessory TGF-β receptor endoglin) have been linked to vascular disorders named *hereditary hemorrhagic telangiectasia*. In addition, knockout mice for the different components of the TGF-β signaling pathway have shown that TGF-β is indispensable for angiogenesis. Recent studies have revealed that TGF-β can regulate vascular homeostasis by balancing the signaling between two distinct TGF-β type I receptors (*ie*, the endothelial-restricted ALK1 and the broadly expressed ALK5 receptors). The activation of these receptors has been shown to induce opposite effects on endothelial cell behavior and angiogenesis. In this review, we will present recent advances in understanding the role of TGF-β signaling in endothelial cells as well as the underlying molecular mechanisms by which perturbation of this pathway can lead to vascular disorders.

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Key words: angiogenesis; endothelial cells; hereditary hemorrhagic telangiectasia; Smad; transforming growth factor-β

Abbreviations: ALK = activin receptor-like kinase receptor; AVM = arteriovenous malformation; HHT = hereditary hemorrhagic telangiectasia; KO = knockout; R-Smad = receptor Smad; TGF = transforming growth factor; TβRI = transforming growth factor-β type I receptor; TβRII = transforming growth factor-β type II receptor; VEGF = vascular endothelial growth factor

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Over 35 distinct transforming growth factor (TGF)-β members have been identified in the human genome.[1] Individual family members are conserved across various species and are structurally related to the prototypical-founding member TGF-β1. All family members have profound effects on developmental processes ranging from left-right asymmetry to the development of soft tissues as well as the development of the skeleton.[1] TGF-β itself was initially identified as a factor that has the ability to induce the anchorage-independent growth of fibroblasts. Later, it became apparent that TGF-β exerts a multitude of cellular effects, including the inhibition of proliferation, the stimulation of extracellular matrix deposition, and the modulation of the immune response (Fig 1). Moreover, aberrant TGF-β signaling has been linked to various diseases such as cancer, pulmonary and liver fibrosis, autoimmune diseases, and vascular disorders (Fig 1).[2]

Signal Transduction of TGF-β Family Members

Members of the TGF-β superfamily exert their effect by binding to specific serine/threonine kinase type I and type II receptor complexes (Fig 2, *top*, *A*). In mammals, seven type I receptors, also termed *activin receptor-like kinase* (ALK) 1 to 7 and five type II receptors have been identified. TGF-β has high affinity for the TGF-β type II receptor (TβRII), and on binding a specific TGF-β type I receptor (TβRI) is recruited. On heteromeric complex formation between type I and type II receptors, the type I receptor is transphosphorylated by the type II receptor (Fig 2, *bottom*, *B*). This results in a conformational change in and activation of the type I receptor, which can subsequently propagate the signal inside the cell by the phosphorylation of specific effectors. In most cells, TGF-β

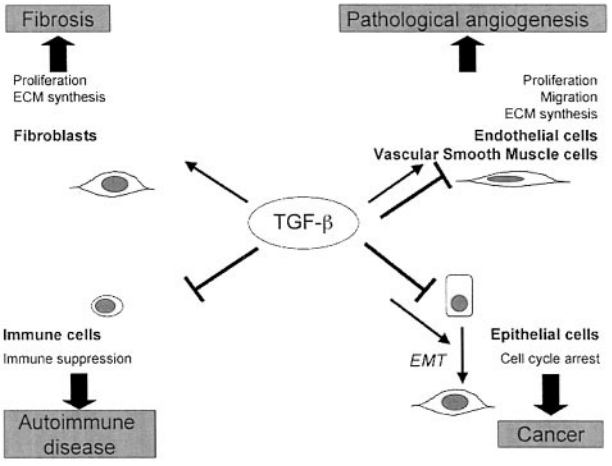

Figure 1. TGF-β is a multifunctional regulator of cell proliferation and differentiation; it regulates many different biological responses in a highly context-dependent manner. The subversion of TGF-β signal transduction has been implicated in many different diseases (large black arrow), including cancer, fibrosis, autoimmune diseases, and vascular disorders.

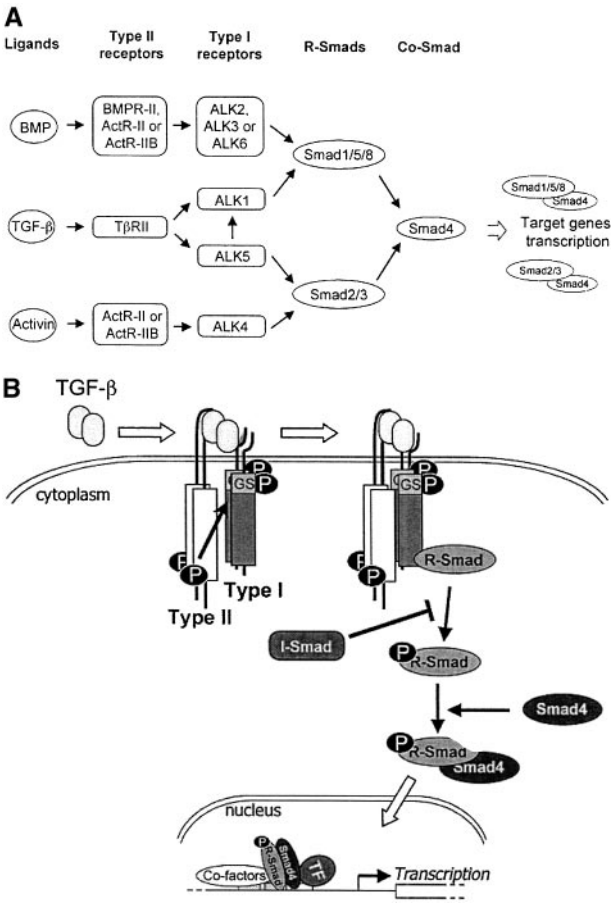

FIGURE 2. *Top*, *A*: divergence and convergence in TGF-β signaling through receptors and Smads. *Bottom*, *B*: schematic representation of TGF-β/Smad signal transduction pathway. TGF-β induces a heteromeric complex of type I and type II serine/threonine kinase receptors. On ligand binding, a type II receptor interacts with a type I receptor and phosphorylates the GS domain of the type I receptor. Then, the activated type I receptor activates R-Smads by phosphorylating their C-terminus. On phosphorylation, R-Smads form heteromeric complexes with Smad4 that translocate into the nucleus. Within the nucleus, the heteromeric Smad complexes, in collaboration with transcription factors (TFs), cofactors (*ie*, coactivators and corepressors) participate in the regulation of target gene expression.

signals via TβRII and TβRI (also termed *ALK5*). In endothelial cells, however, TGF-β has been shown to bind and signal via both ALK5 and ALK1[3] (Fig 2, *top*, *A*). A third type of TGF-β receptors (endoglin and betaglycan) are transmembrane proteins with short intracellular domains that lack an enzymatic motif. Betaglycan can present TGF-β to serine/threonine kinase receptors and thereby facilitates signaling.[4] The role of endoglin is less well understood. Unlike betaglycan, endoglin cannot bind TGF-β in the absence of a type II receptor, and the mechanism by which it regulates signaling is not known.

The signal molecules that play a pivotal role in transducing the TGF-β signal from the membrane-bound receptors to the nucleus are called *Smads*. After activation of the receptor complex, receptor Smads (R-Smads) are presented to the type I receptor and phosphorylated. The common Smad, also known as *Smad4*, subsequently forms heteromeric complexes with R-Smads. These complexes then translocate to the nucleus and modulate gene expression (Fig 2, *bottom*, *B*). The affinity of Smads for DNA is rather weak, and therefore complex formation with transcription factors is required. Two major Smad pathways are induced on ligand binding to the receptor; the Smad 2/3 pathway, which is induced on activation of ALK4, 5 and 7, and the Smad 1/5/8 pathway, which is activated by ALK1, 2, 3, and 6 signaling (Fig 2, *top*, *A*).[4] Thus, in endothelial cells that express both ALK1 and ALK5, TGF-β signaling is mediated by two different pathways, namely, Smad1/5/8 and Smad 2/3 (Fig 2, *bottom*, *B*).

TGF-β and Angiogenesis

Vascular development in the embryo starts with the formation of a primitive vascular network from endothelial precursors (vasculogenesis). Subsequently, this primary plexus is extended and remodeled into a complex vascular network (angiogenesis).[5] The importance of the TGF-β signaling pathway in angiogenesis and vascular remodeling has been highlighted during the last decades by numerous studies.[3] Gene-targeting studies in mice have shown that the a loss of TGF-β signaling components leads in general to abnormal differentiation and maturation of the primitive vascular plexus, resulting in fragile vessels with decreased integrity of the vessel wall (Table 1). Targeted inactivation of TGF-β1 caused midgestation lethality due to defects in the yolk sac vasculature and hematopoietic system.[3] Moreover, the vessels of the TGF-β1 mutant embryos have decreased wall integrity, which is caused by a defect in endothelial cells differentiating into capillary-like tubules. A similar phenotype is exhibited by mice lacking the TβRII as well as in mice with null mutations of the genes encoding the TGF-β-signaling components ALK5, ALK1, endoglin, and Smad5.

However, despite the resemblance of the observed phenotype affecting the formation of mature vessels (angiogenesis), some differences have also been found between these different knockout (KO) mice (Table 1). TGF-β1 and TβRII are critical for both the formation of the primary vascular plexus (vasculogenesis) and the subsequent extension and remodeling into a complex network (angiogenesis). In contrast, the phenotypes of other targeted TGF-β-signaling components (*ie*, ALK5, ALK1, Smad5, and endoglin) showed that they are only critical for the latter step. In addition, in the latter group the vessels of the embryo as well as the vessels of the yolk sac are affected. Moreover, a striking defect in the recruitment of smooth muscle cells at the periphery of the newly formed vessels was observed. However, we found that endothelial-specific KO of ALK5 and TβRII phenocopied the complete TβRII-deficient or ALK5-deficient embryos, indicating that the defects in the recruitment or differentiation of smooth muscle cells in these KO mice are likely to be an indirect consequence of the primary defect in the endothelial cells (Goumans et al; unpublished data). The disruption of ALK1, expressed predominantly in endothelial cells, also leads to an embryonic lethality that was reminiscent of the one seen in ALK5 KO

Table 1—*Vascular and Nonvascular Defects Observed in Heterozygous or Nullizygous Mutant Mice for the TGF-β Signalling Components***

Gene	Lethality	Vascular Defects Observed	Other Described Phenotypes	Adult Heterozygous Mice Vascular Phenotype
TGF-β1	E10.5	Defective yolk-sac vasculogenesis; decreased vessel walls integrity; defect in extraembryonic mesoderm differentiation	Inflammation, autoimmunity; defective yolk sac hematopoiesis	ND
TβRII	E10.5	Defective yolk sac vasculogenesis, distended capillary vessel formation	Defective yolk sac hematopoiesis (secondary effect)	ND
ALK5	E10.5	Defect in vessel formation, impaired endothelial cell migration and proliferation; severe defects in vascular development of the yolk sac and placenta	Absence of circulating RBCs; normal hematopoiesis	ND
ALK1	E11.5	Defective angiogenesis and differentiation and recruitment of vascular smooth muscle cells; arteriovenous malformation; dilated vessels in yolk sac and fusion of vessels in the embryo proper	ND	HHT-like syndrome
Endoglin	E10-E11.5	Defective maturation of the primary plexus of the yolk sac, muscle cells development and recruitment; aberrant organisation of the major vessels in the embryo	Cardiac malformations (enlarged ventricles), reduced number of RBCs (defect in hematopoiesis)	HHT-like syndrome
Smad5	E9.5-E11.5	Aberrant yolk sac vasculature composed of enlarged blood vessels surrounded by decreased numbers of vascular smooth muscle cells; defect of branching of vessels in the embryo proper	Left-right asymmetry, craniofacial abnormalities, malformations of the neurotube, gut, and heart; increased mesenchymal apoptosis	ND

*ND = not determined.

embryos (Table 1). Furthermore, in the ALK1-deficient embryos, the vascular defects observed are associated with an enhanced expression of angiogenic factors like the vascular endothelial growth factor (VEGF) angiopoietin-2, which could cause the excessive growth of endothelial cells and the fusion of the capillary vessels. A feature of the ALK1 KO not observed in the ALK5 KO mice is the presence of arteriovenous malformations (AVMs) in the vascular bed of the embryo proper due to the fusion of major arteries and veins. Interestingly, a marker of arteries in mammals, Ephrin-B2, which is involved in the regulation of boundary formation between arteries and veins, was absent in ALK1-deficient embryos, suggesting that a TGF-β-mediated signaling event involving ALK1 is required for developing distinct arterial and venous vascular beds. Although in the endoglin KO embryos the early loss of anatomic and functional distinctions between arteries and veins has been described, this occurs to a lesser extent than in ALK1 KO mice.[6] The underlying cause of this defect is not completely elucidated as the expression of ephrin-B2 was not affected. Finally, endoglin-KO embryos beside their defects in cardiac development and hematopoiesis also show a failure of endothelial remodeling affecting not only the vessels in the yolk sac but also to a lesser extent the vessels in the embryo proper (Table 1). Smad5-KO embryos have enlarged blood vessels surrounded by decreased numbers of vasculature smooth muscle cells, confirming that it acts as a downstream effector of ALK1 signaling.

Thus, the study of transgenic KO mouse models has revealed that TGF-β signaling is essential for vascular development. It points us to genetic signaling pathways in endothelial cells composed of TGF-β1, TβRII, ALK5, ALK1, endoglin, and Smad5 that are crucial to several steps of the angiogenic process, including the maintenance of the integrity of the vessel wall, the recruitment of smooth muscle cells, the deposition of extracellular matrix, as well as the differentiation of endothelial cells into a more specialized endothelium such as arteries and veins.

Balancing TGF-β Signaling in Endothelial Cells

Angiogenesis can be roughly divided into an activation phase and a resolution phase. Under normal conditions, the endothelium is quiescent due to the stabilization of the vessel by mural cells. During the activation phase, smooth muscle cells detach, and endothelial cells start to proliferate and migrate, and to form a new tube. During the resolution phase, smooth muscle cells will be recruited to cover the new tube and to inhibit the proliferation and migration of the endothelial cells.[5] One of the aspects that has puzzled researchers for years is that TGF-β1 exerts bifunctional effects on endothelial cells *in vitro*; it can both stimulate and inhibit the proliferation of endothelial cells. In addition, low doses of TGF-β stimulate endothelial proliferation, while high doses of TGF-β inhibit it. Other effects of TGF-β on endothelial cells are the inhibition or stimulation of the migration and extracellular matrix formation, depending on the source of the endothelial cells and culture conditions used.[7] Furthermore, TGF-β1 is known to regulate the expression and/or activity of matrix metalloproteases 2 and 9, which are involved in the degradation of extracellular matrix, thereby initiating angiogenesis. On the other hand, TGF-β1 also acts in a paracrine manner by stimulating the chemotaxis of

monocytes and the release of proangiogenic cytokines that can subsequently induce the activation of endothelial cells. Thus, TGF-β1 can stimulate angiogenesis by direct and indirect mechanisms.

To clarify the role of TGF-β on angiogenesis, studies were undertaken in our laboratory to identify the TGF-β receptor-signaling pathways that regulate angiogenesis. Although ALK5 is the predominant receptor that mediates TGF-β signaling, ALK1 can also form complexes with the type II receptor. One indication that ALK1 might be involved in angiogenesis was the observation that its expression is restricted to active sites of angiogenesis in the embryo and coincides with the expression of the TGF-β1-activated endothelium.[3] Moreover, in endothelial cells both ALK1 and ALK5 are expressed and bind TGF-β,[8] which corroborates the partly overlapping phenotypes in the ALK1 and ALK5 KO mice. Therefore, to dissect the role of each of the two signaling pathways in the regulation of angiogenesis *in vitro*, constitutive active type I receptors, as well as the methods to specifically disrupt type I receptor expression in endothelial cells, have been used. By these approaches, it was shown that the ratio between ALK5 and ALK1 activation by TGF-β in the endothelium will eventually determine whether the endothelium is stimulated or remains quiescent.[9–11] Whereas activation of the ALK1-Smad1/5 pathway induces the expression of proangiogenic genes (*ie*, Id1, endoglin, and IL1RL1), the activation of the ALK5-Smad2/3 pathway results in different signaling events associated with the expression of maturation-specific genes (*ie*, connexin 37, βIG-H3, and plasminogen activator inhibitor-1).[9–11] The use of a synthetic kinase inhibitor that inhibits ALK5 kinase activity substantiated these data showing the inhibition of the maturation of endothelial and mural cells.[12] Although it has been postulated that TGF-β may be involved in the transdifferentiation of endothelial cells into smooth muscle cells, as endothelial cells can express early smooth muscle cell markers in the presence of TGF-β or after the overexpression of ALK5,[10,13] it is currently thought that additional factors are required to induce late differentiation markers.[14] Further studies are awaited to further define the role of ALK5 in the recruitment and differentiation of periendothelial cells.

Interestingly, ALK1 and ALK5 not only induce different target genes, they also interact at various levels. First, ALK5 signaling is crucial to the optimal activation of ALK1 since endothelial cells derived from ALK5 KO mice are not able to activate the TGF-β/ALK1 pathway. The following model was postulated (Fig 3): on formation of TGF-β-induced complexes between TβRII and ALK5, ALK1 can be recruited and activated in an ALK5 kinase-dependent manner. In addition to the completely opposite responses of the ALK5-Smad2/3 and ALK1-Smad1/5 pathways, the activation of the ALK1 pathway antagonizes the ALK5-signaling pathway downstream of Smad phosphorylation.[11] Consistent with genetic data that endoglin and ALK1 act in a common pathway, endoglin was recently shown (Lebrin et al; unpublished data) to be essential for TGF-β/ALK1 signaling and to indirectly negative regulate TGF-β/ALK5 signaling.

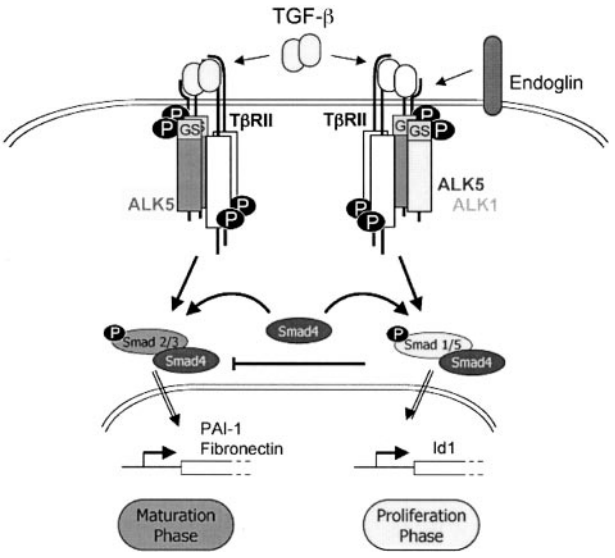

FIGURE 3. Schematic model by which TGF-β switches endothelial cell behavior via two distinct TGF-β type I receptor/Smad pathways. TGF-β first binds to TβRII, which subsequently recruits ALK5 and ALK1 in a common complex. Endoglin is needed for efficient TGF-β/ALK1 signaling. Activated ALK1 and activated ALK5 phosphorylate Smad1/5 and Smad2/3, respectively, which results in the activation of ALK1-specific and ALK5-specific target genes. ALK1 and ALK5 have opposite effects on endothelial cell migration and proliferation. In addition, ALK1 can indirectly inhibit ALK5-induced Smad-dependent transcriptional responses.

TGF-β Functions in Adult Angiogenesis

Angiogenesis occurs not only in developmental processes but also plays an important role during adult life. Adult angiogenesis can have different origins and has been shown to occur in either physiologic situations (*eg*, wound healing or the reproductive cycle) or in pathologic situations (*eg*, tumor angiogenesis, ischemia, or inflammation). Interestingly, TGF-β signaling has been shown to act as a potent activator of tumor progression and metastasis through the stimulation of angiogenesis.[2,10] Several models have highlighted the role of TGF-β and endoglin in tumor angiogenesis. The use of a neutralizing antibody against TGF-β1 has been shown to strongly inhibit angiogenesis.[15] In addition, elevated levels of endoglin, which are positively correlated with tumor metastasis, have been detected in cancer patients.[16] These results suggest a potential role for endoglin as a therapeutic target. More recently, endoglin antibodies have been used in clinical trials to target endothelial cells as an antiangiogenic therapy.[16–18] Further studies will be required to identify the role of TGF-β in tumor angiogenesis and to determine whether the balance between ALK1 and ALK5 signaling, and modulation by endoglin, is also involved in the *in vivo* regulation of this process.

Vascular Disorders Caused by Mutations in TGF-β Family Receptors

Interestingly, TGF-β signaling components have also been related to vascular disorders. Mutations in endoglin

or ALK1, but not in ALK5, TβRII, Smad5, and TGF-β1, have been linked to an autosomal-dominant disorder called hereditary hemorrhagic telangiectasia (HHT). Two major types of HHT have been described (HHT1 and HHT2), and whereas positional cloning was mapped the locus of HHT2 to ALK1, the HHT1 locus was mapped to endoglin. The most common clinical manifestations of HHT, also known as *Rendu-Osler-Weber syndrome*, are nosebleeds and mucocutaneous, small, localized vascular malformations called *telangiectasias*. GI bleeding occurs in more advanced stages, and AVMs in the lung, brain, and liver could also be observed in patients to a lesser extent. Clinical manifestations of HHT1 are associated with a higher incidence of pulmonary AVMs than in HHT2, which generally have a later onset. However, the existence of a high heterogeneity between clinical manifestations and the absence of a correlation between the severity of HHT and the type of mutation has suggested the potential role of modifier genes in the progression of this pathology. Recent observations[19] have indicated a possible involvement of Smad4 mutations in HHT as a subset of the patients experiencing juvenile polyposis have been shown to develop vascular malformations and epistaxis.

Mice carrying the heterozygous mutation for ALK1 or endoglin have a phenotype showing striking similarities with HHT disorder.[20–22] Heterozygous ALK1 mice are prone to develop age-dependent vascular lesions in the same organs as the ones affected in HHT2 patients but to a lesser extent. The high heterogeneity and difference in the severity of the disease observed in patients with HHT was confirmed in endoglin heterozygote mice. Endoglin heterozygous mice generated in the 129/Ola strain all show a vascular pathology, but they display a variation in the severity of the disease.[20] In addition, endoglin heterozygous mice with a different genetic background show a less severe phenotype or no phenotype.[10,21,23] This indicates that both genetic and complementary epigenetic factors are required for the onset of the disease.

HHT is not the only vascular disorder, which has been linked to members of the TGF-β family. Familial primary pulmonary hypertension is a vascular disorder that is caused by a sustained pulmonary arterial pressure. This pathology that involves the uncontrolled remodeling of pulmonary arteries has initially been linked to mutations of the bone morphogenetic protein type II receptor.[24] However, the identification of primary pulmonary hypertension-like syndromes developed in certain HHT2 patients has led to the hypothesis that ALK1 mutations could also have been involved in this pathology.[25]

The defect in smooth muscle cell recruitment that is seen in endoglin and ALK1 mutant embryos might contribute to the pathogenesis of HHT. This hypothesis has recently been confirmed in a study[26] in which the injection of adenoviral VEGF into the brain basal ganglia of endoglin-heterozygous mice resulted in the formation of enlarged vessels. This study showed that additional factors play a role in the local induction of angiogenesis, which could explain the variety of defects seen in HHT patients within one family.

Perspectives

From the generation of KO mice as well as from mutation analysis in patients, it became evident that TGF-β and its signaling components play a pivotal role in the regulation of angiogenesis. Despite the recent advances in the identification of two opposing TGF-β type I receptor-signaling pathways in endothelial cells, many issues remain to be elucidated. The heteromeric complex formation between ALK1 and ALK5 that has been identified in endothelial cells may possibly occur in other cell types and may involve other type I receptors. In addition, in cells that do not express ALK1, TGF-β (or activin) signaling via ALK5 (or ALK4) and bone morphogenetic protein signaling via ALK2, ALK3, or ALK6 may involve mechanisms of crosstalk that are similar to the ones we have observed for ALK5 and ALK1 pathways for TGF-β in endothelial cells.

The mechanism involved in the vascular defects observed in HHT patients could result from an imbalance of TGF-β signaling via ALK5 and ALK1 in endothelial cells. The defects seen in recruitment and differentiation in vascular smooth muscle cells are likely indirectly caused by defects in endothelial cell function. How intercellular communication between endothelial cells and vascular smooth muscle cells is perturbed in HHT patients is an important area of future research. Additional factors such as local inflammation, or the induction of angiogenesis might lead to an inappropriate or incomplete differentiation, giving rise to vascular defects.

In addition, whereas many studies have shown that endoglin is expressed on activated endothelial cells and its expression is highly induced by hypoxia, not much is known about ALK1 in human adult tissues and vascular diseases. Moreover, studies are awaited on the identification of regulators of ALK1 expression. Recently, a regulatory genomic DNA segment was identified[27] that drives ALK1 expression in arteries, which may indicate that TGF-β, besides its role in angiogenesis, is also involved in the remodeling of vessels *in vivo*. This was confirmed by studies[28] on collateral formation in which TGF-β enhanced arteriogenesis. If the hyperactivation of an endoglin/ALK1 pathway can be demonstrated in tumors *in vivo*, then this pathway may be an attractive target for antiangiogenesis therapy. Finally, it is important to note that the stability of vessels is a highly regulated process. The crosstalk of ALK1 and ALK5 type I receptor pathways with angiogenic factors like VEGF, fibroblast growth factor, angiopoietins, as well as antiangiogenic agents like thrombospondin is an interesting area of future research.

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Bone Morphogenetic Protein 4 Promotes Vascular Remodeling in Hypoxic Pulmonary Hypertension*

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Abbreviations: BMP = bone morphogenetic protein; PH = pulmonary hypertension; VSMC = vascular smooth muscle cell

Chronic hypoxia gives rise to structural remodeling of the pulmonary vasculature and is the most common underlying cause of pulmonary hypertension (PH) in humans. Recent studies in patients with familial primary PH suggest that the bone morphogenetic protein (BMP) signaling pathway may be involved in regulating pulmonary vascular remodeling. To explore this further, we have characterized the expression of BMP receptors and their ligands in a murine model of hypoxic PH. The BMP type I and II receptors, Alk3 and BMPR-II, are expressed in pulmonary endothelial cells and vascular smooth muscle cells (VSMCs), while one of their ligands, BMP4, is expressed in endothelial cells and airway epithelia, and is selectively up-regulated following prolonged exposure to hypoxia *in vivo*. To explore the functional role of BMP4 in hypoxic PH, we exposed wild-type mice and *Bmp4* heterozygous null mutant littermates to 10% hypoxia for 3

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