ROLE of TGF-β SIGNALING IN THE PATHOGENESIS OF VASCULAR DISEASES

By

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To my loving wife, parents, and brother

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ROLE of TGF-β SIGNALING IN THE PATHOGENESIS OF VASCULAR DISEASES

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Genetic mutations causing diverse genetic vascular diseases have been found in the genes that encode components of the transforming growth factor-β (TGF-β) signaling pathway, suggesting that TGF-β signaling is essential for homeostasis of the vascular system. To explore the role of TGF-β signaling in the pathogenesis of a couple of vascular diseases, our laboratory developed genetic mouse models for two major vascular diseases, pulmonary arterial hypertension (PAH) and hereditary hemorrhagic telangiectasia (HHT). PAH is a rare but fatal disease that increases pulmonary pressure due to thickening of pulmonary arterial walls. Although deficiency of bone morphogenetic protein type II receptor (BMPR2) in TGF-β signaling is known as a genetic contributor, whether dysfunction of SMAD1, one of the canonical transducers of BMPR2 is critical for PAH development remains unknown. We hypothesized that deficiency of SMAD1 would lead to PAH and tested the hypothesis using *Smad1*-conditional knockout (cKO) mice. The *Smad1* gene was deleted in endothelial cells or smooth muscle cells using L1cre and *Tagln*-cre lines respectively. We discovered that these *Smad1*-cKO mice develop PAH, suggesting that SMAD1 could be a critical downstream mediator of BMPR2 in PAH. HHT2 is a vascular disease with arteriovenous

malformations (AVMs) and visceral hemorrhages due to *ALK1* deficiency. Our previous studies showed that wounding plays an essential role in AVM development in the *Alk1*-deficient context. We investigated the involvement of two major wound-healing responses: inflammation and angiogenesis. We found that either LPS (for inflammation) or VEGF (for angiogenesis) could recapitulate the wound-induced AVM formation in *Alk1*-deficient skin. The inhibition of angiogenesis with VEGF-neutralizing antibody (Ab) significantly inhibited both LPS- and wound-induced AVMs and ameliorated internal bleedings in *Alk1*-deficient mice, suggesting a critical role for angiogenic stimulation in AVM development. These data provide a better scientific basis for the therapeutic effect of a VEGF blockade (Bevacizumab) for epistaxis, GI bleeding, and liver AVMs in HHT patients. Our data also demonstrated that the skin AVM model by *Alk1*-conditional knockout mice is reliable for preclinical screening of drug candidates for epistaxis and GI bleedings.

CHAPTER 1
SMAD1-DEFICIENCY IN EITHER ENDOTHELIAR OR SMOOTH MUSCLE CELLS RESULTS IN PULMONARY ARTERIAL HYPERTENSION

Introduction

Pulmonary Arterial Hypertension (PAH)

Pulmonary Arterial Hypertension (PAH) (formerly “primary pulmonary hypertension-PPH”) is rare but fatal lung vascular disease and is characterized by sustained elevation of mean pulmonary arterial pressure (PAP) and increased pulmonary vascular resistance leading to right heart failure. PAH is one of 5 types of pulmonary hypertension (PH), which is caused by chronic thrombosis, embolization, elevated left ventricular end-diastolic pressure, lung disease/hypoxemia or valve disease. While PH is diagnosed by the sole criterion being a resting mean PAP > 25 mmHg, diagnosis of PAH is required two additional criteria: more than 3 Wood units of pulmonary vascular resistance (PVR), and less than 15 mmHg of pulmonary capillary wedge pressure without other causes of PH. PAH is subclassified into idiopathic PAH (IPAH), heritable PAH (HPAH), and PAH associated with other diseases (APAH). Other diseases include congenital heart defect, portal hypertension, HIV infection, connective tissue disease, appetite suppressant drug use.[1]

Pathology of Pulmonary Arterial Hypertension

The basic pathological phenotype of PAH is a narrowing and thickening of small pulmonary vessels. All PAH patients exhibit pulmonary vascular remodeling of all layers of the vessel: intimal thickening, smooth muscle cell hypertrophy, adventitial fibrosis and occluded vessels by *in situ* thrombosis.[2] The intimal layer is a single-layered lining of endothelial cells (ECs) between the internal elastic lamina and lumen. Normally, ECs are quiescent but in PAH lungs, they are activated, rapidly proliferating and form

neointima layers. Myofibroblast is one of major cell types in the neointima and expresses smooth muscle cell (SMC) markers such as smooth muscle α-actin (αSMA) and vimentin instead of EC makers such as CD31 and the von Willebrand factor (vWF).[3] Although the origin of the myofibroblasts remains unknown, *in vitro* data suggest that myofibroblasts are likely originated from vascular SMC or transdifferentiated from EC.[4, 5] Plexiform lesions are commonly observed in the severe form of PAH showing multiple capillary-like channels in a pulmonary artery.[6] They contain several cell types including ECs, myofibroblast, and connective tissues.[7] ECs are the most responsible cell types for initiation of plexiform lesions through monoclonal proliferation of tumorlet-like clusters of ECs. A study showed that ECs with *BMPR2* mutation are more susceptible to apoptotic stimulus and after repeated apoptosis, the surviving ECs are apoptosis-resistant and undergo considerable proliferation and develop plexiform lesions.[8] Vascular SMCs are a predominant cell type of the medial layer and normally unresponsive to mitogens.[9] Hyperplastic SMCs are a common phenotype in all the different forms of PAH. In precapillary vessels, cells inside internal elastic lamina appear to be involved in differentiation into SMCs[10] and in distal vessels lacking elastic lamina, pericyte and interstitial fibroblast surrounding lung parenchyma seem to contribute to muscularization.[11] Remodeling of adventitia layer by increase of fibroblasts is associated with induction of many proinflammatory cytokines including monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2, interleukin (IL)-1β, IL-6.[12] Endothelial level of prostacyclin (prostaglandin-I_2), an endogenous vasodilator, an inhibitor of platelet aggregation and a suppressor of vascular SMCs proliferation, was shown to be decreased in patients with PAH. [13] The mechanism

involved in this observation could be that lowered expression of prostacyclin synthase impaires balance between prostacyclin and thromboxane A2, a vasoconstrictor and a potent stimulator of platelet aggregation.[14] Thrombotic lesions in PAH are associated with imbalanced ratio of prostacyclin and thromboxane A2 and potential outcome of the serotonin pathway.[15, 16]

Current Treatment Options for PAH

The incidence of PAH is 2-3 cases per million people every year.[17] There is age variability for the initial onset of PAH.[18, 19] Without proper treatments, mortality is predicted within 3 years after diagnosis. Up to now, the following treatments are available and have been used widely.

Prostacyclin (prostaglandin I_2)

One of the most extensively and successfully used therapies for PAH patients is to increase the level of prostacyclin with exogenous prostanoids. Fatty acid cyclooxygenase metabolizes arachidonic acid to prostaglandin H_2, a substrate for both prostacyclin (prostaglandin I_2) synthase and thromboxane synthase. Prostacyclin is expressed by prostacyclin synthase in endothelial cells and works as a vasodilator through stimulation of cyclic AMP (cAMP) and inhibits proliferation of SMCs.[20] On the other hand, thromboxane A2 (TxA2) is produced by throboxane synthase in platelet and endothelial cells and stimulates vasoconstriction and platelet aggregation. PAH patients showed decreased prostacyclin metabolites and increase of TxA2 production,[14] indicating that endothelial dysfunction and platelet activation in PAH might impair the balance between vasodilators and vasoconstrictors. Continuous intravenous infusion of epoprostenol (Floran) started in the early 1980s, has shown to decrease pulmonary vascular resistance (PVR), increased cardiac output, and improved exercise capacity

and overall survival rates.[21-23] Due to poor stability, high expense, and side effects from intravenous treatment of epoprostenol,[24] more stable analogs and alternative delivery of prostacyclin were developed: subcutaneous (treprostinil), oral (beraprost), or inhaled (iloprost) delivery of protacyclin analogs.[25-27] Recent combination therapy of prostanoid and a PDE5 inhibitor was effective in improving pulmonary hemodynamic change and exercise tolerance in PAH.[28, 29]

Endothelin-1 receptor antagonists

Endothelin-1 (ET-1) works as a vasoconstrictor via 2 types of receptors (ETA and ETB) and has a mitogenic effect on SMCs.[30, 31] Both receptors are expressed in SMCs and mediate vasoconstriction while ETB in ECs promotes vasodilation through NO and prostacyclin production.[32, 33] PAH patients exhibited high levels of lung and circulating ET-1.[34, 35] Endothelin receptor antagonist such as Bosentan (a nonselective ET receptor antagonist), sitaxsentan and ambrisentan (selective ETA receptor blocker) were FDA-approved and showed significant but moderate improvement in pulmonary hemodynamics and 6-minute walk distance.[36-39] However, there is no clear evidence showing advantage of selective ETA antagonism over combined antagonism for both ETA and ETA. Liver toxicity and teratogenicity are classic side effects. Despite a report that bosentan monotherapy increased survival,[40] more robust data for survival are necessary for clinical trials. Since the endothelins are produced from pro-endothelin by endothelin-converting enzyme, inhibitors for endothelin-converting enzyme could be an alternative approach to blocking overproduction of endothelin in PAH. Studies with this inhibitor (eg, daglutril) have been conducted in systemic hypertension and heart failure[41] and application for PAH patients are being investigated.

Nitric oxide (NO) and cyclic GMP (cGMP)

NO produced by endothelial NO synthase (eNOS) dilates blood vessel in the pulmonary circulation. Endothelium-derived NO stimulates soluble guanylate cyclase (sGC) to produce intracellular cGMP in SMCs.[42] Increased cGMP activates cGMP-dependent protein kinase (cGKI) and decreases the sensitivity of myosin to calcium-induced contraction and lowers concentration of calcium released from the sarcoplasmic reticulum.[42] Phosphodiesterase type 5 (PDE5), the target of sildenafil opposes NO-dependent vessel dilation by suppressing the rise of NO-induced cGMP.[43] NOS expression and NO bioavailability were shown to be reduced in the lungs of PAH patients.[44-46] Furthermore, PDE5 is upregulated in the hypoxia-mediated animal model for PAH, which worsens cGMP availability.[47,48] Endogenous NOS inhibitors, asymmetrical and symmetrical dimethylarginines (ADMA and SDMA) appeared to be more abundant in PAH.[49, 50] Thus, Inhaled NO gas dilates pulmonary arteries, which lowers vascular resistance, PA pressure, and RV afterload.[51, 52] The oral delivery of sildenafil citrate, an inhibitor of PDE5, improved exercise capacity and hemodynamics in PAH patients.[53]

Calcium channel blocker

Intracellular calcium and calcium-mediated signals play a crucial role in smooth muscle contraction.[54, 55] A study has shown that a high dose of calcium channel blockers have a beneficial effect on the survival of some PAH patients.[56] However, it should be used very carefully because calcium channel blockers are only convincingly effective in the 5% of patients who showed an acute vasoactive response to vasoreactivity testing.[56, 57]

In summary, current treatments for this disease alleviate symptoms and improve heart functions, but are not a cure of the disease. Better understanding on the pathogenetic mechanisms of disease would allow development of drugs targeting the cure.

Pathologic Mechanism of PAH

Involvement of BMP signaling based on genetic studies

Genetic studies have found the linkage of a locus for the gene, named PPH1, to chromosome 2q31–32 in 1997[58, 59] and later showed that Bone Morphogenetic Protein type 2 Receptor (BMPR2), one of the receptors in transforming growth factor β (TGF-β) superfamily signaling, is responsible for heritable PAH in an autosomal dominant manner.[60-63] A heterozygous BMPR2 mutation was found in nearly 70% of HPAH patients and also in 25% of sporadic IPAH patients.[63, 65-67] The levels of *BMPR2* mRNA and proteins are markedly reduced in the lungs of PAH patients with heterozygous *BMPR2* mutations,[64] indicating that BMPR2 mutations are associated with haploinsufficiency. Penetrance of disease is low and disease expressivity varies even within members of a family. Estimates showed that only about 20% of individuals with *BMPR2* mutation develops PAH during their entire life. This low penetrance in PAH suggests that additional factors such as inflammation may be necessary for clinical manifestations of PAH in addition to the genetic predisposition. While *Bmpr2*$^{+/-}$ mice displayed mild PAH phenotype, adenovirus-mediated pulmonary overexpression of 5-lipoxygenase (5-LO), a mediator of inflammation, or a chronic infusion of serotonin (5HT) developed full-blown PAH in *Bmpr2*$^{+/-}$ mice.[68, 69] Interestingly, BMPR2 is downregulated in the lung tissues and cells from idiopathic PAH patients without *Bmpr2* mutation,[64, 70] implying a wide-range of influence of BMPR2 deficiency to other forms of

PAH. Furthermore, BMPR2 signaling plays an important role in the proliferation of local endothelial cells and the migration and local proliferation of smooth muscle cells.[8, 71] Taken together, impaired BMP signaling due to BMPR2 deficiency would be a considerable contributor for PAH development.

TGF-β superfamily signaling

TGF-β is a large cytokine family that contributes to diverse cellular processes including cell proliferation, migration, apoptosis, pattern formation, and immunosupression. TGF-β family members include TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins/inhibins, and müllerian inhibiting substance (MIS)/ anti-Müllerian hormone (AMH).[72-74] TGF-β signal transduction is initiated by binding of ligands to heteromeric complex of transmembrane serine/threonine type 2 and type 1 receptors. Once ligands bind to a type 2 receptors, the ligand-type 2 receptor complex recruits and trans-phosphorylates type 1 receptor, which, in turn, activates receptor-regulated SMADs (R-SMADs): SMAD2/3 for TGF-βs and SMAD1/5/8 for BMPs. R-SMADs then form a complex with a common partner, SMAD4 (Co-SMAD) and enter the nucleus and initiate transcription of target genes. On the other hand, there is mounting evidence demonstrating that independently of SMADs, TGF-β signaling can be transduced through mediators other than SMADs such as the mitogen-activated protein kinases (MAPKs), including p38MAPK, p42/44MAPK (ERK1/2), and c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK).[75, 76] For instance, exogenous BMP ligands stimulate phosphorylation of p38MAPK and p42/44MAPK, and affect proliferation of SMCs.[70, 77]

Endothelial dysfunction in PAH

The endothelium is the cells lining the interior surface of blood vessels in the entire circulatory system. Endothelial cells function in many aspects of vascular biology: vasoconstriction and vasodilation, and hence the control of blood pressure, blood clotting (thrombosis & fibrinolysis), atherosclerosis, formation of new blood vessels (angiogenesis),inflammation and swelling (oedema) and also control of the passages of materials and blood cells (permeability). Thus, endothelial dysfunction may result in increased coagulation, proliferation, and vasoconstriction.[78-81] Endothelial dysfunction seems to play an integral role in mediating the structural changes in the pulmonary vasculature. Disordered endothelial cell proliferation along with concurrent neoangiogenesis results in the formation of glomeruloid structures known as the plexiform lesions, which were found in the pulmonary vessels of patients with severe PAH.[82] *In vitro* studies with endothelial cells suggest a plausible mechanism of angioproliferative change of ECs in PAH. Loss of BMPR2 function cannot protect ECs from apoptosis, representing a possible initiating step and increased apoptosis of ECs promoted appearance of the apoptosis-resistant cells.[8, 83] In addition, an altered production of various endothelial vasoactive mediators such as Nitric oxide (NO), prostacylin, endothelin-1 (ET-1), serotonin, and thromboxane, has been increasingly recognized in patients with PAH.[14, 34, 84-86] Since change of these mediators can affect the growth of the smooth muscle cells, alteration in their production may facilitate the development of pulmonary vascular hypertrophy. Thus, it is conceivable that the beneficial effects of currently available treatments for PAH, such as prostacyclin, NO, and ET antagonists, result in part from restoring the balance between these mediators. Furthermore, endothelial dysfunction may bring about a change in the EC permeability

and allow direct contact of serum proliferative mediators with the subendothelium, leading to cell proliferation in the medial and advential layers.[87-89]

Compromised immune response and inflammation

Pulmonary hypertension has been associated with connective tissue disease,[90] human immunodeficiency virus (HIV),[91] and auto-antibodies.[92] Mononuclear cell infiltration was often observed in the PAH vascular lesions.[64] Inflammatory cells including macrophages and lymphocytes are highly accumulated in the plexiform lesions of hypertensive pulmonary vessels.[7] Induction of many proinflammatory cytokines and chemokines, such as monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, interleukin (IL)-1β, IL-6, RANTES, fractalkine has been implicated in PAH.[12, 93-97] In the classical PH animal model, Monocrotalline (MCT, a pyrrolizidine alkaloid plant toxin)-injected rats elevated pulmonary pressure with apoptosis of ECs and SMCs and massive mononuclear infiltration into the perivascular regions of pulmonary arterioles.[98, 99] Therefore, the observations from a variety of human and animal studies suggest a compelling conclusion that intact immune system is required to maintain homeostasis of pulmonary circulation. In other words, dysregulated immunity could be an environmental second hit for clinical manifestation of PAH in individuals with *Bmpr2* mutation.

Role of TGF-β/BMP Balance in Pulmonary Vascular Homeostasis

Only 20% disease penetrance in individuals with *Bmpr2* mutation implies that the *Bmpr2* mutation is necessary but not sufficient alone for clinical manifestation of PAH. This fact suggests that other genetic or environmental modifiers are necessary for initiation and progression of PAH. TGF-β could be a possible candidate of modifiers in PAH.

BMP and TGF-β signaling form an opposing balance in pulmonary vascular homeostasis.[100] Thus, BMPR2 deficiency may result in predominant TGF-β signaling which favors fibrosis and smooth muscle cell growth. Recent studies suggest that an imbalance between TGF-β and BMP signaling leads to PAH development. Activation of BMP signaling suppresses proliferation of pulmonary vascular smooth muscle cells.[64] Furthermore, TGF-β polymorphisms that increase TGF-β activity are associated with early onset of PAH and increased penetrance of heritable PAH.[101] Zaiman *et al*. reported that increased TGF-β signaling participates in the pathogenesis of monocrotaline-induced PH.[102] Long *et al*. showed that suppression of TGF-β signaling via activin receptor-like kinase-5 inhibitor prevents development and progression of PAH in the monocrotaline model.[103] Impaired BMP signaling from BMPR2 deficiency would not be sufficient to develop PAH and when in conjunction with predominance of TGF-β, finally would elevate pulmonary pressure.

Is SMAD Important Signaling Mediator for PAH Caused by BMPR2-Deficiency?

Human genetic studies have identified various *BMPR2* mutations throughout the exons coding for the BMPR2 protein.[104] These data imply that deficiency of BMPR2 is a crucial genetic factor in the PAH development. However, which downstream signaling molecules of BMPR2 such as MAPKs or SMADs contribute to PAH is still unknown. Mutations in the kinase domain (about 50% of total mutations) suggested that downstream SMAD signaling play a role for PAH development because of its reduced SMAD-dependent transcriptional activity.[105] However, mutations in cytoplamic tail domain (about 20% of total mutations) such as R899X leave SMAD signaling intact, indicating that the cytoplasmic tail of BMPR2 may not be essential for transduction of BMP signals through SMADs.[106, 107] In other words, SMAD-deficiency may not be

associated with the pathogenesis of PAH. James West *et al*. addressed this question using transgenic mice expressing $BMPR2^{R899X}$ transgene in smooth muscle.[108] In these mice, truncated BMPR2 proteins with a tail domain mutation were overexpressed in smooth muscle cells by doxycycline treatment from 4 weeks of age. They elevated right ventricular systolic pressure, associated with extensive pruning, muscularization of pulmonary arterioles and perivascular infiltration of immune cells, not affecting SMAD activity. These *in vivo* results suggest that the SMAD-deficiency may not be associated with PAH caused by BMPR2-deficiency.

Endothelial BMPR2-Deficient PAH Mouse Model

Previously we produced *Bmpr2*-conditional knockout (cKO) mice [L1cre(+);$Bmpr2^{f/f}$ or $^{+/f}$] by deleting the *Bmpr2* gene in the pulmonary endothelium using the novel L1cre line.[109] A subset of mice exhibited elevated right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH) which are the representative phenotypes of pulmonary hypertensive (PH) mice [110] and these PH mice showed increased muscularization of pulmonary arterioles and thickening of vessel wall compared to non-PH mice and controls.

Deletion of *Smad1* Gene in ECs or SMCs

SMAD1 is a canonical signal transducer of BMPR2 and its reduced activity has been associated with PAH. Yang *et al*. showed that phosphorylation of SMAD1 was reduced in the pulmonary arterial SMCs of PAH patients with *BMPR2* mutation.[70] Thus, we hypothesized that SMAD1 is a contributing downstream mediator of BMPR2 in the pathogenesis of PAH. To test this hypothesis, we produced *Smad1* cKO mice by deleting *Smad1* gene in the pulmonary endothelial cells (pECs) or smooth muscle cells (SMCs) using L1cre and *Tagln*-cre lines, respectively and found that *Smad1* deletion in

pECs or SMCs can develop PAH in mice with muscularization of pulmonary vessels, suggesting that SMAD1 may be a critical downstream molecule in PAH. Recent studies reported that enhanced TGF-β activity is associated with PAH pathology as well as impaired BMP signaling.[103, 111-113] In *Bmpr2*-deleted pulmonary endothelial cells, we found that a reduced BMP response resulted in a predominant TGF-β response, supporting the role of a balance between TGF-β and BMP signaling in the homeostasis of pulmonary blood pressure.[100]

Results

SMAD1/5/8 Phosphorylation was Impaired in the Lungs of Pulmonary Hypertensive L1cre(+);*Bmpr2*$^{2f/2f}$ Mice.

We have previously shown that about 40% of mice in which *Bmpr2* gene was specifically deleted in endothelial cells by L1cre exhibited pulmonary hypertensive phenotype.[110] To assess the extent to which the conditional *Bmpr2* deletion affects on its downstream SMAD1/5/8 signaling, lungs of cre-negative controls, Non-PH L1cre(+);*Bmpr2*$^{2f/2f}$, PH L1cre(+);*Bmpr2*$^{2f/2f}$ mice were immunostained with anti-phospho-SMAD1/5/8 antibodies (Fig. 1-1A through 1-1F). While pSMAD1/5/8 positive cells were readily detected in the cre-negative controls (Fig. 1-1A through 1-1B) and Non-PH *Bmpr2* mutants (Fig. 1-1C), they appeared to be much less in the PH *Bmpr2* mutants (Fig. 1-1D through 1-1F). Consistent with this immunostaining result, the levels of SMAD1/5/8 phosphorylation in the whole lung samples were significantly reduced in the PH *Bmpr2* mutants compared to the cre-negative controls and Non-PH *Bmpr2* mutants (Fig. 1-1G, H). These data suggested to us that impaired SMAD1 signaling may play a pivotal role in the pathogenesis of pulmonary arterial hypertension associated with *Bmpr2* deficiency.

Smad1 Deletion in Pulmonary ECs or SMCs by L1cre or *Tagln*-cre

To investigate the role of SMAD1 in the pathogenesis of PAH, we exploited conditional knockout approaches for deleting the *Smad1* gene in endothelial cells or smooth muscle cells by L1cre or *Tagln*-cre lines, respectively, because *Smad1*-null mice were embryonic lethal.[114] Both L1cre(+);*Smad1*$^{f/f}$ and *Tagln*-cre(+);*Smad1*$^{2f/2f}$ mice were viable and normal compared to their cre-negative littermates. We analyzed the Cre-activity by detecting the *Smad1*-null allele in several organs of 2 month-old mice, including the lung, heart, liver, kidney, and spleen. Consistent with our expectations based on our previous reports,[109, 110] the Cre-mediated *Smad1* deletion was detected primarily in the lungs of L1cre(+);*Smad1*$^{f/f}$ mice, and found in most organs of *Tagln*-cre(+); *Smad1*$^{f/f}$ mice (Fig 1-2).

Some Mice with the *Smad1* Deletion in Pulmonary ECs or SMCs Exhibited Elevated Pulmonary Pressure and Right Ventricular Hypertrophy.

To assess the pulmonary pressure, we measured the right ventricular systolic pressure (RVSP) of L1cre(+);*Smad1*$^{f/f}$, *Tagln*-cre(+);*Smad1*$^{f/f}$, and their age-matched cre(-) control mice. While RVSPs of controls were clustered in a 20-27 mmHg range, those of L1cre(+);*Smad1*$^{f/f}$ and *Tagln*-cre(+);*Smad1*$^{f/f}$ mice were scattered in a wide range (Fig. 2A) from 22 to 45 mmHg. About 40% (14/35) of L1cre (+);*Smad1*$^{f/f}$ mice and 12% (4/33) of *Tagln*-cre(+);*Smad1*$^{f/f}$ mice had their RVSPs greater than 30 mmHg, and we designated them as pulmonary hypertensive (PH) group (Fig. 1-3A). The mean RVSPs of L1cre(+);*Smad1*$^{f/f}$ (28.1 mmHg) was significantly higher than that of cre(-);*Smad1*$^{2f/2f}$ (23.9 mmHg). Fulton index, the ratio of RV free wall weight over septum plus left ventricular free wall weight, was used to estimate RV hypertrophy. The Fulton index of L1cre(+);*Smad1*$^{f/f}$ and *Tagln*-cre(+);*Smad1*$^{f/f}$ mice was significantly greater

than that of cre(-);*Smad1*$^{f/f}$ controls (Fig. 1-3B). It was greater in the PH mice than that in N-PH mice, indicating that sustained elevation of pulmonary pressure might have resulted in RV hypertrophy in the PH groups (Fig. 1-3C). There was no difference in systemic blood pressure among three groups (Fig. 1-3D).

The Number of αSMA-Positive Distal Arteries and Medial Wall Thickness Were Increased in the *Smad1* Mutant Mice.

To examine whether the elevated RVSP and RV hypertrophy in the *Smad1* mutants is associated with pulmonary vascular remodeling, anti-smooth muscle α-actin (αSMA)-positive pulmonary arteries ranging from 30-70 μm in diameter were counted and the wall thickness was measured. The PH group L1cre(+);*Smad1*$^{f/f}$ mice showed higher number of αSMA-positive pulmonary arterioles and thicker arterial walls compared to the N-PH group and the cre(-);*Smad1*$^{f/f}$ (Fig. 1-4B, E, F). In *Tagln*-cre(+);*Smad1*$^{f/f}$ mice, however, both PH and N-PH groups showed a higher number of muscularized vessels and thicker walls compared to the cre(-);*Smad1*$^{f/f}$ controls.

Isolation of Pulmonary Endothelial Cells Carrying *R26*$^{creER/+}$;*Bmpr2*$^{2f/2f}$ Allele

It has been hypothesized that an opposing balance between TGF-β and BMP signalings is critical for homeostasis of pulmonary vasculature, and that imbalance of TGF-β/BMP signalings may contribute to the pathogenesis of PAH.[100] In order to investigate this hypothesis and to examine the extent to which Bmpr2 deficiency impact on this balance, we established an in vitro model as follows. We isolated pulmonary ECs (pECs) from the lung of *R26*$^{creER/+}$;*Bmpr2*$^{2f/2f}$ mouse, in which the conditional "2f" allele can be converted to null "1f" allele by tamoxifen treatment that activates the Cre recombinase function. Three days of culture of the pECs with medium containing 1 μM 4-hydroxy-tamoxifen (OH-TM) efficiently deleted exons 4 and 5 of the *Bmpr2* gene (Fig.

1-5A). When the *Bmpr2*-deleted cells were subsequently cultured with OH-TM-free media for 10 days, no overgrowth of undeleted cell populations were observed (Fig. 1-5B). Henceforth, *Bmpr2*-intact and -deleted cells will be designated as *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells, respectively.

To examine whether OH-TM treatment and *Bmpr2* deletion affected the expression of other genes involved in TGF-β pathways or altered general EC characteristics, semi-quantitative RT-PCR analyses were performed. Expression of EC-specific markers including Nos3, Tie2, Eng, and Flk1 were maintained in *Bmpr2*$^{1f/1f}$ pECs (Fig. 1-5D). *Bmpr2* transcript level was undetectable while transcripts for other TGF-β/BMP receptors were unchanged in *Bmpr2*$^{1f/1f}$ pECs (Fig. 1-5C).

Deletion of *Bmpr2* Gene and Impaired BMP Signaling

In order to assess the extent to which *Bmpr2*-deletion affects BMP signaling, BMP4 or BMP7 was added to *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells at various doses (0, 5, 25, 50 ng/ml) after 15 hr serum starvation. First, we examined the protein level of BMPR2 in these cells. While BMPR2 was readily detected in *Bmpr2*$^{2f/2f}$ cells, BMPR2 protein was undetectable in *Bmpr2*$^{1f/1f}$ cells (Fig 1-6A, B). Level of phosphorylation of SMAD1/5/8 was augmented in a dose-dependent manner in *Bmpr2*$^{2f/2f}$ cells whereas it was suppressed at all BMP4 doses in *Bmpr2*$^{1f/1f}$ cells (Fig. 1-6A, C), indicating that the BMP4 signaling is impaired in *Bmpr2*$^{1f/1f}$ pECs. BMP7 signaling can be compensated by ACVR2A in BMPR2-depleted pulmonary artery SMCs (PASMCs).[77] As shown in Fig. 1-6D, phosphorylation of SMAD1/5/8 was elevated in a dose-dependent manner in both *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells, suggesting that BMP7 signaling can be compensated in *Bmpr2*$^{1f/1f}$ pECs.

Enhanced TGF-β Signaling in BMPR2-Deficient pECs

To investigate whether impaired BMP signaling affects TGF-β signaling, we first examined the basal level of SMAD2 phosphorylation in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells cultured in medium containing 10% fetal bovine serum (Fig. 1-7A). The level of SMAD2 phosphorylated in *Bmpr2*$^{1f/1f}$ cells was much higher than that in *Bmpr2*$^{2f/2f}$ cells. To test if *Bmpr2*-deficient cells are more sensitive to TGF-β, we examined SMAD2 phosphorylation as a response to TGF-β1 (0, 1, and 2 ng/ml) (Fig. 1-7B). While both *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells showed a dose-dependent augmentation of pSMAD2, the level of SMAD2 phosphorylation was significantly higher in *Bmpr2*$^{1f/1f}$ cells at 1 and 2 ng/ml TGF-β1 compared to *Bmpr2*$^{2f/2f}$ cells, suggesting that impaired BMP signaling may potentiate the TGF-β signaling.

Opposing Balance Between TGF-β and BMP Signalings in pECs

To investigate whether BMP and TGF-β signaling form an opposing balance in pECs, we examined whether TGF-β-induced SMAD2 phosphorylation is suppressed by BMP treatment in pECs. TGF-β1 (0, 0.1, 1, and 2 ng/ml) and BMP4 (25 ng/ml) or BMP7 (25 ng/ml) were treated for 30 min after serum starvation in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells. The level of SMAD2 phosphorylation by 1 and 2 ng/ml of TGF-β1 treatment in *Bmpr2*$^{2f/2f}$ cells was decreased by either BMP4 or BMP7, implying that BMP and TGF-β have a competitive relationship by opposing each other (Fig. 1-8). However, TGF-β-mediated SMAD2 phosphorylation in *Bmpr2*$^{1f/1f}$ cells was not affected by BMP4 treatment, suggesting that BMP4 signaling is mediated mainly through BMPR2 (Fig. 1-8A). Interestingly, BMP7 could suppress overactivated TGF-β signaling in *Bmpr2*-deficient pECs as well as *Bmpr2*-intact pECs, suggesting that BMP7 signaling is not mediated

mainly through BMPR2 and also mediated by other type 2 receptors such as ACVR2a (Fig. 1-8B)

Discussion

In this study, we showed that genetic ablation of *Smad1* in pECs or SMCs predisposes mice to PAH, suggesting that impaired SMAD1 signaling is relevant to the pathogenesis of PAH. Furthermore, the findings of reduced SMAD1/5/8 activation and the overactivation of TGF-β signaling in the BMPR2-deficient pECs suggests that a predominance of TGF-β signaling, gained from the genetically-disturbed balance between TGF-β and BMP signaling, may be an important factor triggering PAH development.

SMAD1 is a Downstream Mediator of BMP Signaling in Pathogenesis.

SMAD1 is a canonical downstream mediator of the BMP pathway that functions in a variety of cellular and developmental events. Therefore, reduced BMP activity in association with the reduced expression of its receptors is accompanied by decreased phosphorylation of SMAD1/5/8 in the development of diseases. Liu *et al*. found that pSMAD1/5/8 and BMPR1b were decreased in malignant glioma tissues compared with normal brain tissues.[115] Yang *et al*. reported that the activated form of SMAD1 is deficient in the pulmonary vasculature of patients with a *BMPR2* mutation, suggesting that inactivation of SMAD1 plays a role in the PAH pathogenesis.[70] Our data demonstrate that *Smad1* deletion in either ECs or SMCs can induce PH in mice, implying that SMAD1 may be an important downstream signaling molecule of BMPR2 in PAH development.

SMAD1 Involvement in the Pathogenesis of PAH

Our data showing the role of SMAD1 in PAH may appear to be conflict with the previous data from James West's group demonstrated that overexpression of tail domain *BMPR2* mutant (R899X) not affecting the SMAD1 activation resulted in PAH.[108] However, frame shift and nonsense mutations consist of 70% of total mutations of *BMPR2* gene and interestingly, approximately one-quarter of this type mutations are collected in the cytoplamic tail domain. Nonsense mutation decay (NMD) is a cellular mechanism to destroy defective RNA transcripts with nonsense mutation to block the production of truncated proteins.[116] Thus, most of *BMPR2* mRNAs with the cytoplasmic tail mutation are destroyed through NMD-mediated processing, leading to BMPR2 deficiency.[104, 117] In James West's studies, truncated BMPR2 was not destroyed by NMD and showed normal SMAD1 activation. Thus, their mutant mice may not represent human PAH patients carrying a cytoplasmic tail muation of *BMPR2*. Otherwise, this discrepancy suggests that some BMPR2-associated PAH might be SMAD-dependent (mutation in the kinase domain) and some are not (mutation in the tail domain). Mutations in the tail domain have been shown to interrupt interactions between BMPR2 and the dynein light chain Tctex-1 as well as LIMK1, a key regulator of actin dynamics.[118, 119] Thus, the cytoplamic tail mutation may contribute to PAH development by disrupting these interactions with potentially critical signaling molecules in SMAD-independent manner. As an alternative interpretation, SMAD1 signaling is critical in ECs, not in SMCs for PAH development. Hansmann *et al*. presented a novel anti-proliferative axis – BMP2/BMPR2/PPARγ/ApoE in PAH.[120] Mice with targeted deletion of PPARγ in SMCs spontaneously developed PAH with elevated RVSP, RVH, and increased muscularization of the distal pulmonary arteries. This was independent of SMAD1/5/8

phosphorylation. Li *et al*. suggested a novel role of NOTCH3 in controlling proliferation of SMCs and in maintaining SMCs in an undifferentiated state.[121] They found that the severity of disease in human PAH patients and rodent PH models correlated with the amount of NOTCH3 protein in vascular SMCs of the lung, suggesting that NOTCH3 signaling pathway in SMCs is crucial for the development of PAH. Even though it still remains unknown whether NOTCH3 and PPARγ signaling in ECs are also critical contributors to PAH pathogenesis, up to now, our data with *Smad1* cKO mice demonstrated that SMAD1 deficiency in ECs is a important contributing factor for spontaneous PAH development.

SMAD1 Deficiency in ECs has a Greater Impact on PAH Than That in SMCs

Mice with endothelial *Smad1* deletion showed 40% penetrance while mice with *Smad1*-deficiency in SMCs displayed 13% penetrance. Why did endothelial deletion make a greater influence on PAH pathogenesis compared to deletion in SMCs? Malfunctioning of SMCs usually increase their growth rate, leading to thickening of vessel wall, whereas endothelial dysfunction contributes to PAH pathogenesis in various ways. Disordered endothelial proliferation forms neoinitimal layers[82] and disrupted balance of vasoactive mediators exacerbates vasoconstriction[14, 34, 84-86] and increased cell permeability allows serum growth factors to affect SMC proliferation and release of mitogen such as serotonin from ECs induces smooth muscle hyperplasia[122] and the number of occluded vessels are increased by *in situ* thrombosis.[2] Therefore, because of these various effects of ECs, *Smad1* deletion in ECs may accelerate PAH development compared to *Smad1* deletion in SMCs.

Incomplete Penetrance of PAH in *Smad1* Conditional KO Mice

All the mice with endothelial or smooth muscle-*Smad1* deletion did not develop PAH. Only a subset of mice showed PH phenotypes including elevation of RVSP and thickening of smooth muscle layer. First, SMAD1/5/8 could be functionally redundant and compensate for the absence of other molecules. Cre-mediated genetic ablation of either *Smad1* or *Smad5* in ovarian granulosa cells results in normal reproductive function but that combined loss of *Smad1* and *Smad5* results in fertility defects and granulosa cell tumors.[123] SMAD1, SMAD5, and SMAD8 function redundantly in activating regression of the Müllerian duct mesoepithelium.[124] Second, additional environmental insult would be required for the manifestation of PAH with genetic predisposition. Inflammation has been associated with PAH as an influencing environmental factor. Inflammatory cells including macrophages and lymphocytes were accumulated and many proinflammatory cytokines were induced in the PAH vascular lesions.[7, 12, 64, 93-97] Thus, if treated with inflammatory stimulants such as IL-6[125] and 5-lipoxygenase (5-LO)[68], *Smad1* cKO mice may show increased penetrance. Conversely, blocking inflammatory activation could be therapeutically beneficial for PAH patients. Studies with animal models support this. Platelet-activating factor (PAF) antagonists, an anti-inflammatory drug inhibited pulmonary vascular remodeling induced by hypobaric hypoxia in rats.[126] Inhibition of 5-lipoxygenase-activating protein (FLAP) suppressed hypoxia-induced pulmonary vasoconstriction *in vitro* and the development of chronic hypoxic pulmonary hypertension in rats.[127]

Imbalance in TGF-β and BMP Signalings in PAH Pathogenesis

Many studies have been focused on impaired BMP signaling to explain PAH pathogenesis. However, as dysfunction of BMP pathway alone is not sufficient to

develop PAH, recent studies suggest a role of imbalance in TGF-β/BMP signaling for PAH development because BMP and TGF-β signaling form an opposing balance in pulmonary vascular homeostasis.[128] BMPR2-deficient pulmonary vessels lose control of overactivation of TGF-β signaling leading to predominance of TGF-β signaling.[101-103, 129] Based on these reports, we hypothesized that BMPR2 deficiency results in overactivated TGF-β signaling in pECs. However, it is difficult to demonstrate that BMPR2 deficiency directly elevates TGF-β signaling *in vivo* system because even though our *Bmpr2* or *Smad1* cKO mice exhibit TGF-β activation such as increased pSMAD2/3, it could be a secondary effect of PAH. Hence, we isolated and immortalized pECs from a *R26*$^{creER+/-}$;*Bmpr2*$^{2f/2f}$ mouse, in which tamoxifen treatment induces *Bmpr2* deletion. This inducible system is advantageous because we can exclude strain and individual difference using the same parental cells. *Bmpr2*-deleted pECs showed the low level of pSMAD1/5/8 by BMP4 treatment and enhanced phosphorylation of SMAD2 not only at the basal level but also by TGF-β1 treatment compared with *Bmpr2*-intact pECs, supporting our hypothesis that BMPR2 deficiency overactivates TGF-β signaling in pECs. In addition, we found a marked reduction of total SMAD2 with an accompanying high level of pSMAD2. This may have arisen because activated SMAD2 is multi-ubiquitinated and is degraded by the proteosome.[130-132]

These results from pECs present the possibility of the future therapeutic options to restore TGF-β/BMP imbalance. Recent reports support our results and suggest potential candidates for inhibitor of TGF-β pathway. Inhibition of TGF-β signaling by ALK5 inhibitor, IN-1233 prevented PAH in the monocrotalin-treated rat, which showed increased TGF-β activity.[103] The angiotensin II type 1 receptor (AT1) blocker, losartan

averted aortic aneurysm in a mouse model of Marfan syndrome (MFS), which is associated with increased TGF-β signaling.[133] In the aspect of BMP activation, Irrespective of BMPR2 deficiency, pECs activated SMAD1/5/8 in response to BMP7. This intact BMP7 response was also shown in SMCs with *Bmpr2* deletion.[77] Therefore, restoration of pSMAD1/5/8 by BMP7 could be beneficial to PAH patients with BMPR2 deficiency.

Thickening of the Smooth Muscle Layer Might not be Sufficient to Lead to High Pulmonary Pressure.

The PH group in L1cre(+);*Smad1*$^{2f/2f}$ mice showed thickened vessel walls, implying that high pulmonary pressure was associated with the muscularization of pulmonary arterioles. However, *Tagln*-cre(+);*Smad1*$^{2f/2f}$ mice including Non-PH and PH groups showed thickening of the vascular smooth muscle layer, suggesting that SMAD1 dysfunction in SMCs directly affected proliferation of vascular smooth muscles. Yang *et al*. reported that SMAD1 dysfunction promoted MAPK signaling leading to aggressive proliferation of PASMCs.[70] More importantly, our result suggests that thickening of the smooth muscle layer might not be sufficient to lead to high pulmonary pressure in the absence of related endothelial dysfunction. We speculate that SMAD1 deficiency in pECs results in endothelial dysfunction. Pulmonary endothelial dysfunction induces sustained constriction of blood vessels due to an increase of vasoconstrictors such as endothelin or decrease of vasodilators such as eNOS or prostacyclin. These alterations of vasoactive molecules were readily observed in PAH patients[14, 44, 134, 135] and PH animal models.[136-138] Continued vascular constriction leads to high pulmonary pressure and results in thickening of the vascular smooth muscle layer. However, although *Smad1* deletion in SMCs induces thickening of smooth muscle layer, unaffected

endothelial cells may enable vessels to dilate more in response to the high blood pressure gained from thickened blood vessels. Therefore, thickening of the smooth muscle layer might not be sufficient to sustain high pulmonary blood pressure.

Smad1 cKO Mice and Inducible *Bmpr2* KO pECs are Useful Resources for Future Mechanism Research, Drug Screening and Preclinical Study.

In this study, we developed two useful resources for PAH studies; *Smad1* cKO mice and inducible *Bmpr2* KO pECs. In the future, these mice and pECs will be usefully utilized for mechanistic research to find downstream targets of BMPR2/SMAD1 pathway for novel PAH therapy and when candidate drugs are developed or preexisting medicines with potential effect are not tried to PAH patients, we can get valuable information about efficacy and effectiveness of candidate drugs for PAH using these materials.

Table 1-1. Primers for genomic and semi-quantitative PCR reaction

Genomic PCR			
Smad1-A	CACCTGTGCCCCCTCCAAGT	Smad1-B	GAGCTCTGCTCCGCCACTCA
Alk1-F	CAGCACCTACATCTTGGGTGGAGA	Alk1-R	ACTGTTCTTCCTCGGAGCCTTGTC
Bmpr2-2A	CACACCAGCCTTATACTCTAGATAC	Bmpr2-6R	CACATATCTGTTATGAAACTTGAG
Bmpr2-2C	TTATTGTAAGTACACTGTTGCTGTC		
L1cre-F	GTTTTCCTTTGAAAAACACGATGA	L1cre-R	ATCAGGTTCTTGCGAACCTCATCA
Tagln-cre-F	CTCCTTCCAGTCCACAAACGAGC	Tagln-cre-R	GGGCGATCCCTGAACATGTCC
R26R-F	GTCGTTTTACAACGTCGTGACT	R26R-R	GATGGGCGCATCGTAACCGTGC
RT-PCR			
Gapdh-F	CAATGCATCCTGCACCACCAA	Gapdh-R	GTCATTGAGAGCAATGCCAGC
Bmpr2-F	GTTGACAGGAGACCGGAAACAG	Bmpr2-R	GGAGACTCAGATATTTGCACAG
Tgfbr2-F	TTGCCTGTGTGACTTCGGGCT	Tgfbr2-R	CTATTTGGTAGTGTTCAGCGA
Acvr2a-F	CGTTCGCCGTCTTTCTTATC	Acvr2a-R	AGGATTTGAAGTGGGCTGTG
Alk1-F	TCATGGTGCACAGTGGTGCTG	Alk1-R	CAAATCCCGCTGCTTCTCCTG
Alk2-F	AGTCATGGTTCAGGGAGACG	Alk2-R	TGCAGCACTGTCCATTCTTC
Alk3-F	TAAAGGCCGCTATGGAGAAG	Alk3-R	CCAGGTCAGCAATAAGCAA
Alk6-F	CACTCCCATTCCTCATCAAA	Alk6-R	TTCCAATCTGCTTCACCATC
Tgfβ1-F	CGGAAGCGCATCGAAGCCATCC	Tgfβ1-R	GCAAGCGCAGCTCTGCACGG
Nos3-F	TTCCGGCTGCCACCTGATCCTAA	Nos3-R	AACATATGTCCTTGCTCAAGGCA
Tie2-F	CTCATCTGTGGACGCTGGATG	Tie2-R	GGCACTGAGTGGATGAAGGAG
Eng-F	TGCACTCTGGTACATCTATTC	Eng-R	TGGATTGGGCAGTTCTGTAAA
Flk1-F	AGAACACCAAAAGAGAGGAACG	Flk1-R	GCACACAGGCAGAAACCAGTAG

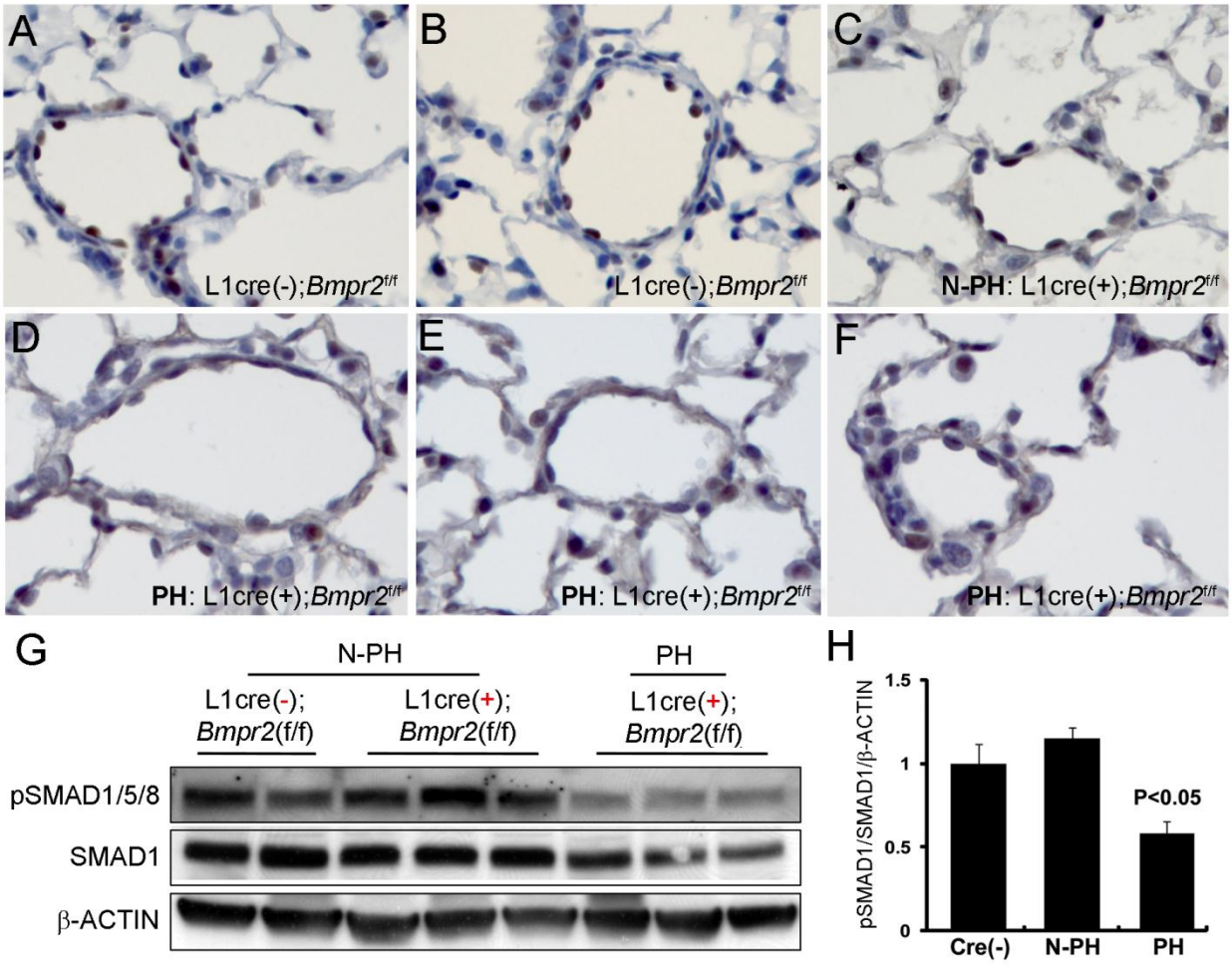

Figure 1-1. BMP response was severely impaired in the lungs of PH mice [L1cre(+);*Bmpr2*$^{f/f}$]. (A-F) The levels of pSMAD1/5/8 and SMAD1 were examined to compare BMP response among lungs of cre-negative controls (A,B), N-PH (C), and PH mice (D-F). (G-H) The level of pSMAD1/5/8 was significantly decreased in the PH lungs compared to controls and N-PH lungs.

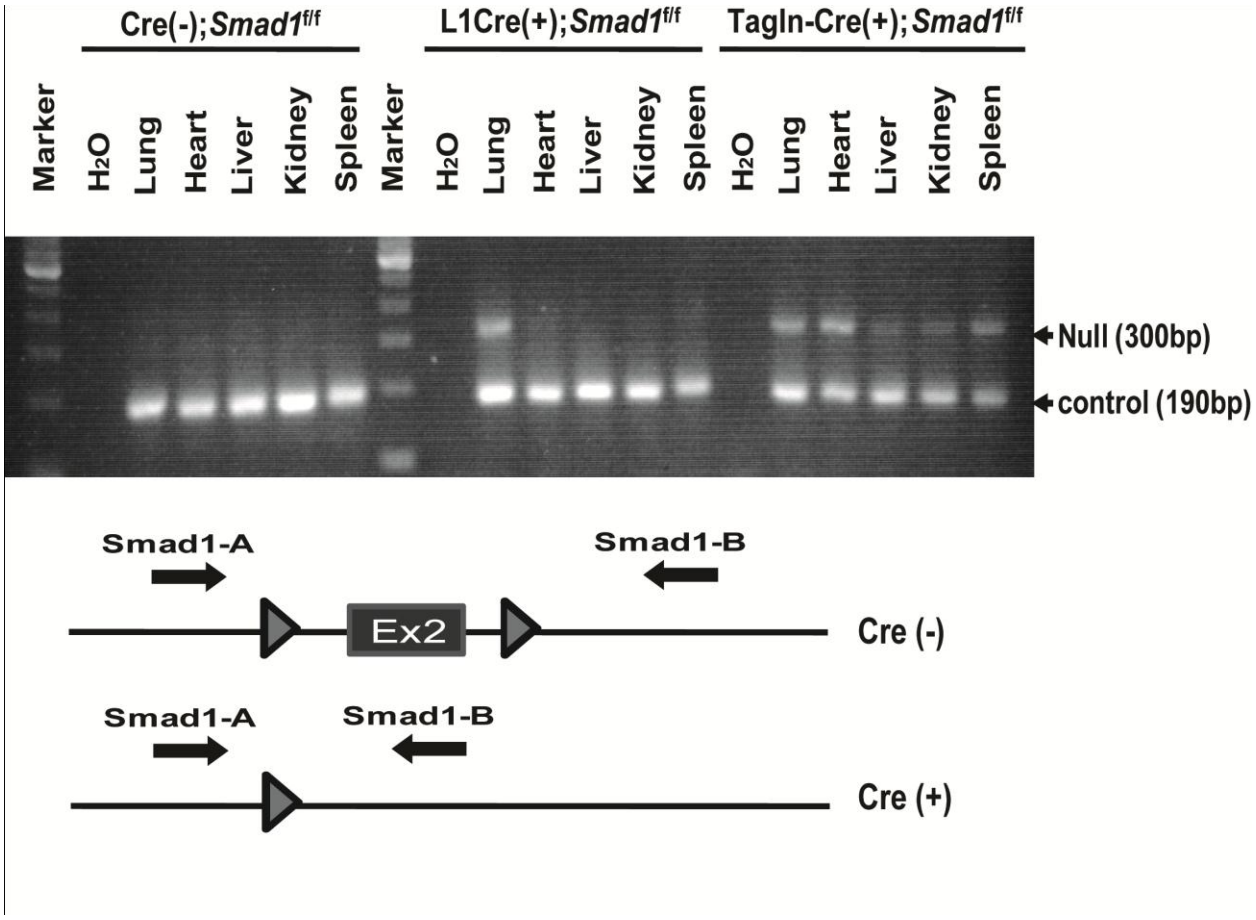

Figure 1-2. *Smad1* deletion in L1cre(+);*Smad1*$^{f/f}$ and *Tagln*-cre(+);*Smad1*$^{f/f}$ mice. Deleted *Smad1* allele was examined in various organs of *Smad1*-conditional KO (cKO) mice and cre-negative control mice by genomic PCR analysis. Null *Smad1* was detected at 300 bp. A primer set amplifying the *Alk1* locus (190 bp) was used as a control for the PCR amplification. Genomic segment between 2 loxp sites including exon2 was deleted by cre recombinase activated by tamoxifen leading to production of PCR product by a primer set (Smad1-A and Smad1-B).

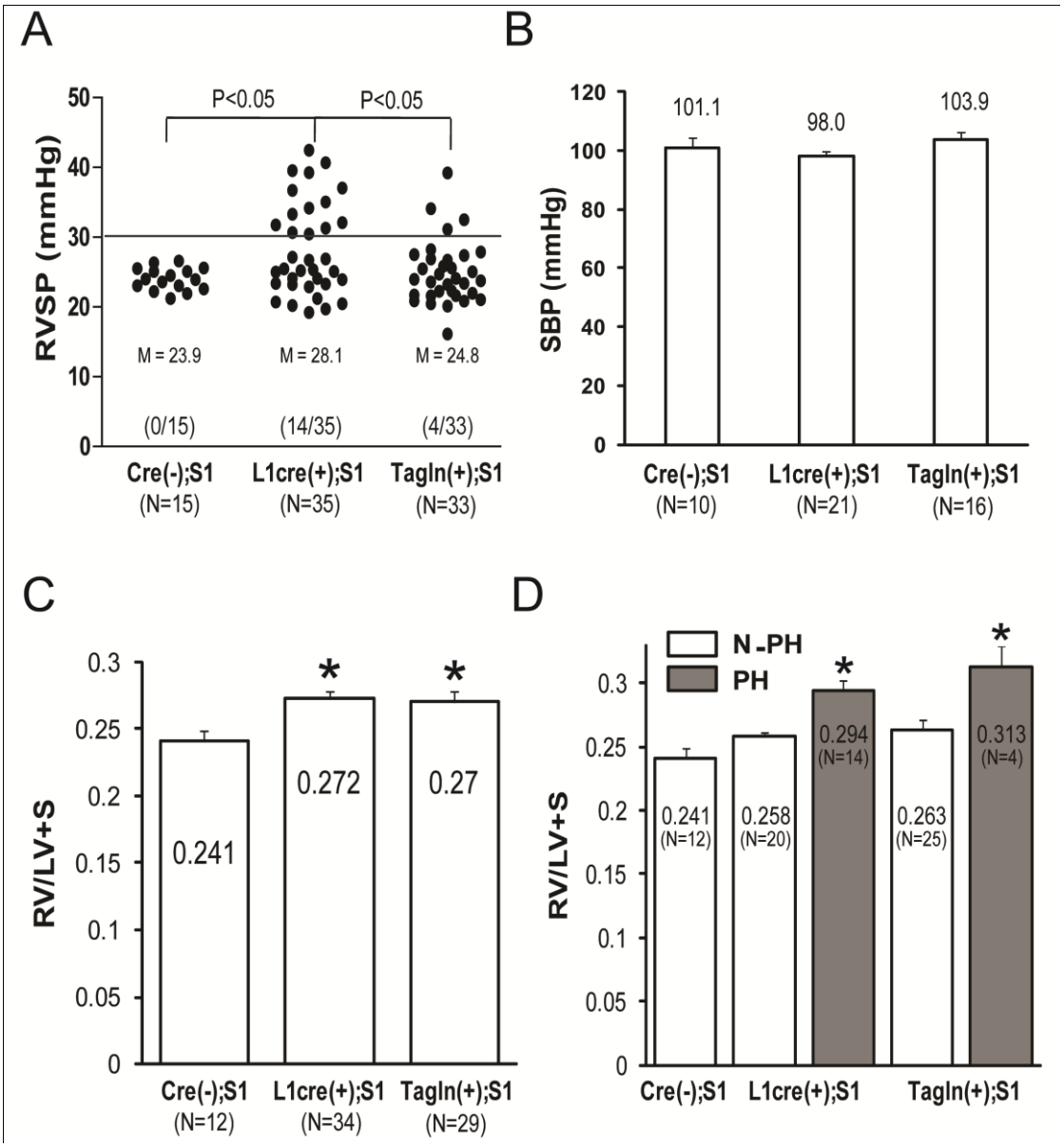

Figure 1-3. *Smad1* deletion in SMCs and ECs resulted in elevation of RVSP and RV hypertrophy. (A) closed circles indicate RVSP of each mouse. More than 30 mmHg of RVSP was designated as pulmonary hypertensive pressure. 40% of L1cre(+);Smad1f/f mice and about 12% of Tagln-cre(+);Smad1f/f mice were pulmonary hypertensive. RVSP of L1cre(+);Smad1f/f was significantly higher compared to either that of controls or that of Tagln-cre(+);Smad1f/f . (B) Systemic blood pressure was not significantly different by genotypes of mice. (C) Both Smad1-cKO mouse lines showed significant RV hypertrophy compared to cre-negative controls. (D) When mice were divided into non-pulmonary hypertensive (N-PH) and pulmonary hypertensive (PH) groups, RV hypertrophy of PH mice was significantly higher than cre-negative controls and N-PH mice of each cKO group. Statistical differences ($p<0.05$) were indicated by asterisks above each bar.

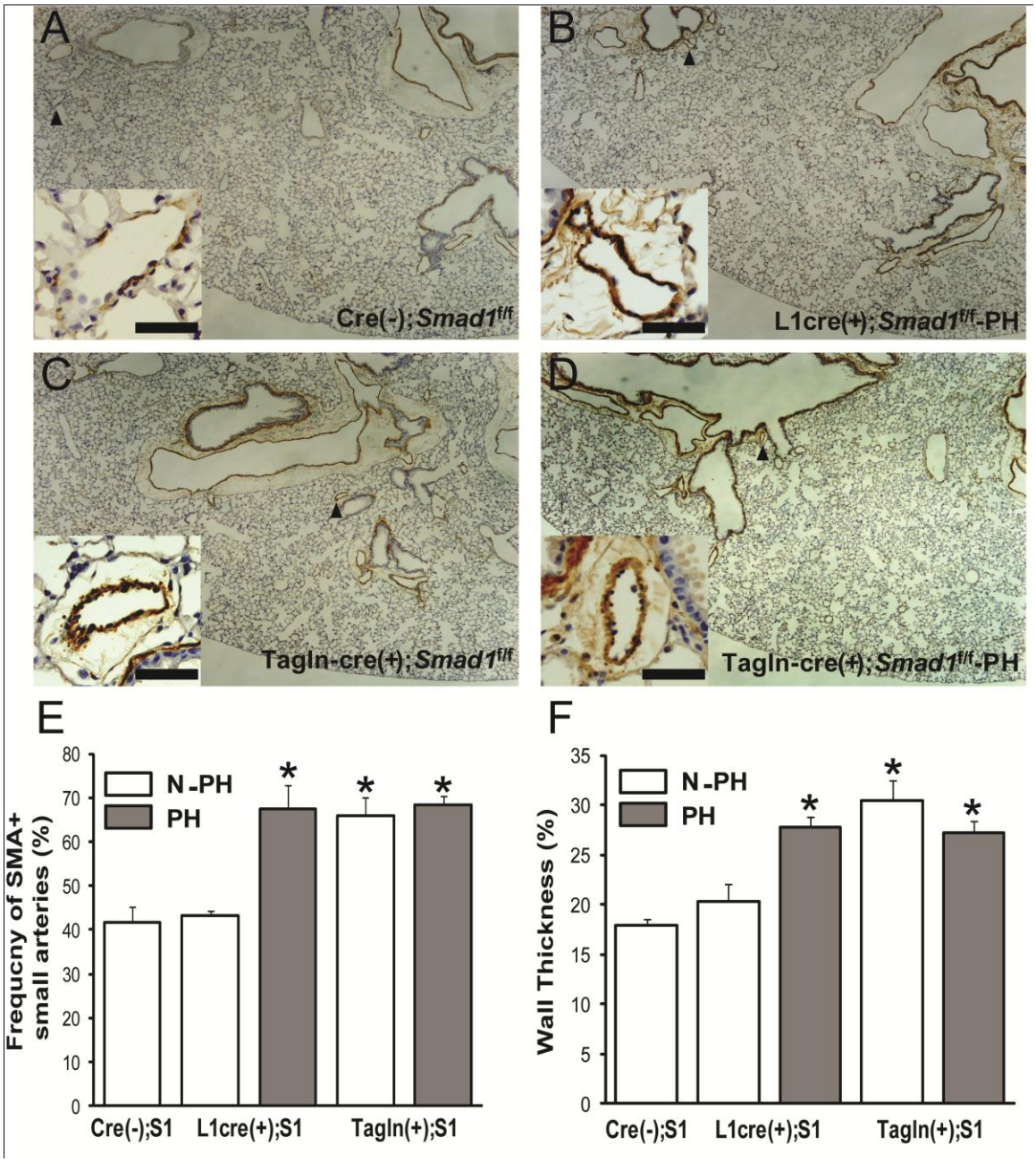

Figure 1-4. Elevated pulmonary pressure is associated with pulmonary vascular remodeling. (A-D), pulmonary arterioles were visualized by staining with anti-αSMA antibody. A representative vessel from each group (indicated by arrowheads) is shown at higher magnification at the left bottom corner of each frame. Scale bars in insets represent 50 μm. PH mice of each *Smad1*-cKO mice showed muscularized vessels (E) and thickened vessel walls (F) compared with cre-negative controls. Interestingly, N-PH group of *Tagln*-cre(+);*Smad1*$^{f/f}$ also exhibited muscularized vessels with thickened vessels walls. * indicates significant statistical difference ($p<0.05$) compared to cre-negative controls.

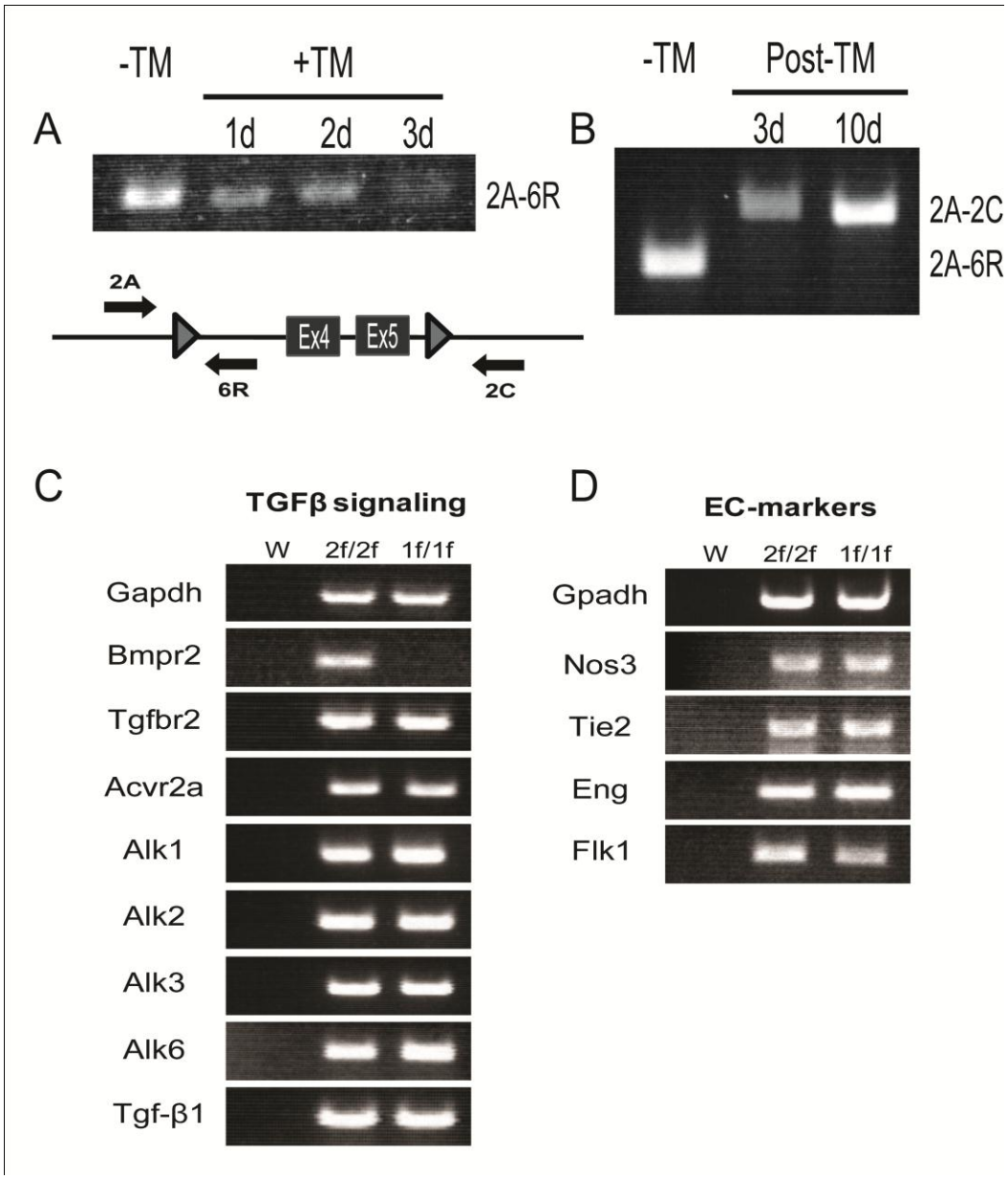

Figure 1-5. Deletion of *Bmpr2* gene in pulmonary endothelial cells (pECs). (A) pECs were treated with tamoxifen (TM) for 3 days. As shown in the diagram, TM enables cre recombinase to loop out the genomic segment containing 6R primer binding sequence and exon 4-5, which is flanked by two loxp sites (triangles). This deleted allele can produce a PCR product with the 2A-2C primer pair. Deletion of *Bmpr2* gene was examined using the 2A-6R primer pair. (B) Following growth in TM-free media, reappearance of an intact *Bmpr2* gene in TM-treated pECs was examined using primer pairs for the deleted allele [2A-2C] and the intact allele [2A-6R]. The mRNA levels of TGF-β signaling molecules (C) and endothelial markers (D) were examined by semi-quantitative RT-PCR to determine whether tamoxifen treatment for *Bmpr2* deletion affected expression of other molecules in TGF-β pathway and EC characteristic.

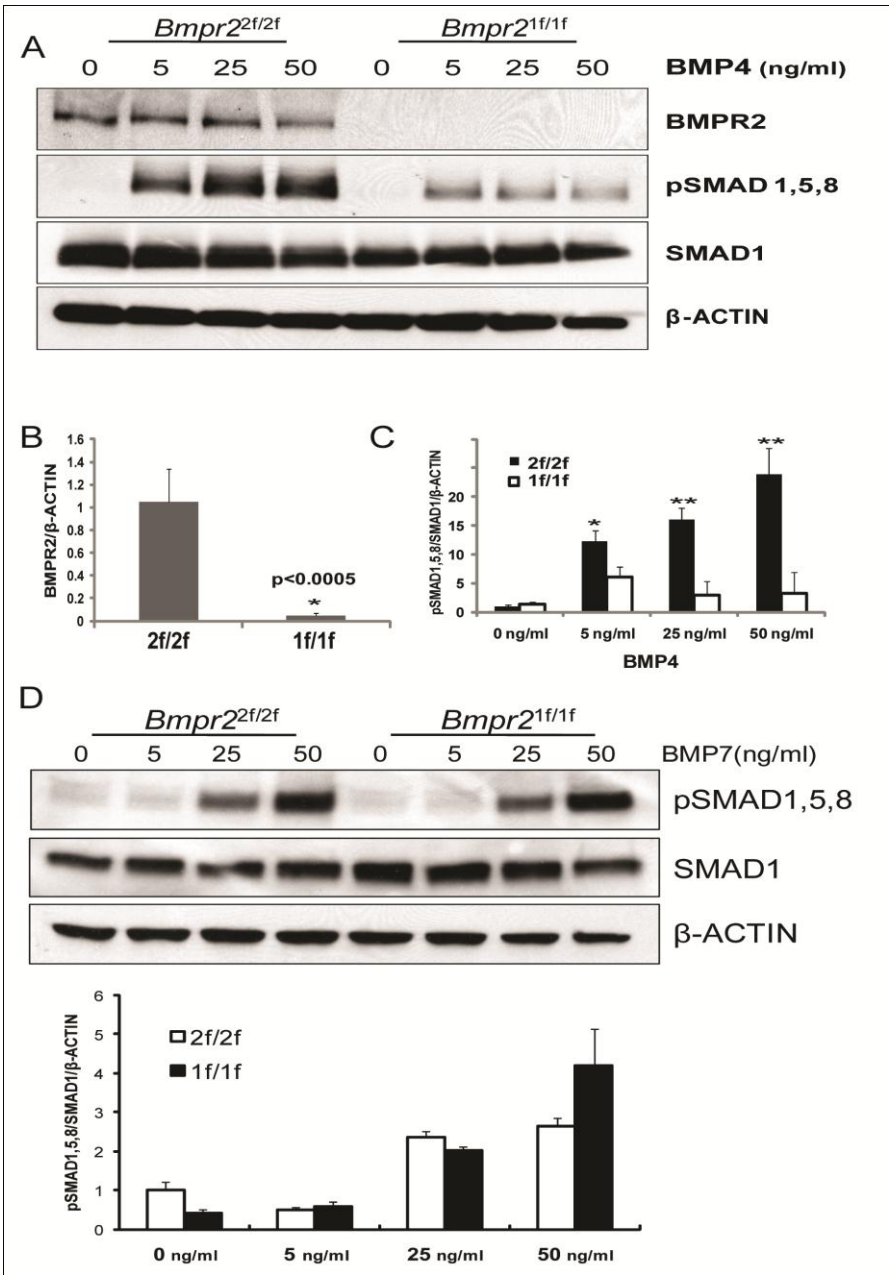

Figure 1-6. *Bmpr2*-deleted pECs displayed a reduced response to BMP4 but not BMP7. (A) BMP4 (0 – 50 ng/ml) was added to *Bmpr2*-intact (*Bmpr2*$^{2f/2f}$) and *Bmpr2*-deleted (*Bmpr2*$^{1f/1f}$) cells for 30 minutes after serum starvation. The levels of pSMAD1/5/8 were examined to evaluate the BMP4 response. (B) The protein levels of *Bmpr2* were determined for *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells. (C) BMP4 response was quantified by the ratio of pSMAD1,5,8/SMAD1/β-actin in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells. (D) BMP7 (0 – 50 ng/ml) was added to *Bmpr2*-intact (*Bmpr2*$^{2f/2f}$) and *Bmpr2*-deleted (*Bmpr2*$^{1f/1f}$) cells for 30 minutes after serum starvation. The levels of pSMAD1/5/8 were determined to evaluate differences in response to BMP7. The BMP7 response was quantified by the ratio of pSMAD1,5,8/SMAD1/β-actin in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells.

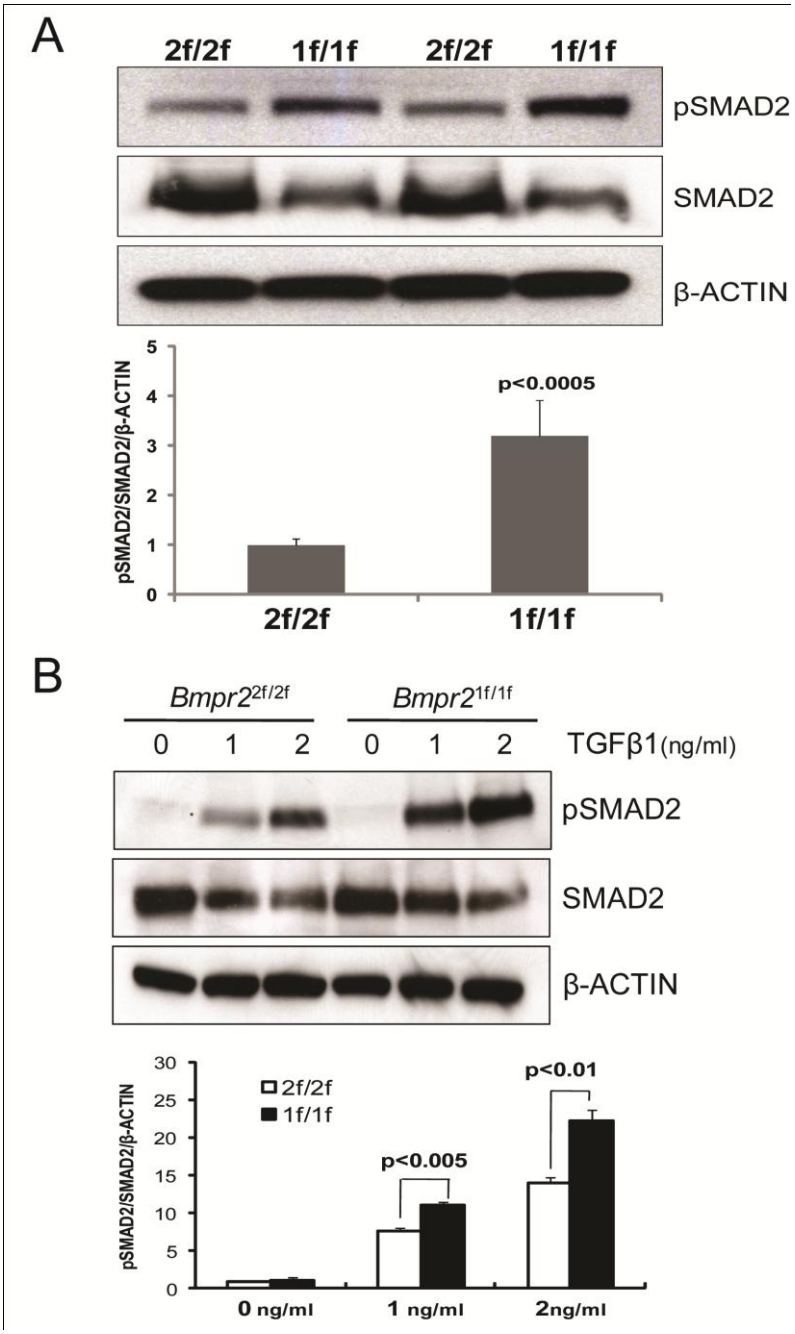

Figure 1-7. Reduced BMP response induced overactivation of TGF-β signaling. (A) The levels of pSMAD2 were examined to compare basal TGF-β activation in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells under normal media condition without additional cytokine treatment. TGFβ activation was quantified by the ratio of pSMAD2/SMAD2/β-actin in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells. (B) In order to compare TGF-β response in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells, TGF-β1 (0-2 ng/ml) was added for 30 minutes after serum starvation. TGF-β response was quantified by the ratio of pSMAD2/SMAD2/β-actin in *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells.

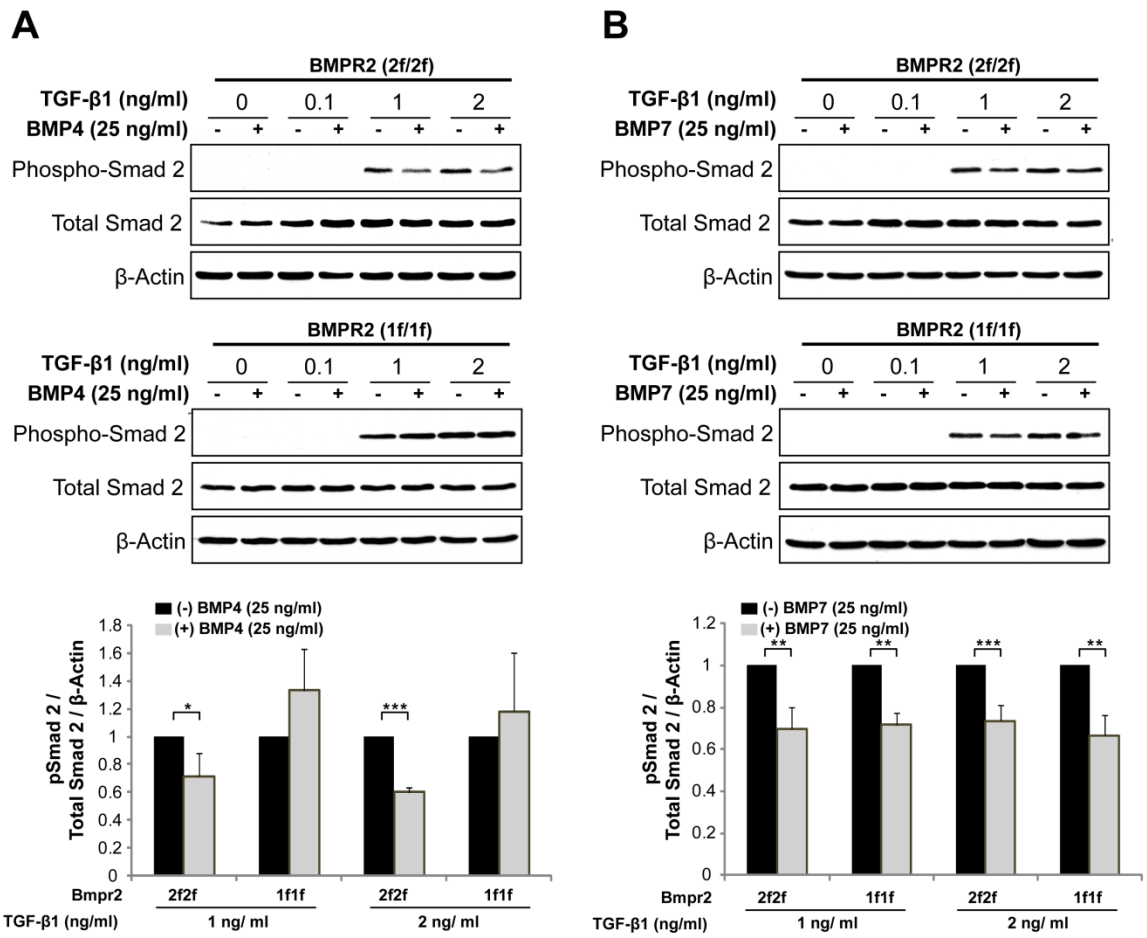

Figure 1-8. BMP and TGF-β signaling form an opposing balance in pECs (A) Activation of TGF-β signaling was suppressed by either BMP4 or BMP7 in *Bmpr2*-intact pECs. TGFβ-1 (0-2 ng/ml) was treated on *Bmpr2*$^{2f/2f}$ and *Bmpr2*$^{1f/1f}$ cells for 30 minutes after serum starvation. TGF-β response was quantified by the ratio of pSMAD2/SMAD2/β-actin. However, (B) this suppression was induced only by BMP7, not by BMP4 in *Bmpr2*-deficient pECs.

CHAPTER 2
THERAPEUTIC EFFECT OF VEGF BLOCKADE ON ARTERIOVENOUS MALFORMATIONS (AVMS) IN AN ANIMAL MODEL FOR HEREDITARY HEMORRHAGIC TELANGIECTASIA

Introduction

Hereditary Hemorrhagic Telangiectasia (HHT)

Hereditary Hemorrhagic Telangiectasia (HHT) is an inherited autosomal dominant vascular disease affecting 1 in 5,000~8,000 individuals worldwide.[139-141] The disease is characterized by epistaxis (spontaneous and recurrent nosebleeds), mucocutaneous telangiectases as well as arteriovenous malformations (AVMs) in the brain, lungs, and visceral organs including the liver and GI tract.[142-145] An AVM is a direct connection between arteries and veins without intervening capillaries[142] and is an underlying defect associated with recurrent nose bleeding, telangiectases, and visceral hemorrhaging. In AVMs, high velocity-arterial blood invades venous vascular beds creating turbulent and swirling flows leading to tortuous and irregular vascular remodeling. When these blood vessels rupture, it results in chronic and severe anemia due to massive blood loss. Brain and lung hemorrhages are life-threatening and the major cause of death in young patients.[146, 147] To compensate for the lost blood from GI and nose bleeding, patients receive regular blood transfusions and/or iron supplementation therapy. More than 90% of HHT patients experience recurrent nosebleeds by their 60s[148] and this affliction impacts profoundly on the patients quality of life.[149, 150] All the patients receiving Young's procedure for completely blocking nostrils said that the quality of their lives was improved following the surgery.[151]

Limitation of Current Therapeutic Interventions in HHT

Therapeutic treatments are available to alleviate telangiectasia and visceral lesions. Embolization can prevent blood flow to AVM lesions by occluding blood vessels with metal coils and is effective for pulmonary and cerebral AVMs.[152, 153] For acute nosebleeds, packing is a common method and in chronic and recurrent nose bleeding, humidification with topical lubricant, laser therapy, and surgeries including Young's procedure are applied depending on the unique condition of the patient.[154] Systemic hormonal treatments or prothrombotic strategies using antifibronolytic agents have been useful in limiting excessive hemorrhaging.[155, 156] However, the mechanism of hormonal efficacy remains unknown and the negative impacts of prothrombogenic agents must be considered before treatment.[157] Thus, the development of therapies targeting the pathogenic mechanisms underlying AVM formation remains an urgent issue.

Genetic Studies in HHT

Heterozygous mutations in HHT-causing genes have been identified: *ENG* for HHT1,[158-160] *ALK1* for HHT2,[161, 162] and *SMAD4* for Juvenile Polyposis and HHT (JP-HHT).[163] Over 80% of HHT patients possess heterozygous mutations in one of these genes, while mutations at additional loci are responsible for the remaining 20%. These loci include the HHT3 locus (chromosome 5)[164] and the HHT4 locus (chromosome 7)[165] even though causative genes remain unidentified.

Genetic Mouse Models for HHT

The role of ALK1 in HHT pathogenesis has been explored using genetically modified mouse models.[166, 167] Heterozygous *Alk1* null mice displayed HHT-like phenotypes such as age-dependent subcutaneous and mucocutaneous vascular lesions as well as hemorrhages in the lungs, GI tract, liver, brain and spleen.

Penetrance, however, was not complete and symptoms when observed were not as severe as observed in humans. Homozygous *Alk1* knockout (KO) mice died *in utero* displaying vascular defects such as hyper-dilated blood vessels and AVMs.[166, 168] Targeted *Alk1*-deletion in endothelial cells of transgenic mice resulted in AVMs and hemorrhages in the vascular beds of brain, lung and GI tract.[109] Recently, our lab demonstrated that the conditional deletion of *Alk1* in adult mice using the tamoxifen-inducible RosacreER driver recapitulated HHT phenotypes common in human patients, such as severe hemorrhaging and low hemoglobin levels.[169]

Secondary Hits are Required for AVM Development.

AVM lesions in HHT patients and *Alk1* mutant mice appear in selective organs such as the lung, GI tract, liver and brain despite the global presence of *ALK1* or *ENG* mutations. Even in the same organ, some vascular beds form AVMs and hemorrhage while other vessels remain normal in appearance. This suggests that genetic predisposition is not sufficient to cause disease and other genetic or environmental factors play a role. When excisional wounds were performed on the dorsal skin and ear of *Alk1*-deficient adult mice, vascular dysplasia, or "*de novo* AVM" was induced around to the wounded area.[169] Vessels removed from the wounded area were unaffected. This was evidence to suggest that ALK1 deficiency and injury combined to induce the vascular lesion. Angiogenesis is variably regulated by processes associated with wound-healing. These include the actions of infiltrating inflammatory cells, the turnover of extracellular matrix via matrix metalloproteinase (MMP) secretion, and the local activity of growth factors and cytokines produced by endogenous and recruited cells.[170] Which event is most critical for AVM formation in *Alk1*-deficient blood vessels is a question we addressed in this study. We focused on LPS-induced inflammation and

growth factor-induced angiogenesis. Mucocutaneous telangiectasia occurs frequently on the lips, tongue, and nose which are constantly exposed to infection. Increased levels of inflammatory markers factors such as MMP9 and interleukin-6 (IL-6) have been demonstrated in brain AVM tissue.[171, 172] Inflammatory cells including neutrophils and macrophages/microglia were frequently identified in the vascular walls of AVM tissue.[173] In addition, the tissues and plasma of HHT patients contain high levels of vascular endothelial growth factor (VEGF). Vessel density was also increased.[174, 175] Brain AVMs usually develop congenitally during vascularization. Some case reports showed that anti-angiogenesis therapy using bevazicumab and thalidomide worked well to prevent epistaxis and liver AVMs.[176, 177]

In this report, we demonstrate that inflammation or direct angiogenic stimulation can induce AVMs in *Alk1*-deficient subdermal vessels. Angiogenic stimulation is a necessary sequella to inflammation in the induction of AVM formation. Then, angiogenesis blockade using a VEGF-neutralizing antibody is effective in alleviating wound-induced AVMs and internal bleeding in GI tract and lungs in ALK1-deficient adult mice. Taken together, our findings suggest that angiogenesis blockades could be promising and fundamental medication in future clinical trials for HHT patients.

Results

Vascular Endothelial Growth Factor (VEGF) or Lipopolysaccharide (LPS) can Induce De Novo AVMs in *Alk1*-Deficient Subdermal Vessels.

In order to examine whether inflammation or angiogenesis can induce AVM formation in *Alk1*-deficient mice, VEGF or LPS was administered to mice in the form of PLGA particles implanted subdermally under the dorsal skin. LPS-PLGA and VEGF-PLGA particles were injected into cre-negative mice as a control to assess whether

either agent alone could induce vascular lesions in the absence of an *Alk1* deficiency. After 7 days, blue latex dye was infused through the left heart to visualize the blood vessels and to locate arterial-venous (AV) shunts. The dye should be prohibited from traversing capillary beds unless an AV shunt is present. The particles formed an orange round aggregation on the subdermal area because of rhodamine. After removing particle-aggregates, the skin was cleared to reveal the vascular network. In *Alk1* wild type mice, a higher vascular density was evident around LPS and VEGF particle-injected areas compared to neighboring regions, but vascular malformation such as AV shunts were not observed (Fig. 2-1A, B). PLGA particles themselves occasionally induced higher vascular densities around implantation sites in *Alk1*-deficient mice but did not induce AV shunts. This was demonstrated in tamoxifen-treated *R26*$^{creER/+}$;*Alk1*$^{2f/2f}$ mice in which PBS-PLGA was implanted into the dorsal skin. PBS-PLGA had no affect on subdermal vessels (Fig. 2-1C) or, on rare occasions, induced a moderate level of abnormal vasculature (Fig. 2-1D). When VEGF-PLGA was injected into *Alk1*-deficient skin, tortuous, irregular, and excessive vasculature developed around the particles and surrounding veins were dilated and contained latex dye, indicating the presence of AV shunts (Fig. 2-1E). LPS-PLGA particles also induced a comparable level of tortuous vessels and AV shunting (Fig. 2-1F).

VEGF and LPS Stimulated Angiogenesis.

VEGF is a strong angiogenic stimulator in vascularization[178, 179] and tumors[180] and LPS has been to stimulate angiogenesis.[181, 182] Therefore, we questioned whether VEGF or LPS released from PLGA particles elicited an angiogenic response. We observed the vascular morphology around VEGF- or LPS-PLGA-injected area 4 days following PLGA-particle injection. Sprouting blood vessels were seen to be drawn

towards the VEGF and LPS particles (Fig. 2-1G, H). This result was consistent with angiogenesis being a common, and necessary, element to both VEGF and LPS-induced AVM formation. It could also be a critical factor in wound-induced AVMs.

VEGF-Blockade Suppressed Wound-Induced AVM in Alk1-Deficient Subdermal Vessels.

To test the participation of VEGF in the development of inflammation-induced or wound-induced AVM formation, experiments were repeated in the presence of VEGF-neutralizing antibody to block angiogenesis. *Alk1*-deficient and wounded mice were simultaneously treated with VEGF antibody or saline alone. The local vasculature was evaluated 9 days following the application of the wound. Saline-treated control mice exhibited a typical AV shunts with excessive, tortuous, and dilated vessels surrounding the wounded area (Fig. 2-2A, top panels). In contrast, AV shunts were only occasionally observed in VEGF antibody-treated animals and there were fewer tortuous and irregular vessels near the wound (Fig. 2-2A, bottom panels). Blood vessels positive for latex dye were quantified to measure the severity of AVMs in saline-treated and VEGF antibody-treated groups. The VEGF antibody-treated group (mean: 17.38 % vessel area) displayed a reduction in vessel content of approximately 40% in mean vessel area relative to saline-treated group (17.38 % versus 29.04 %, respectively). Thus VEGF antibody was capable of suppressing AVM formation in *Alk1*-deficient subdermal vessels responding to a wound (Fig. 2-2B).

VEGF-Blockade Alleviated Internal Bleeding and Low Hemoglobin Occurring in *Alk1*-Deficient Mice.

Alk1-deleted mice (tamoxifen-treated $R26^{creER/+};Alk1^{2f/2f}$ mice) die within 2-3 weeks of tamoxifen treatment displaying severe GI, lung, and uterine hemorrhage.[169] Since AVMs are a major cause of bleeding in HHT, we questioned whether

angiogenesis blockade would suppress visceral bleeding in *Alk1*-deficient mice. VEGF antibody (or saline, as a control) was injected into *R26*$^{creER/+}$;*Alk1*$^{2f/2f}$ mice following tamoxifen treatment. After 9 days, the concentration of blood hemoglobin was determined as an indirect measure of internal hemorrhaging. Normal mice had hemoglobin levels at 15-17 g/dl (data not shown). *Alk1*-deficient mice treated with saline exhibited significantly lower hemoglobin levels (mean: 8.6 g/dl) indicative of severe hemorrhaging. On the other hand, VEGF antibody-treated mice displayed near normal levels of hemoglobin (13.8 g/dl), suggesting that internal bleeding was greatly reduced by VEGF antibody treatment (Fig. 2-3A). Thoracic and abdominal cavities were explored for direct evidence of hemorrhaging. In addition, heart size was compared as well because poorly oxygenated blood due to severe hemorrhage would force the heart to work harder to maintain oxygen levels, leading to enlargement of the heart. The relative severity of GI and lung bleeding and heart enlargement was categorized as weak, moderate, or severe (Fig. 2-7). The saline-treated group showed moderate to severe bleeding in the lungs and GI tract (11/12 animals;Table 2-1, Fig. 2-3B). The majority of the VEGF antibody-treated group displayed weak GI and lung hemorrhaging (9/12 mice;Table 2-1, Fig. 2-3B). The hearts of saline-treated group were more enlarged compared to the VEGF antibody-treated group (Fig. 2-3B) suggestive that severe hemorrhaging in the mock-protected group adversely affected the size of the heart.

VEGF-Blockade Suppressed LPS-Induced AVMs in *Alk1*-Deficient Subdermal Vessels.

To determine whether inflammation alone, in the absence of angiogenesis, could induce AVMs in *Alk1*-deficient mice, LPS particles were implanted in subdermal sites and animals were simultaneously treated with VEGF-antibody to block angiogenesis.

VEGF-antibody suppressed AVM formation associated with LPS implantation and mock treatment with vehicle (saline; Fig. 2-4A through 2-4D). This result indicates that LPS-induced AVM formation is linked to angiogenic stimulation.

TNF-α did not induce severe AVM formation compared to LPS.

Inflammatory stimulation largely independent of an angiogenic response was evaluated with the implantation of TNF-α-PLGA particles into *Alk1*-deficient mice. TNF-α is less actively involved in the monocyte-induced angiogenesis compared to other inflammatory cytokines such as IL-8.[183] A higher vascular density was observed around TNF-α particles, indicating that TNF-α induced neovascularization. However, it did not provoke severe form of AVMs shown around LPS-particles (Fig. 2-5D through 2-5F)

Discussion

Although HHT is a genetic disease, vascular lesions appear only in limited vascular beds, indicating that localized secondary genetic or environmental insult is involved in development of AVMs. Recently, we have demonstrated that wound could induce AVMs in the subdermal vessels of a mouse model of HHT2.[169] In the present study, we showed that angiogenic stimulation by VEGF or LPS could mimic the wound effect for *de novo* AVM formation in subdermal vessels of ALK1-deficient mice (Fig. 2-6). We further demonstrated that treatment with VEGF-neutralizing antibody could inhibit both wound- and LPS-induced AVMs (Fig. 2-6), and could alleviate the internal bleeding in the *Alk1* conditional KO model. These data attest to the use of angiogenesis blockade for treating epistaxis and GI bleeding in HHT patients.

Therapeutic Potential of Anti-Angiogenic Drugs in HHT

Recent anecdotal clinical evidence suggests that anti-angiogenic drugs can be an effective therapy for HHT. The first ground-breaking report came from an Australian

group showing VEGF-neutralizing antibody, Bevacizumab (Avastin) treatment effectively regressed hepatic AVMs.[177] More recently, Bevacizumab treatment was shown to be effective for pancreatic AVMs,[184] GI bleeding,[185, 186] and epistaxis[187-190] in HHT patients. Lebrin et al showed that thalidomide, known to have anti-angiogenic and anti-inflammatory functions, enhanced mural cell recruitment in Eng+/- mouse model, and alleviated the frequency and severity of nosebleeds in HHT patients.[176] Various side effects of Bevacizumab including cardiac failure, GI bleeding, wound-healing complication, and arterial thrombo embolic events have been reported.[191] In order to reduce side effects through systemic administration of Bevacizumab, topical application to nasal area using spray has been tried and shown to be effective.[192, 193]

Therapeutic Potential of Anti-Inflammatory Intervention

More than 90% of patients experience nosebleeds that stem from nasal Telangiectasia,[194] indicating that the nasal mucosa is very susceptible to AVM formation. We speculate that chronic inflammation, infection and immune activation in the nose might be related to the high susceptibility. Inflammatory cytokines gene expression was implicated in AVM formation as the promoter polymorphisms in IL-1β, IL-6, TNF-α and APOEε2 are associated with AVM susceptibility and clinical presentation of intracranial hemorrhage.[171, 195-197] More recently, Torsney et al showed that inflammation in the ears and eyelids almost invariably caused bleeding from thin-walled dilated vessels in the *Eng*$^{+/-}$ mouse.[198] Our skin wound model demonstrated that AVMs form only in the wound areas in *Alk1*-deficient mice,[169] and here we showed that LPS could mimic the wound effect.

Inflammatory Stimulation Associated with Angiogenesis is Important for Inducing AVM Formation.

LPS is an endotoxin that provokes an acute inflammatory response by releasing proinflammatory cytokines including tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8.[199] TNF-α is a cytokine that promotes a strong inflammatory response by recruiting immune cells, induces additional proinflammatory cytokines such as IL-1 and IL-6 and inhibits apoptosis of inflammatory cells.[200, 201] We show that LPS can induce angiogenic stimulation in the context of an inflammatory response. Neovascularization and recruitment of sprouting blood vessels occurred around LPS-injected sites. Mattsby-Baltzer et al have suggested that endotoxin-mediated neovascularization is a component of inflammation and wound healing.[181] LPS is associated with increased angiogenesis, vascular permeability, tumor cell invasion, and VEGF expression in macrophages.[182, 202] TNF-α also induced angiogenesis via macrophage stimulation.[203] Activation of the Eck RPTK by its inducible ligand B61 is involved in TNF-α-induced angiogenesis.[204] We showed that the AVMs formed by LPS were more severe than those by TNF-α. Perhaps it happened because LPS might have provoked stronger inflammatory stimulation by releasing various proinflammatory cytokines (including TNF-α) compared to TNF- α-induced inflammation, leading to more intensive angiogenic stimulation. For instance, it was shown that production of IL-8 which may be play a role in a more lasting myeloid cell-driven angiogenesis could be induced by LPS, but not TNF-α.[183]

Taken together, our data suggest that blocking angiogenesis or inflammation could be an effective therapy for epistaxis and GI bleeding in HHT patients. Number of FDA approved drugs inhibiting angiogenesis or inflammation is rapidly increasing. In addition

to angiogenesis blockade and inflammatory inhibitors, drugs targeting some other aspect of AVM formation such as antifibrinolytic agent (Tranexamic acid),[156, 205] antioxidants (N-Acetyl Cystein),[206] estrogen analogs (Raloxifene),[207] have been suggested for treating HHT. The preclinical model presented here would be an invaluable resource with which these potential drugs can be screened for effectiveness in preventing skin de novo AVMs and GI bleeding. Thus, inflammation prevention could be therapeutic for HHT patients suffering nosebleeds.

Table 2-1. The number of mice with hemorrhage in saline- and VEGF-antibody-treated groups

		Saline (n=12)	VEGF-Ab (n=12)
GI bleeding	Weak	1	7
	Moderate	6	3
	Severe	5	2
Lung bleeding	Weak	2	9
	Moderate	5	2
	Severe	5	1
Liver AVM	Weak	11	10
	Moderate	0	1
	Severe	1	1

Lung, GI bleeding and liver AVM of each mouse were categorized into three groups (weak, moderate, severe) and the number of groups was counted in saline-treated (n=12) and VEGF-treated (n=12) Alk1-deficient mice.

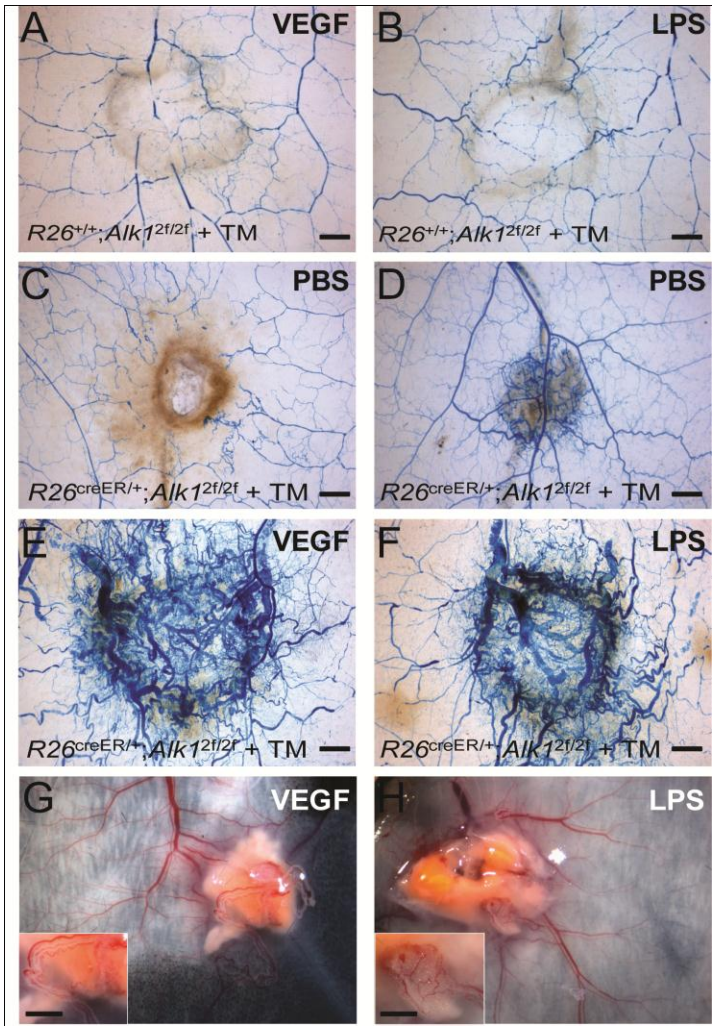

Figure 2-1. Angiogenic or inflammatory stimulation induces AVMs in *Alk1*-deficient adult subdermal vessels but not *Alk1*-wild type vessels. VEGF-PLGA and LPS-PLGA were implanted under dorsal skin as an angiogenic or inflammatory stimulus, respectively. Tamoxifen (TM) was intraperitoneally injected to effect *Alk1*-deletion. After 7 days, blue latex dye was infused through the heart to visualize blood vessels in the dorsal skin and particle-injected skins were cleared (see methods). (A-B) VEGF (A) and LPS (B) particles did not induce AVMs in Cre-negative controls ($R26^{+/+}$;$Alk1^{2f/2f}$ + TM) bearing wild type *Alk1* alleles. (C-D) PBS-PLGA was implanted to investigate whether PLGA itself can induce AVM formation in *Alkl*-deficient mice ($R26^{creER/+}$;$Alk1^{2f/2f}$ + TM). PBS-PLGA did not induce AVMs (C) or rarely formed abnormal vasculature (D). (E-F) VEGF (E) and LPS (F) particles induced severe AVMs and tortuous vessels in ALK1-depleted vessels ($R26^{creER/+}$;$Alk1^{2f/2f}$ +TM). VEGF (G) and LPS (H) particles induced angiogenesis by recruiting sprouting vessels toward particles as seen 4 days post-implantation of loaded particles. At left bottom are higher magnifications of recruited sprouting vessels. Scale bars in each panel indicate 1 mm.

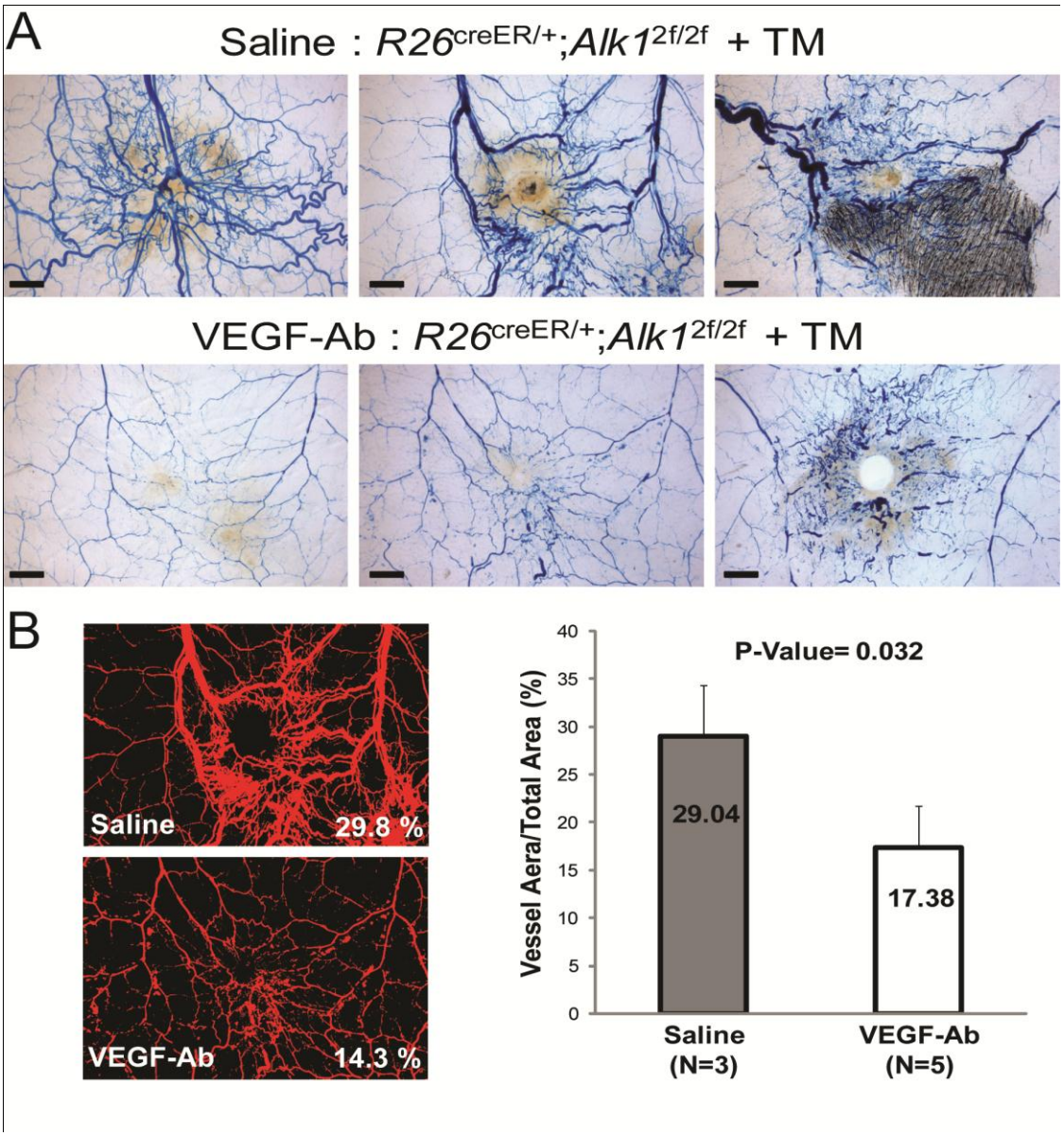

Figure 2-2. Angiogenesis blockade (VEGF-neutralizing antibody) suppressed wound-induced AVM formation in Alk1-deficient adult subdermal vessels. (A) Saline or VEGF-antibody (Ab) were injected into Alk1-deficient adult mice (R26creER/+;Alk12f/2f) to block angiogenesis. AVMs were induced by incisional wound of dorsal skin. VEGF-Ab suppressed wound-induced AVMs compared to saline-treated group. Tamoxifen (TM) was treated to effect Alk1 deletion. VEGF-Ab (bottom panels) suppressed wound-induced AVMs compared to saline-treated animals (top panels). (B) The severity of AVMs formed surrounding wound sites was calculated as the percentage of vessel area containing latex dye (MATLAB program). Panels on the left are representative of one experimental comparison. The graph on the right is a compilation of all data showing a significant reduction in the percentage of vessel area in VEGF-treated group compared to the saline-treated group. Scale bars in each panel indicate 1 mm.

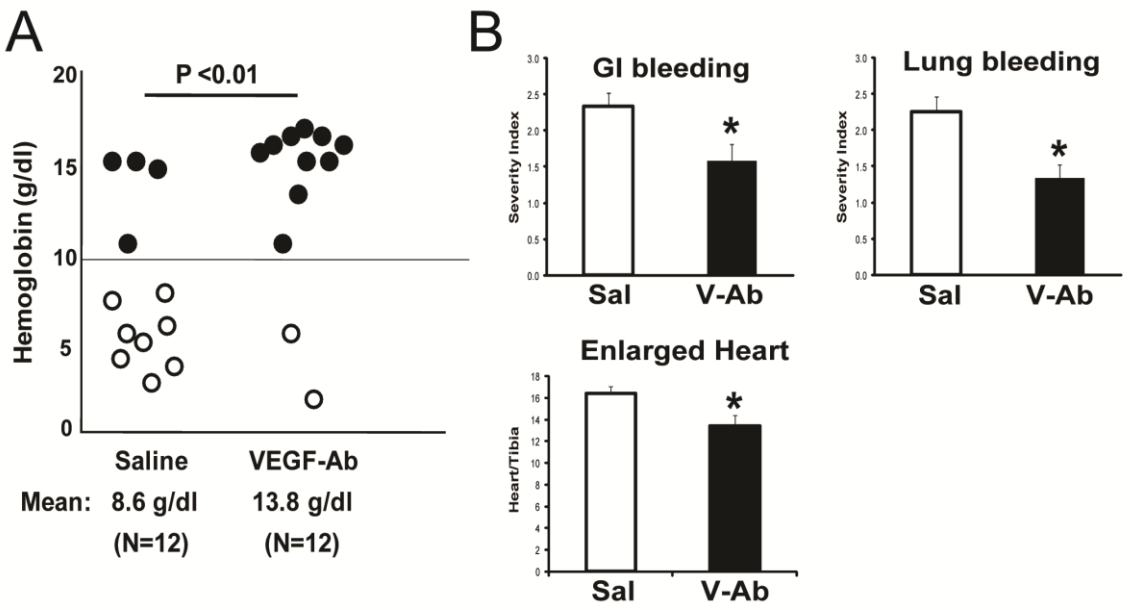

Figure 2-3. Angiogenesis blockade improved hemoglobin levels and visceral hemorrhage in *Alk1*-deficint adult mice. (A) Hemoglobin concentration was measured in saline-treated (n=12) and VEGF-treated (n=12) *Alk1*-deficient mice 9 days after tamoxifen treatment to effect *Alk1* deletion. VEGF-blockade improved hemoglobin levels in *Alk1*-deficient mice relative to the saline-treated group. (B) The severity of GI and lung bleeding (top) was expressed as a severity index based on direct observation and categorization. Hypertrophy of the heart (bottom) was expressed as a ratio of heart volume and tibia length (μl/cm). VEGF-blockade significantly suppressed related hemorrhaging and heart enlargement in *Alk1*-deficient mice.

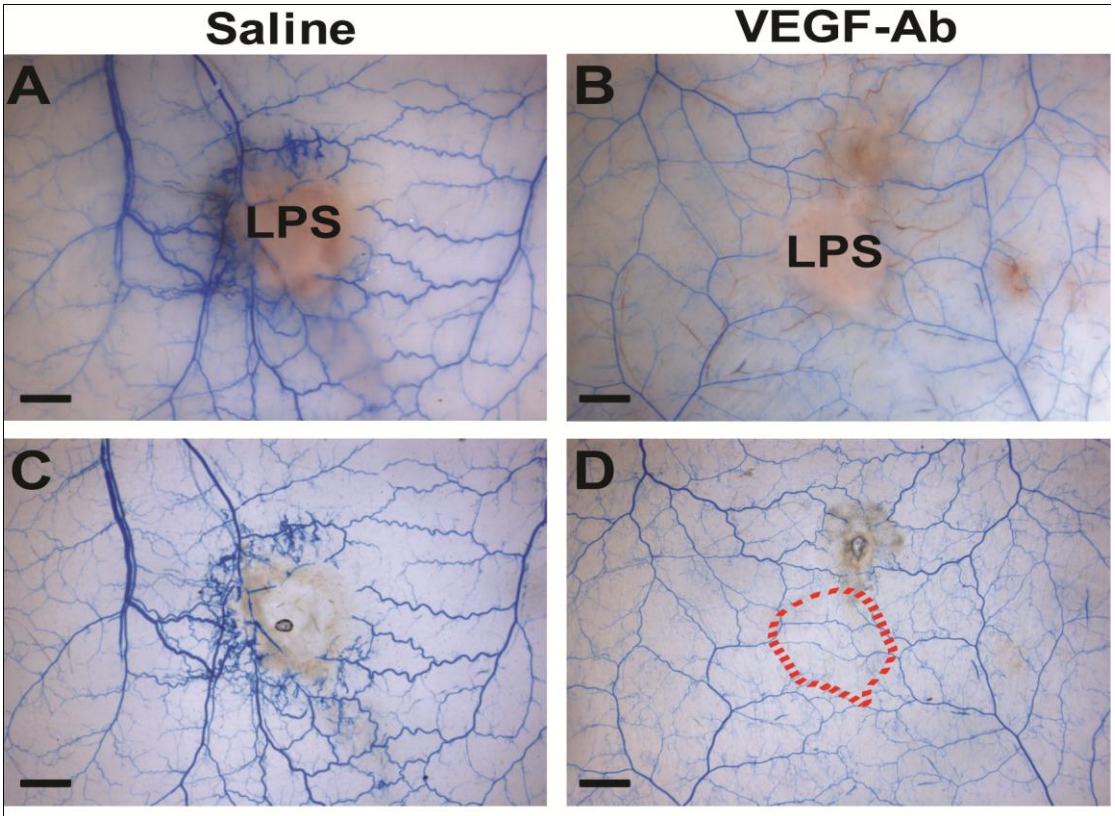

Figure 2-4. Angiogenesis blockade suppressed LPS-induced AVMs in *Alk1*-deficient mice. LPS particles formed aggregates on subdermal surfaces in both saline-treated (A) and VEGF-antibody-treated (B) *Alk1*-deficient mice. After skins were cleared to enhance the visualization of blood vessels, AVMs were apparent in saline-treated mice (C) but not mice treated with VEGF antibody (D). Scale bars in each panel indicate 1 mm.

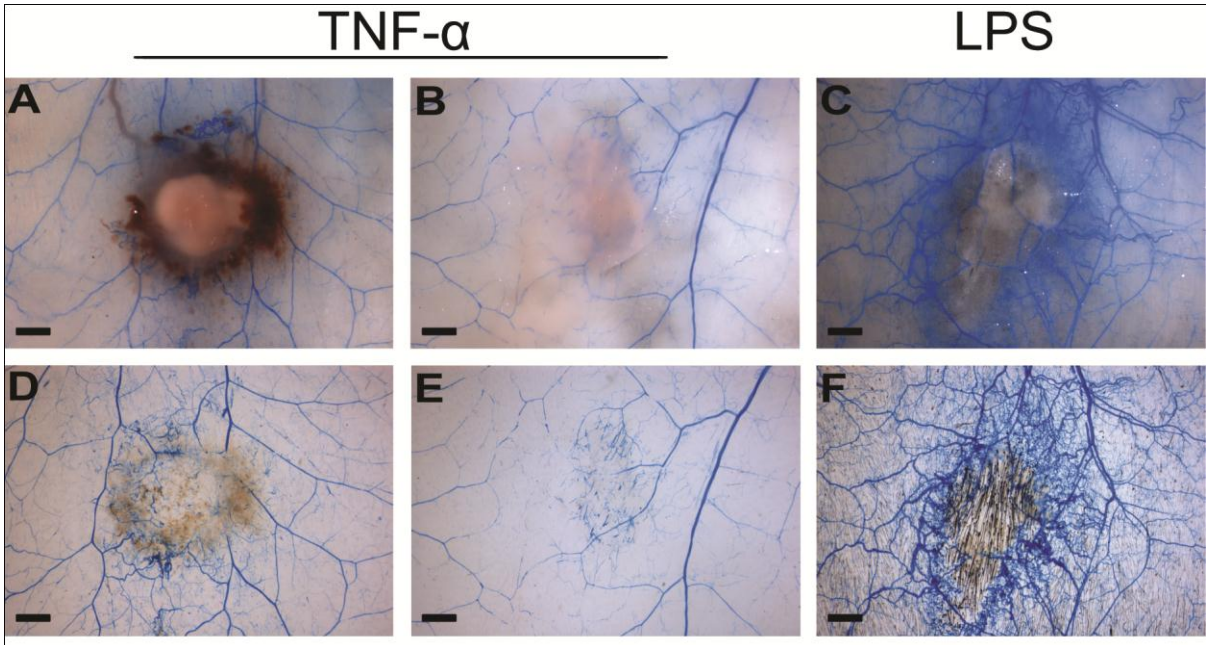

Figure 2-5. TNF-α induced mild AVM formation in *Alk1*-deficient mice. TNF-α PLGA particles were implanted under dorsal skin in tamoxifen-treated *Alk1*-deficient mice (*R26*$^{creER/+}$;*Alk1*$^{2f/2f}$). Vessels were observed after 7 days with the latex dye. TNF-α-treated mice (A, B and D, E) are compared with an LPS-treated mouse (C and F) before (A-C) and after (D-F) the clearing of aggregated particles. TNF-α induced neovascularizaion around particle aggregate. However, AVMs formed by TNF-α (D, E) were less numerous than those induced by LPS (F). Scale bars in each panel indicate 1 mm.

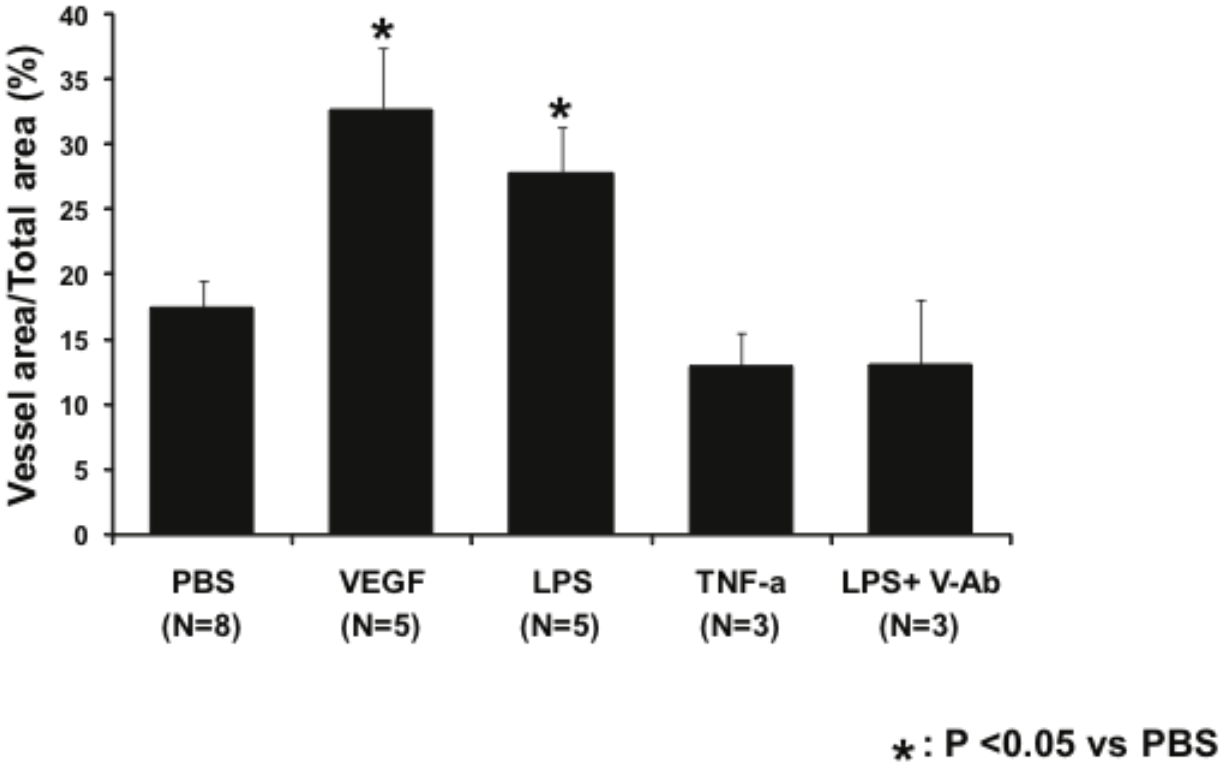

Figure 2-6. Quantification of AVM formation by various stimuli in Alk1-deficient subdermal vessels. VEGF and LPS-treated groups significantly promoted AVM formation compared to PBS-treated group. TNF-α did not significantly induce AVM formation in Alk1-deficient mice. LPS-induced AVM formation was suppressed by VEGF-neutralizing antibody (V-Ab).

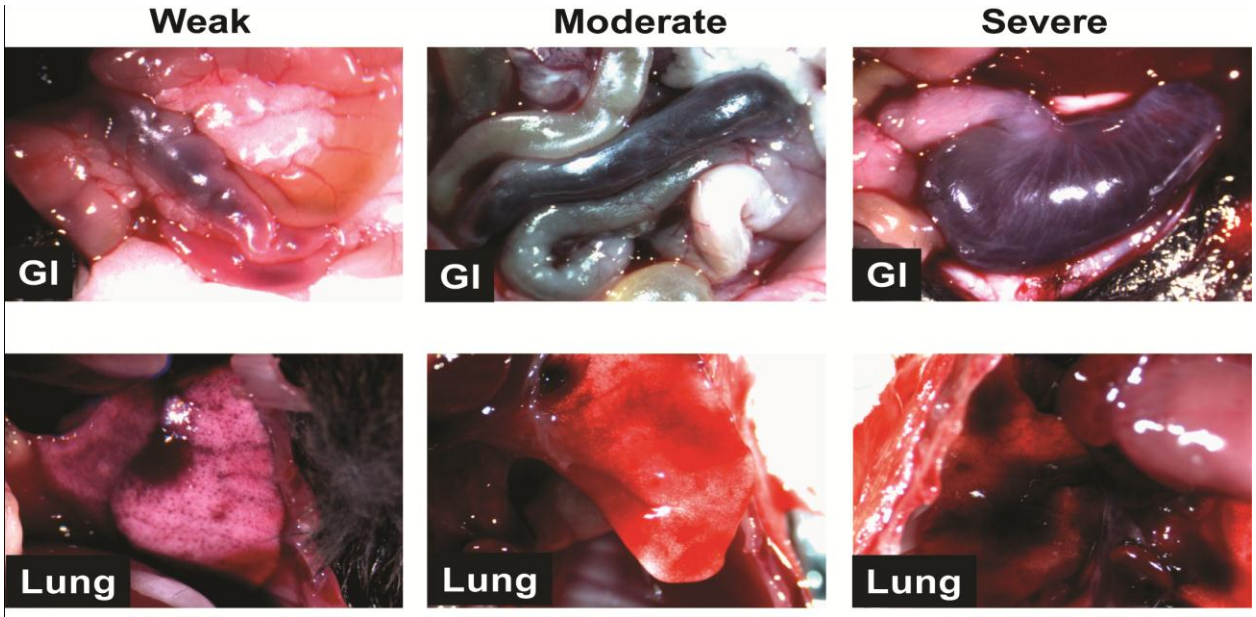

Figure 2-7. Lung (top) and GI bleeding (bottom) was categorized into three groups (weak, moderate, and severe) depending on the severity. Partial or focal hemorrhaging belonged to the weak level (left). Vast areas of hemorrhaging were categorized as severe (right). Intermediate levels of hemorrhaging were grouped as moderate (middle).

CHAPTER 3
MATERIALS AND METHODS

Smad1-Conditional Knockout Mice

Generation of a conditional *Smad1* allele (*Smad1*^{f}) was previously described.[208] Generation of the Tg(*Alk1*-cre)-L1 (L1cre) line has also been described.[109] Tg(*Tagln*-cre) and R26R mice were purchased from the Jackson Laboratory. *Smad1*$^{f/f}$ mice were intercrossed with L1cre or *Tagln*-cre lines. The L1cre(+);*Smad1*$^{f/+}$ or L1cre(+);*Smad1*$^{+/2f}$;R26R males were further intercrossed with *Smad1*$^{f/f}$;R26R females to produce L1cre(-);*Smad1*$^{f/f}$ and L1cre(+);*Smad1*$^{f/f}$. *Tagln*-cre(-);*Smad1*$^{f/f}$ and *Tagln*-cre(+);*Smad1*$^{f/f}$ were produced by the same breeding scheme. More than half of the control and experimental mice contained the R26R allele to monitor the Cre activity. PCR primer sets and conditions for detecting the conditional as well as null alleles of *Smad1* were previously described.[208] Primer sets for the genotype of L1cre, *Tagln*-cre, or R26R are shown in Table 1-1.

Hemodynamic Analysis

To evaluate pulmonary artery pressure, right ventricular systolic pressure (RVSP) was measured by right heart catheterization through the right jugular vein. Each mouse was anesthetized by isoflurane (1-2 %) and placed in the supine position. A 1-2 cm incision was made in the neck to expose the right jugular vein. The Mikro-Tip pressure transducer (SPR-835, Millar Instrument) was inserted into right external jugular vein and advanced into the right ventricle. Systemic blood pressure was recorded non-invasively using the tail-cuff method. A pneumatic pulse sensor was placed on the tail distal to an occlusion cuff controlled by a Programmed Electro-Sphygmomanometer (PE-300, Narco Bio-Systems), which is connected to the Powerlab system (ADinstrument). All

electrical outputs from the tail cuff, the pulse sensor and transducer were recorded and analyzed by the Powerlab 8/30 data acquisition system and associated Chart software (ADinstrument).

Right Ventricular Hypertrophy (RVH)

After hemodynamic analysis, mice were euthanized and using syringe-generated flow, the pulmonary circulation was perfused with PBS containing heparin (3 units/ml). The hearts were isolated and outflow tracts and atria were removed. The right ventricle was cut out from the heart by the spring scissor and the right ventricle and the remaining left ventricle (LV) plus septum (S) were weighed. Right ventricular hypertrophy was determined by the ratio of RV/LV+S.

Pulmonary Vessel Morphometry

After the hemodynamic analysis, the left lung was inflated with PBS for 20 minutes followed by formalin at a constant inflation pressure of 23cmH2O, fixed with 4% paraformaldehyde overnight, and paraffin-embedded. Each lung sample was transversely sliced into 5 μm thick sections and subjected to immunostaining with anti-smooth muscle α-actin (αSMA) antibody (Sigma-aldrich, mouse monoclonal, 1:800) using M.O.M kit (Vector laboratories) to visualize the vascular smooth muscle layer. To determine muscularization of pulmonary vessels, peripheral blood vessels ranging from 30-70 μm in diameter were counted in at least four fields at 20X magnification with a Zeiss Axioplan-2 optical microscope. The counted vessels were categorized as as fully muscularized (75-100% of medial layer covered by anti-αSMA staining), partially muscularized (1-74% of medial layer is covered by anti-αSMA staining), or nonmuscularized vessels at the level of alveolar ducts. The percentage of pulmonary vessels in each category was calculated by dividing the number of vessels by the total

number of counted vessels in the same field. To calculate the percentage of wall thickness (WT), circular and fully muscularized vessels were selected. WT1 (the thickness between the outer boundary and the inner boundary of αSMA positive medial layer) was measured at one point of the vessel wall and WT2 at the point which was diametrically opposite, guided by Openlab 5.03 Beta software (Improvision Inc.). External diameter (ED) was also measured at the same vessel. The percentage of medial wall thickness was calculated as (WT1 + WT2) x100/ED.

Establishment of Immortalized Pulmonary Endothelial Cells

Whole lungs were removed from an eight-week-old *R26*$^{CreER/+}$*; Bmpr2*$^{2f/2f}$ mouse and washed in HEPES followed by additional washing in DMEM. Lung tissues were finely minced using a sterile scalpel. The chopped tissues were subjected to serial digestion using 2 ml of a 1X trypsin solution [0.25% trypsin, 0.5 M EDTA (pH 8.0) in DMEM] at 37°C, with frequent shaking, for three times at 8 minutes each. Trypsin digestion was inactivated by adding 6 ml of normal endothelial cell media (ECM)[10% fetal bovine serum, 1 mg/ml heparine , 0.1 mg/ml endothelial mitogen (Biomedical technologies), 1 mM non-essential aminoacids (Cellgro), 1 mM sodium pyruvate, 50 units/ml penicillin/streptomycin]. After the large debris sinking down, the supernatant was carefully collected and plated into 2 wells of a 6-well culture plate. For immortalization, when the culture reached 50% confluency post isolation, they were transfected with 4.0 µg of SV40 DNA: ATCC (VRMC-3), pUCSV40-B2E[209, 210] using Lipofectamine (Invitrogen) following the manufacturer's protocol. After a couple of passages, the endothelial cells were sorted out by Fluorescence-Activated Cell Sorting (FACS) using Dio-Ac-LDL (Biomedical technologies) and lectin (Sigma-aldrich). For deleting the *Bmpr2* gene, the immortalized pulmonary ECs were plated on 6-well plates

($2x10^5$ cells/well) and cultured with medium containing 1 μM of 4-hydroxy tamoxifen (Sigma-aldrich) for 3 days. Thereafter the cells were cultured with 4-hydroxy tamoxifen-depleted growth medium. Primers (table 1) detecting null allele of *Bmpr2* (*Bmpr2*$^{1f/1f}$) were used to confirm the deletion of *Bmpr2* by tamoxifen.

Semi-Quantitative RT-PCR

To determine the levels of transcripts of endothelial cell-specific markers and genes involved in TGF-β superfamily signaling, *Bmpr2*-intact (*Bmpr2*$^{2f/2f}$) and *Bmpr2*-deleted (*Bmpr2*$^{1f/1f}$) pECs were harvested at 100% confluency from a 25 cm^2 culture flask. Total RNAs from the cells were extracted using the NucleoSpin RNA purification kit (Clontech). One μg of RNA was used for reverse transcription (RT) reaction. The cDNAs were synthesized using SuperScript III First-Stand synthesis kit (Invitrogen). 2 μl of cDNA was used as a template for PCR amplification for 25 cycles: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 1 minute. The transcription level of each gene was normalized to Gapdh expression. The primers used for RT-PCR analysis are shown in Table 1-1.

Western Blotting

Pulmonary ECs were harvested with a Chemicon lysis buffer containing 50 mM Tris (pH 6.8), 1 mM EDTA and 2% SDS and sonicated. Lysates were spun down at 13000 rpm for 15 minutes. The concentration of protein was determined using the Bio-rad DC Assay kit. 50 μg of total protein was fractionated by 10% of SDS-PAGE and transferred to nitrocellulose membranes (Bio-rad). Membranes were incubated with the primary antibody followed by the horseradish peroxidase-linked secondary antibody. A chemiluminescent detection reagent (ECL PlusTM, Amersham Pharmacia Biotech Inc.) was used to visualize proteins. The antibodies used for Western blotting analysis are

the following: SMAD1 (rabbit polyclonal, 1:1000), SMAD2 (mouse monoclonal, 1:1000), pSMAD2 (rabbit polyclonal, 1:1000), pSMAD1/5/8 (rabbit polyclonal, 1:1000) from Cell Signaling, β-actin (mouse monoclonal, 1:10,000) from Sigma-aldrich, BMPR2 (mouse monoclonal, 1:500) from BD Transduction Laboratories, Secondary antibodies include: mouse (1:5000) and rabbit (1:5000) from Sigma-aldrich.

Alk1-Conditional Knockout Mice

Establishment of the *Alk1*^{2f} allele in laboratory mice was described previously.[109] *R26*$^{creER/+}$ allele-carrying mice were purchased from The Jackson Laboratory. By crossing *R26*$^{creER/+}$ line with *Alk1*$^{2f/2f}$ mice, *R26*$^{creER/+}$;*Alk1*$^{2f/2f}$ mice were produced and 2-4 month-old mice were used in all experiments. The inactivation of the *Alk1* gene in adult mice was accomplished by treating animals as previously described.[169] All *in vivo* procedures were conducted in accordance with animal use guidelines established by the University of Florida Institutional Animal Care and Use Committee.

Preparation of PLGA Microparticles

Poly (d,l lactide-co-glycolide) (PLGA), 50:50 composition with inherent viscosity 0.55-0.75 dl/g in hexafluoroisopropanol, HFIP (Lactel) was used to generate the particles. The emulsion stabilizer, poly-vinyl alcohol (PVA) (MW ~ 100,000 g/mol) was purchased from Fisher Science. Phosphate buffered saline (PBS) solution (Hyclone) was used as the aqueous phase to form the emulsions while methylene chloride (Fisher Scientific) was used as an organic solvent to dissolve PLGA polymer. Microparticles were formed using a standard water-oil-water solvent evaporation technique.[211, 212] Briefly, a 3% solution of PLGA polymer in methylene chloride was generated. 1 ml of 3% PLGA solution was emulsified with 10 µl of 10 mg/ml Rhodamine 6G (Sigma-Aldrich) in PBS, 100 µl of 1% bovine serum albumin (Fisher Scientific) in PBS, and either 100 µl of 100

μg/ml VEGF (R&D systems) in PBS for VEGF-PLGA, 100 μl of 100 μg/ml TNF-α (R&D systems) in PBS for TNF-α-PLGA or 100 μl of 1 mg/ml LPS (Sigma-Aldrich) in PBS for LPS-PLGA at 26,500 rpm for 60 seconds using a tissue-miser homogenizer (Fisher Scientific) to form the primary emulsion. The primary emulsion was added to 10 ml of 5% PVA solution in PBS and the homogenizing was continued at 19,500 rpm for 120 seconds to form the secondary emulsion. Then, the secondary emulsion was added to 100 ml of 0.5% PVA made in dIH_2O. The particles thus formed were agitated using a magnetic stirrer for 16 hours to evaporate residual methylene chloride. The remaining solution was centrifuged at 10,000 rpm for 10 minutes to collect microparticles which were subsequently washed three times with PBS. The particle suspension in 5 ml of PBS was then flash-frozen in liquid nitrogen and stored at -20^0C until used.

Injection of PLGA Particles into Subdermal Area

The recipient mice (*R26*$^{creER/+}$;*Alk1*$^{2f/2f}$ and *R26*$^{+/+}$;*Alk1*$^{2f/2f}$) were anesthetized by placing them within an induction chamber and introducing 4.0% isoflurane, and out of the chamber, anesthesia was maintained by 2.0%–3.0% isoflurane using a nose cone. The hairs on the back of the mice were shaved and 100 μl of PLGA particles containing 200 ng of VEGF, 2 μg of LPS, or 200 ng of TNF-α were implanted into the subdermal area of the mid-dorsal skin using 29-gauge 0.3-ml syringe (Monoject). Tamoxifen (TM, Sigma-aldrich) was injected intraperitoneally at a dose of 2.5 mg/25 g of body weight for *Alk1* gene deletion. Then, 0.6 ml of the blue latex dye (Connecticut Valley Biological Supply Co.) was injected via the left heart 7 days after TM injection to visualize the blood vessels.

Skin Wound Generation and VEGF-Antibody Treatment

The recipient mice (*R26*$^{creER/+}$;*Alk1*$^{2f/2f}$) were anesthetized as described above. The hairs on the back of the mice were shaved and one 2 mm-diameter full-thickness excisional wounds were inflicted on the mid-dorsum using a 2 mm-dermal biopsy punch (Miltex). The wounds were left unsutured, and betadine was applied to the wounds. No analgesics or antibiotics were given in order not to affect the wound healing process. TM was injected intraperitoneally at a dose of 2.5 mg/25 g of body weight and VEGF-neutralizing antibody (100 µl, 5 mg/kg of body weight, Genentech) or the same volume of saline was serially injected on the first, 4th, and 7th day of post-wounding. On the 9th day of post-wounding, 0.6 ml of the blue latex dye was injected via the left heart.

Latex Dye Injection and Image Processing

Mice were anesthetized with intraperitoneal injection of ketamine/xylazine (100 mg/15 mg/kg of body weight) and abdominal and thoracic cavities were opened. To perfuse all the blood out, heparin (200 unit/ml, Sigma-aldrich) in 10 ml PBS was infused through the left ventricle of the heart at 120 ml/hour using a syringe pump (KD scientific) after a small cut was made on the left atria. Then, 10 ml of PBS-dilator mixture containing heparin (10 unit/ml, Sigma-Aldrich), papaverine (0.04 mg/ml, Sigma-Aldrich), and sodium nitroprusside (100 µM, Sigma-aldrich) was infused through the same hole made on the left ventricle to maximize dilation of blood vessels. To fix the dilated vessels, 10 ml of 10% formalin was infused through the same hole on the left ventricle. Then, 0.6 ml of the blue latex dye was slowly and steadily injected into the hole of left ventricle with a 26-gauge 1-ml syringe. Then, the hair removal cream (Veet) was put on the shaved back skin of dye-injected mice and the hairs were completely removed after 10 minutes incubation. The mice were washed briefly in water and fixed with 10%

formalin overnight. After fixation, the dorsal skin was peeled off, stretched, and flattened on the Styrofoam. The flattened skin was dehydrated by methanol series (20%, 50%, 75%, and 100%, each for 20 minutes) and cleared with organic solvent (benzyl alcohol/benzyl benzoate, 1:1; Sigma-aldrich). Blood vessels containing the latex dye in the cleared skin were imaged via a CCD camera (Leica) and processed by MATLAB (MathWorks) as described previously.[169]

Hemoglobin Concentration and Hemorrhage Index

2-3 drops of blood were collected from the tails of VEGF-antibody-treated (N=12) and saline-treated mice (N=12) and soaked up by microcuvettes (STANBIO Laboratory). The concentration of hemoglobin was measured by the hemoglobin photometer (Hemopoint H2, STANBIO Laboratory) using the blood-soaked microcuvettes. Then, after anesthesia with ketamine/xylazine, abdominal and thoracic cavities were opened and hemorrhages of lungs and gastrointestinal tract and the size of the heart were observed and categorized into three levels: weak, moderate, and severe conditions according to the degree of severity (Figure 2-6). Hemorrhage levels were graded: 1.0 for weak, 2.0 for moderate, and 3.0 for severe level. The mean values were expressed as severity index.

Hypertrophy of the heart

After latex-dye injection through the heart, hearts were taken and all the blood vessels connecting to the heart were removed. Hearts were submerged in 1.5 ml tube containing 500μl PBS and increased volume of PBS was measured as a volume of heart. Right legs of mice were cut out in the middle of femur and were skinned off and were put in 1M NaOH overnight for digestion of tissues surrounding bones. After digestion, tissues were easily removed and the foot bone and broken femur were

separated from the tibia by slowly pulling them. The length of whole tibia was measured by the caliper.

Statistical Analysis

T-test was used to determine a statistical significance between two groups, and one-way Anova was used for more than 3 groups, and multiple pairwise comparisons were made by post-hoc tests (Tukey) using the Sigmaplot.

CHAPTER 4
CONCLUDING REMARKS AND FUTURE DIRECTIONS

Current therapeutic interventions for both PAH and HHT diseases are restricted to alleviate severe symptoms because they are not based on mechanistic pathology. There is a pressing need for more effective and pathology-specific therapies.

In PAH studies, I showed that loss of SMAD1 function in ECs or SMCs predisposes mice to pulmonary hypertension and Impaired BMP signaling makes TGF-β signaling overactivated and leads to an imbalance of TGF-β and BMP signaling in pECs, which might be a critical factor for PAH development. From these results, I can suggest several therapeutic targets. First, reduced SMAD1 activity may be associated with PH development. Therefore, inhibiting phosphatase activity could be effective to maintain SMAD1 phosphorylation and its activity although it should be specific to SMAD1/5/8, not SMAD2/3. Second, recovering from imbalance state of TGF-β/BMP signaling would be therapeutic for PAH patients by suppressing overactivated TGF-β. TGF-β antagonists such as the angiotensin II type 1 receptor (AT1) blocker, Losartan[133] or the activin recetor-like kinase 5 inhibitor, SB525334[213] may have therapeutic benefit. In addition to this, I found that BMP7 response was intact in *Bmpr2*-deleted pECs. Thus, treatment of BMP7 ligand could compensate for reduced level of pSMAD1/5/8 from BMPR2 deficiency.

To determine whether overactivation of TGF-β derived from reduced BMP response is a critical factor for PAH development *in vivo*, we are planning to test whether inhibition of TGF-β signaling prevent PAH manifestation in our PH mouse models-*Smad1* or *Bmpr2* cKO mice by additionally deleting genes involved in TGF-β

signaling such as *Tgfbr2* and *Smad4* or treatment with inhibitors blocking TGF-β signaling, such as Activin Receptor Like Kinase 5 (ALK5) inhibitor.[103]

Endothelial *Smad1* deletion did not result in 100% penetracne in PH, indicating that environmental or genetic second hits are required for PAH development. As shown in Figure 1-1, I observed the marked reduction of SMAD1/5/8 phosphorylation in the lungs of PH mice, suggesting that the complete loss of SMAD1 activity in the whole lung may be essential to manifest PAH phenotype. Thus, by mating L1cre(+);*Smad1*$^{f/f}$ with *Tagln*-cre(+);*Smad1*$^{f/f}$, we can delete *Smad1* gene both in endothelial and smooth muscle cells. Although these double cre KO mice did not block SMAD1 activity in the whole lung, we can expect synergistic effect of smooth muscle thickening and endothelial dysfunction and may expect increase of penetrance.

In HHT studies, I demonstrated that inflammation or angiogenesis are sufficient to induce AVMs as second hits for ALK1 deficiency. In terms of therapeutic aspect, antagonizing inflammatory or angiogenic pathway may be effective for HHT patients. I showed that VEGF-neutralizing antibody can prevent AVM formation and internal bleeding in *Alk1*-deficient adult mice. Even though effective anti-inflammatory reagents are not available, IL-9-neutralizing antibody could be a useful reagent to test because IL-9 is essential factor for inflammation-induced angiogenesis.

Up to now, many studies have focused on assessing increased response to angiogenic stimulation in *Alk1*-deleted endothelial cells. However, what makes this increased sensitivity as a result of *Alk1* deficiency remains unclear. To address this question, we have many useful resources: pulmonary endothelial cells (pECs), mice carrying *Alk1*$^{2f/2f}$ and *Alk1*$^{1f/1f}$ allele respectively, and microarray data from pECs

(*Alk1*$^{2f/2f}$ and *Alk1*$^{1f/1f}$). I showed VEGF signaling is required for AVM formation with *Alk1*-deficiency. With above materials, examining whether *Alk1* can suppress downstream of VEGF signaling or modulate interaction of VEGF and VEGF receptors and other possible hypotheses, would be interesting and invaluable research to find novel therapeutic targets.

LIST OF REFERENCES

1. Simonneau G, Galie N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, Gibbs S, Lebrec D, Speich R, Beghetti M, Rich S, Fishman A. Clinical classification of pulmonary hypertension. *J Am Coll Cardiol*. 2004;43:5S-12S

2. Highland KB. Pulmonary arterial hypertension. *Am J Med Sci*. 2008;335:40-45

3. Yi ES, Kim H, Ahn H, Strother J, Morris T, Masliah E, Hansen LA, Park K, Friedman PJ. Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension. A morphometric and immunohistochemical study. *Am J Respir Crit Care Med*. 2000;162:1577-1586

4. DeRuiter MC, Poelmann RE, VanMunsteren JC, Mironov V, Markwald RR, Gittenberger-de Groot AC. Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. *Circ Res*. 1997;80:444-451

5. Arciniegas E, Sutton AB, Allen TD, Schor AM. Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J Cell Sci*. 1992;103 (Pt 2):521-529

6. Pietra GG, Edwards WD, Kay JM, Rich S, Kernis J, Schloo B, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al. Histopathology of primary pulmonary hypertension. A qualitative and quantitative study of pulmonary blood vessels from 58 patients in the national heart, lung, and blood institute, primary pulmonary hypertension registry. *Circulation*. 1989;80:1198-1206

7. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol*. 1994;144:275-285

8. Teichert-Kuliszewska K, Kutryk MJ, Kuliszewski MA, Karoubi G, Courtman DW, Zucco L, Granton J, Stewart DJ. Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: Implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension. *Circ Res*. 2006;98:209-217

9. Stenmark KR, Mecham RP. Cellular and molecular mechanisms of pulmonary vascular remodeling. *Annu Rev Physiol*. 1997;59:89-144

10. Meyrick B, Reid L. The effect of continued hypoxia on rat pulmonary arterial circulation. An ultrastructural study. *Lab Invest*. 1978;38:188-200

11. Jones R, Jacobson M, Steudel W. Alpha-smooth-muscle actin and microvascular precursor smooth-muscle cells in pulmonary hypertension. *Am J Respir Cell Mol Biol*. 1999;20:582-594

12. Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, Perrella MA, Mitsialis SA, Kourembanas S. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. *Proc Natl Acad Sci U S A*. 2001;98:8798-8803

13. Said SI. Mediators and modulators of pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2006;291:L547-558

14. Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, Loyd JE. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med*. 1992;327:70-75

15. Perros F, Dorfmuller P, Humbert M. Current insights on the pathogenesis of pulmonary arterial hypertension. *Semin Respir Crit Care Med*. 2005;26:355-364

16. Herve P, Humbert M, Sitbon O, Parent F, Nunes H, Legal C, Garcia G, Simonneau G. Pathobiology of pulmonary hypertension. The role of platelets and thrombosis. *Clin Chest Med*. 2001;22:451-458

17. Gaine S. Pulmonary hypertension. *Jama*. 2000;284:3160-3168

18. Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Morrell NW, Berg J, Manes A, McGaughran J, Pauciulo M, Wheeler L. Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. *N Engl J Med*. 2001;345:325-334

19. Dorigo U, Fontvieille D, Humbert JF. Spatial variability in the abundance and composition of the free-living bacterioplankton community in the pelagic zone of lake bourget (france). *FEMS Microbiol Ecol*. 2006;58:109-119

20. Clapp LH, Finney P, Turcato S, Tran S, Rubin LJ, Tinker A. Differential effects of stable prostacyclin analogs on smooth muscle proliferation and cyclic amp generation in human pulmonary artery. *Am J Respir Cell Mol Biol*. 2002;26:194-201

21. Higenbottam T, Wheeldon D, Wells F, Wallwork J. Long-term treatment of primary pulmonary hypertension with continuous intravenous epoprostenol (prostacyclin). *Lancet*. 1984;1:1046-1047

22. Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH, et al. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. The primary pulmonary hypertension study group. *N Engl J Med*. 1996;334:296-302

23. Sitbon O, Humbert M, Nunes H, Parent F, Garcia G, Herve P, Rainisio M, Simonneau G. Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: Prognostic factors and survival. *J Am Coll Cardiol*. 2002;40:780-788

24. Sitbon O, Humbert M, Simonneau G. Primary pulmonary hypertension: Current therapy. *Prog Cardiovasc Dis*. 2002;45:115-128

25. Humbert M, Sanchez O, Fartoukh M, Jagot JL, Sitbon O, Simonneau G. Treatment of severe pulmonary hypertension secondary to connective tissue diseases with continuous iv epoprostenol (prostacyclin). *Chest*. 1998;114:80S-82S

26. Okano Y, Yoshioka T, Shimouchi A, Satoh T, Kunieda T. Orally active prostacyclin analogue in primary pulmonary hypertension. *Lancet*. 1997;349:1365

27. Hoeper MM, Schwarze M, Ehlerding S, Adler-Schuermeyer A, Spiekerkoetter E, Niedermeyer J, Hamm M, Fabel H. Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. *N Engl J Med*. 2000;342:1866-1870

28. Ghofrani HA, Wiedemann R, Rose F, Olschewski H, Schermuly RT, Weissmann N, Seeger W, Grimminger F. Combination therapy with oral sildenafil and inhaled iloprost for severe pulmonary hypertension. *Ann Intern Med*. 2002;136:515-522

29. Simonneau G, Rubin LJ, Galie N, Barst RJ, Fleming TR, Frost AE, Engel PJ, Kramer MR, Burgess G, Collings L, Cossons N, Sitbon O, Badesch DB. Addition of sildenafil to long-term intravenous epoprostenol therapy in patients with pulmonary arterial hypertension: A randomized trial. *Ann Intern Med*. 2008;149:521-530

30. Alberts GF, Peifley KA, Johns A, Kleha JF, Winkles JA. Constitutive endothelin-1 overexpression promotes smooth muscle cell proliferation via an external autocrine loop. *J Biol Chem*. 1994;269:10112-10118

31. Rich S, McLaughlin VV. Endothelin receptor blockers in cardiovascular disease. *Circulation*. 2003;108:2184-2190

32. Hosoda K, Nakao K, Hiroshi A, Suga S, Ogawa Y, Mukoyama M, Shirakami G, Saito Y, Nakanishi S, Imura H. Cloning and expression of human endothelin-1 receptor cdna. *FEBS Lett*. 1991;287:23-26

33. Ogawa Y, Nakao K, Arai H, Nakagawa O, Hosoda K, Suga S, Nakanishi S, Imura H. Molecular cloning of a non-isopeptide-selective human endothelin receptor. *Biochem Biophys Res Commun*. 1991;178:248-255

34. Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med*. 1993;328:1732-1739

35. Wagner OF, Vierhapper H, Gasic S, Nowotny P, Waldhausl W. Regional effects and clearance of endothelin-1 across pulmonary and splanchnic circulation. *Eur J Clin Invest*. 1992;22:277-282

36. Channick RN, Simonneau G, Sitbon O, Robbins IM, Frost A, Tapson VF, Badesch DB, Roux S, Rainisio M, Bodin F, Rubin LJ. Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: A randomised placebo-controlled study. *Lancet*. 2001;358:1119-1123

37. Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, Pulido T, Frost A, Roux S, Leconte I, Landzberg M, Simonneau G. Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med*. 2002;346:896-903

38. Barst RJ, Rich S, Widlitz A, Horn EM, McLaughlin V, McFarlin J. Clinical efficacy of sitaxsentan, an endothelin-a receptor antagonist, in patients with pulmonary arterial hypertension: Open-label pilot study. *Chest*. 2002;121:1860-1868

39. Barst RJ, Langleben D, Frost A, Horn EM, Oudiz R, Shapiro S, McLaughlin V, Hill N, Tapson VF, Robbins IM, Zwicke D, Duncan B, Dixon RA, Frumkin LR. Sitaxsentan therapy for pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2004;169:441-447

40. McLaughlin VV, Sitbon O, Badesch DB, Barst RJ, Black C, Galie N, Rainisio M, Simonneau G, Rubin LJ. Survival with first-line bosentan in patients with primary pulmonary hypertension. *Eur Respir J*. 2005;25:244-249

41. Tabrizchi R. Slv-306. Solvay. *Curr Opin Investig Drugs*. 2003;4:329-332

42. Griffiths MJ, Evans TW. Inhaled nitric oxide therapy in adults. *N Engl J Med*. 2005;353:2683-2695

43. Mehta S. Sildenafil for pulmonary arterial hypertension: Exciting, but protection required. *Chest*. 2003;123:989-992

44. Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med*. 1995;333:214-221

45. Ghofrani HA, Pepke-Zaba J, Barbera JA, Channick R, Keogh AM, Gomez-Sanchez MA, Kneussl M, Grimminger F. Nitric oxide pathway and phosphodiesterase inhibitors in pulmonary arterial hypertension. *J Am Coll Cardiol*. 2004;43:68S-72S

46. Archer SL, Djaballah K, Humbert M, Weir KE, Fartoukh M, Dall'ava-Santucci J, Mercier JC, Simonneau G, Dinh-Xuan AT. Nitric oxide deficiency in fenfluramine- and dexfenfluramine-induced pulmonary hypertension. *Am J Respir Crit Care Med*. 1998;158:1061-1067

47. Cohen AH, Hanson K, Morris K, Fouty B, McMurty IF, Clarke W, Rodman DM. Inhibition of cyclic 3'-5'-guanosine monophosphate-specific phosphodiesterase selectively vasodilates the pulmonary circulation in chronically hypoxic rats. *J Clin Invest*. 1996;97:172-179

48. Jernigan NL, Resta TC. Chronic hypoxia attenuates cgmp-dependent pulmonary vasodilation. *Am J Physiol Lung Cell Mol Physiol*. 2002;282:L1366-1375

49. Bulau P, Zakrzewicz D, Kitowska K, Leiper J, Gunther A, Grimminger F, Eickelberg O. Analysis of methylarginine metabolism in the cardiovascular system identifies the lung as a major source of adma. *Am J Physiol Lung Cell Mol Physiol*. 2007;292:L18-24

50. Pullamsetti S, Kiss L, Ghofrani HA, Voswinckel R, Haredza P, Klepetko W, Aigner C, Fink L, Muyal JP, Weissmann N, Grimminger F, Seeger W, Schermuly RT. Increased levels and reduced catabolism of asymmetric and symmetric dimethylarginines in pulmonary hypertension. *FASEB J*. 2005;19:1175-1177

51. Frostell CG, Blomqvist H, Hedenstierna G, Lundberg J, Zapol WM. Inhaled nitric oxide selectively reverses human hypoxic pulmonary vasoconstriction without causing systemic vasodilation. *Anesthesiology*. 1993;78:427-435

52. Frostell C, Fratacci MD, Wain JC, Jones R, Zapol WM. Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. *Circulation*. 1991;83:2038-2047

53. Galie N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M, Simonneau G. Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med*. 2005;353:2148-2157

54. Golovina VA, Platoshyn O, Bailey CL, Wang J, Limsuwan A, Sweeney M, Rubin LJ, Yuan JX. Upregulated trp and enhanced capacitative ca(2+) entry in human pulmonary artery myocytes during proliferation. *Am J Physiol Heart Circ Physiol*. 2001;280:H746-755

55. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature*. 1994;372:231-236

56. Rich S, Kaufmann E, Levy PS. The effect of high doses of calcium-channel blockers on survival in primary pulmonary hypertension. *N Engl J Med*. 1992;327:76-81

57. Sitbon O, Humbert M, Jais X, Ioos V, Hamid AM, Provencher S, Garcia G, Parent F, Herve P, Simonneau G. Long-term response to calcium channel blockers in idiopathic pulmonary arterial hypertension. *Circulation*. 2005;111:3105-3111

58. Nichols WC, Koller DL, Slovis B, Foroud T, Terry VH, Arnold ND, Siemieniak DR, Wheeler L, Phillips JA, 3rd, Newman JH, Conneally PM, Ginsburg D, Loyd JE. Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. *Nat Genet*. 1997;15:277-280

59. Morse JH, Barst RJ. Detection of familial primary pulmonary hypertension by genetic testing. *N Engl J Med*. 1997;337:202-203

60. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA. Familial primary pulmonary hypertension (gene pph1) is caused by mutations in the bone morphogenetic protein receptor-ii gene. *Am J Hum Genet*. 2000;67:737-744

61. Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, 3rd, Loyd JE, Nichols WC, Trembath RC. Heterozygous germline mutations in bmpr2, encoding a tgf-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet*. 2000;26:81-84

62. Newman JH, Wheeler L, Lane KB, Loyd E, Gaddipati R, Phillips JA, 3rd, Loyd JE. Mutation in the gene for bone morphogenetic protein receptor ii as a cause of primary pulmonary hypertension in a large kindred. *N Engl J Med*. 2001;345:319-324

63. Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols WC. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding bmpr-ii, a receptor member of the tgf-beta family. *J Med Genet*. 2000;37:741-745

64. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type ii bone morphogenetic protein receptor. *Circulation*. 2002;105:1672-1678

65. Aldred MA, Vijayakrishnan J, James V, Soubrier F, Gomez-Sanchez MA, Martensson G, Galie N, Manes A, Corris P, Simonneau G, Humbert M, Morrell NW, Trembath RC. Bmpr2 gene rearrangements account for a significant proportion of mutations in familial and idiopathic pulmonary arterial hypertension. *Hum Mutat*. 2006;27:212-213

66. Cogan JD, Vnencak-Jones CL, Phillips JA, 3rd, Lane KB, Wheeler LA, Robbins IM, Garrison G, Hedges LK, Loyd JE. Gross bmpr2 gene rearrangements constitute a new cause for primary pulmonary hypertension. *Genet Med*. 2005;7:169-174

67. Cogan JD, Pauciulo MW, Batchman AP, Prince MA, Robbins IM, Hedges LK, Stanton KC, Wheeler LA, Phillips JA, 3rd, Loyd JE, Nichols WC. High frequency of bmpr2 exonic deletions/duplications in familial pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2006;174:590-598

68. Song Y, Jones JE, Beppu H, Keaney JF, Jr., Loscalzo J, Zhang YY. Increased susceptibility to pulmonary hypertension in heterozygous bmpr2-mutant mice. *Circulation*. 2005;112:553-562

69. Long L, MacLean MR, Jeffery TK, Morecroft I, Yang X, Rudarakanchana N, Southwood M, James V, Trembath RC, Morrell NW. Serotonin increases susceptibility to pulmonary hypertension in bmpr2-deficient mice. *Circ Res*. 2006;98:818-827

70. Yang X, Long L, Southwood M, Rudarakanchana N, Upton PD, Jeffery TK, Atkinson C, Chen H, Trembath RC, Morrell NW. Dysfunctional smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circ Res*. 2005;96:1053-1063

71. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol*. 2004;43:13S-24S

72. Shi Y, Massague J. Mechanisms of tgf-beta signaling from cell membrane to the nucleus. *Cell*. 2003;113:685-700

73. Massague J. How cells read tgf-beta signals. *Nat Rev Mol Cell Biol*. 2000;1:169-178

74. Derynck R, Zhang YE. Smad-dependent and smad-independent pathways in tgf-beta family signalling. *Nature*. 2003;425:577-584

75. Nohe A, Keating E, Knaus P, Petersen NO. Signal transduction of bone morphogenetic protein receptors. *Cell Signal*. 2004;16:291-299

76. Massague J. Integration of smad and mapk pathways: A link and a linker revisited. *Genes Dev*. 2003;17:2993-2997

77. Yu PB, Beppu H, Kawai N, Li E, Bloch KD. Bone morphogenetic protein (bmp) type ii receptor deletion reveals bmp ligand-specific gain of signaling in pulmonary artery smooth muscle cells. *J Biol Chem*. 2005;280:24443-24450

78. Aird WC. Endothelial cell heterogeneity. *Crit Care Med*. 2003;31:S221-230

79. Aird WC. Endothelial cell dynamics and complexity theory. *Crit Care Med*. 2002;30:S180-185

80. Titheradge MA. Nitric oxide in septic shock. *Biochim Biophys Acta*. 1999;1411:437-455

81. Vallance P, Chan N. Endothelial function and nitric oxide: Clinical relevance. *Heart*. 2001;85:342-350

82. Cool CD, Stewart JS, Werahera P, Miller GJ, Williams RL, Voelkel NF, Tuder RM. Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers. Evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth. *Am J Pathol*. 1999;155:411-419

83. Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, Voelkel NF, Tuder RM. Inhibition of the vegf receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J*. 2001;15:427-438

84. Fagan KA, Fouty BW, Tyler RC, Morris KG, Jr., Hepler LK, Sato K, LeCras TD, Abman SH, Weinberger HD, Huang PL, McMurtry IF, Rodman DM. The pulmonary circulation of homozygous or heterozygous enos-null mice is hyperresponsive to mild hypoxia. *J Clin Invest*. 1999;103:291-299

85. Steudel W, Ichinose F, Huang PL, Hurford WE, Jones RC, Bevan JA, Fishman MC, Zapol WM. Pulmonary vasoconstriction and hypertension in mice with targeted disruption of the endothelial nitric oxide synthase (nos 3) gene. *Circ Res*. 1997;81:34-41

86. Geraci MW, Gao B, Shepherd DC, Moore MD, Westcott JY, Fagan KA, Alger LA, Tuder RM, Voelkel NF. Pulmonary prostacyclin synthase overexpression in transgenic mice protects against development of hypoxic pulmonary hypertension. *J Clin Invest*. 1999;103:1509-1515

87. Christou H, Yoshida A, Arthur V, Morita T, Kourembanas S. Increased vascular endothelial growth factor production in the lungs of rats with hypoxia-induced pulmonary hypertension. *Am J Respir Cell Mol Biol*. 1998;18:768-776

88. McQuaid KE, Keenan AK. Endothelial barrier dysfunction and oxidative stress: Roles for nitric oxide? *Exp Physiol*. 1997;82:369-376

89. Bogatcheva NV, Garcia JG, Verin AD. Molecular mechanisms of thrombin-induced endothelial cell permeability. *Biochemistry (Mosc)*. 2002;67:75-84

90. Fisher MR, Mathai SC, Champion HC, Girgis RE, Housten-Harris T, Hummers L, Krishnan JA, Wigley F, Hassoun PM. Clinical differences between idiopathic and scleroderma-related pulmonary hypertension. *Arthritis Rheum*. 2006;54:3043-3050

91. Mehta NJ, Khan IA, Mehta RN, Sepkowitz DA. Hiv-related pulmonary hypertension: Analytic review of 131 cases. *Chest*. 2000;118:1133-1141

92. Isern RA, Yaneva M, Weiner E, Parke A, Rothfield N, Dantzker D, Rich S, Arnett FC. Autoantibodies in patients with primary pulmonary hypertension: Association with anti-ku. *Am J Med*. 1992;93:307-312

93. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G, Emilie D. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *Am J Respir Crit Care Med*. 1995;151:1628-1631

94. Dorfmuller P, Zarka V, Durand-Gasselin I, Monti G, Balabanian K, Garcia G, Capron F, Coulomb-Lhermine A, Marfaing-Koka A, Simonneau G, Emilie D, Humbert M. Chemokine rantes in severe pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2002;165:534-539

95. Monti G, Magnan A, Fattal M, Rain B, Humbert M, Mege JL, Noirclerc M, Dartevelle P, Cerrina J, Simonneau G, Galanaud P, Emilie D. Intrapulmonary production of rantes during rejection and cmv pneumonitis after lung transplantation. *Transplantation*. 1996;61:1757-1762

96. Fartoukh M, Emilie D, Le Gall C, Monti G, Simonneau G, Humbert M. Chemokine macrophage inflammatory protein-1alpha mrna expression in lung biopsy specimens of primary pulmonary hypertension. *Chest*. 1998;114:50S-51S

97. Balabanian K, Foussat A, Dorfmuller P, Durand-Gasselin I, Capel F, Bouchet-Delbos L, Portier A, Marfaing-Koka A, Krzysiek R, Rimaniol AC, Simonneau G, Emilie D, Humbert M. Cx(3)c chemokine fractalkine in pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2002;165:1419-1425

98. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, Grimminger F. Reversal of experimental pulmonary hypertension by pdgf inhibition. *J Clin Invest*. 2005;115:2811-2821

99. Ghofrani HA, Seeger W, Grimminger F. Imatinib for the treatment of pulmonary arterial hypertension. *N Engl J Med*. 2005;353:1412-1413

100. Newman JH, Phillips JA, 3rd, Loyd JE. Narrative review: The enigma of pulmonary arterial hypertension: New insights from genetic studies. *Ann Intern Med*. 2008;148:278-283

101. Phillips JA, 3rd, Poling JS, Phillips CA, Stanton KC, Austin ED, Cogan JD, Wheeler L, Yu C, Newman JH, Dietz HC, Loyd JE. Synergistic heterozygosity for tgfbeta1 snps and bmpr2 mutations modulates the age at diagnosis and penetrance of familial pulmonary arterial hypertension. *Genet Med*. 2008;10:359-365

102. Zaiman AL, Podowski M, Medicherla S, Gordy K, Xu F, Zhen L, Shimoda LA, Neptune E, Higgins L, Murphy A, Chakravarty S, Protter A, Sehgal PB, Champion HC, Tuder RM. Role of the tgf-beta/alk5 signaling pathway in monocrotaline-induced pulmonary hypertension. *Am J Respir Crit Care Med*. 2008;177:896-905

103. Long L, Crosby A, Yang X, Southwood M, Upton PD, Kim DK, Morrell NW. Altered bone morphogenetic protein and transforming growth factor-beta signaling in rat models of pulmonary hypertension: Potential for activin receptor-like kinase-5 inhibition in prevention and progression of disease. *Circulation*. 2009;119:566-576

104. Machado RD, Aldred MA, James V, Harrison RE, Patel B, Schwalbe EC, Gruenig E, Janssen B, Koehler R, Seeger W, Eickelberg O, Olschewski H, Elliott CG, Glissmeyer E, Carlquist J, Kim M, Torbicki A, Fijalkowska A, Szewczyk G, Parma J, Abramowicz MJ, Galie N, Morisaki H, Kyotani S, Nakanishi N, Morisaki T, Humbert M, Simonneau G, Sitbon O, Soubrier F, Coulet F, Morrell NW, Trembath RC. Mutations of the tgf-beta type ii receptor bmpr2 in pulmonary arterial hypertension. *Hum Mutat*. 2006;27:121-132

105. Machado RD, Pauciulo MW, Thomson JR, Lane KB, Morgan NV, Wheeler L, Phillips JA, 3rd, Newman J, Williams D, Galie N, Manes A, McNeil K, Yacoub M, Mikhail G, Rogers P, Corris P, Humbert M, Donnai D, Martensson G, Tranebjaerg L, Loyd JE, Trembath RC, Nichols WC. Bmpr2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet*. 2001;68:92-102

106. Nishihara A, Watabe T, Imamura T, Miyazono K. Functional heterogeneity of bone morphogenetic protein receptor-ii mutants found in patients with primary pulmonary hypertension. *Mol Biol Cell*. 2002;13:3055-3063

107. Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (bmp) receptor oligomerization determines different bmp-2 signaling pathways. *J Biol Chem*. 2002;277:5330-5338

108. West J, Harral J, Lane K, Deng Y, Ickes B, Crona D, Albu S, Stewart D, Fagan K. Mice expressing bmpr2r899x transgene in smooth muscle develop pulmonary vascular lesions. *Am J Physiol Lung Cell Mol Physiol*. 2008;295:L744-755

109. Park SO, Lee YJ, Seki T, Hong KH, Fliess N, Jiang Z, Park A, Wu X, Kaartinen V, Roman BL, Oh SP. Alk5- and tgfbr2-independent role of alk1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2. *Blood*. 2008;111:633-642

110. Hong KH, Lee YJ, Lee E, Park SO, Han C, Beppu H, Li E, Raizada MK, Bloch KD, Oh SP. Genetic ablation of the bmpr2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension. *Circulation*. 2008;118:722-730

111. Akhurst RJ. Tgf beta signaling in health and disease. *Nat Genet*. 2004;36:790-792

112. Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC, Carter ND, Spector TD. Genetic control of the circulating concentration of transforming growth factor type beta1. *Hum Mol Genet*. 1999;8:93-97

113. Drumm ML, Konstan MW, Schluchter MD, Handler A, Pace R, Zou F, Zariwala M, Fargo D, Xu A, Dunn JM, Darrah RJ, Dorfman R, Sandford AJ, Corey M, Zielenski J, Durie P, Goddard K, Yankaskas JR, Wright FA, Knowles MR. Genetic modifiers of lung disease in cystic fibrosis. *N Engl J Med*. 2005;353:1443-1453

114. Lechleider RJ, Ryan JL, Garrett L, Eng C, Deng C, Wynshaw-Boris A, Roberts AB. Targeted mutagenesis of smad1 reveals an essential role in chorioallantoic fusion. *Dev Biol*. 2001;240:157-167

115. Liu S, Tian Z, Yin F, Zhang P, W Y, Ding X, Wu H, Wu Y, Peng X, Yuan J, Qiang B, Fan W, Fan M. Expression and functional roles of smad1 and bmpr-ib in glioma development. *Cancer Invest*. 2009;27:734-740

116. Khajavi M, Inoue K, Lupski JR. Nonsense-mediated mrna decay modulates clinical outcome of genetic disease. *Eur J Hum Genet*. 2006;14:1074-1081

117. Austin ED, Loyd JE, Phillips JA, 3rd. Genetics of pulmonary arterial hypertension. *Semin Respir Crit Care Med*. 2009;30:386-398

118. Machado RD, Rudarakanchana N, Atkinson C, Flanagan JA, Harrison R, Morrell NW, Trembath RC. Functional interaction between bmpr-ii and tctex-1, a light chain of dynein, is isoform-specific and disrupted by mutations underlying primary pulmonary hypertension. *Hum Mol Genet*. 2003;12:3277-3286

119. Foletta VC, Lim MA, Soosairajah J, Kelly AP, Stanley EG, Shannon M, He W, Das S, Massague J, Bernard O. Direct signaling by the bmp type ii receptor via the cytoskeletal regulator limk1. *J Cell Biol*. 2003;162:1089-1098

120. Hansmann G, de Jesus Perez VA, Alastalo TP, Alvira CM, Guignabert C, Bekker JM, Schellong S, Urashima T, Wang L, Morrell NW, Rabinovitch M. An antiproliferative bmp-2/ppargamma/apoe axis in human and murine smcs and its role in pulmonary hypertension. *J Clin Invest*. 2008;118:1846-1857

121. Li X, Zhang X, Leathers R, Makino A, Huang C, Parsa P, Macias J, Yuan JX, Jamieson SW, Thistlethwaite PA. Notch3 signaling promotes the development of pulmonary arterial hypertension. *Nat Med*. 2009;15:1289-1297

122. Eddahibi S, Guignabert C, Barlier-Mur AM, Dewachter L, Fadel E, Dartevelle P, Humbert M, Simonneau G, Hanoun N, Saurini F, Hamon M, Adnot S. Cross talk between endothelial and smooth muscle cells in pulmonary hypertension: Critical role for serotonin-induced smooth muscle hyperplasia. *Circulation*. 2006;113:1857-1864

123. Pangas SA, Li X, Umans L, Zwijsen A, Huylebroeck D, Gutierrez C, Wang D, Martin JF, Jamin SP, Behringer RR, Robertson EJ, Matzuk MM. Conditional deletion of smad1 and smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. *Mol Cell Biol*. 2008;28:248-257

124. Orvis GD, Jamin SP, Kwan KM, Mishina Y, Kaartinen VM, Huang S, Roberts AB, Umans L, Huylebroeck D, Zwijsen A, Wang D, Martin JF, Behringer RR. Functional redundancy of tgf-beta family type i receptors and receptor-smads in mediating anti-mullerian hormone-induced mullerian duct regression in the mouse. *Biol Reprod*. 2008;78:994-1001

125. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB. Interleukin-6 overexpression induces pulmonary hypertension. *Circ Res*. 2009;104:236-244, 228p following 244

126. Ono S, Westcott JY, Voelkel NF. Paf antagonists inhibit pulmonary vascular remodeling induced by hypobaric hypoxia in rats. *J Appl Physiol*. 1992;73:1084-1092

127. Voelkel NF, Tuder RM, Wade K, Hoper M, Lepley RA, Goulet JL, Koller BH, Fitzpatrick F. Inhibition of 5-lipoxygenase-activating protein (flap) reduces pulmonary vascular reactivity and pulmonary hypertension in hypoxic rats. *J Clin Invest*. 1996;97:2491-2498

128. Eickelberg O, Morty RE. Transforming growth factor beta/bone morphogenic protein signaling in pulmonary arterial hypertension: Remodeling revisited. *Trends Cardiovasc Med*. 2007;17:263-269

129. Ruiz-Ortega M, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J. Tgf-beta signaling in vascular fibrosis. *Cardiovasc Res*. 2007;74:196-206

130. Lo RS, Massague J. Ubiquitin-dependent degradation of tgf-beta-activated smad2. *Nat Cell Biol*. 1999;1:472-478

131. Lin X, Liang M, Feng XH. Smurf2 is a ubiquitin e3 ligase mediating proteasome-dependent degradation of smad2 in transforming growth factor-beta signaling. *J Biol Chem*. 2000;275:36818-36822

132. Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL. Tgf-beta induces assembly of a smad2-smurf2 ubiquitin ligase complex that targets snon for degradation. *Nat Cell Biol*. 2001;3:587-595

133. Habashi JP, Judge DP, Holm TM, Cohn RD, Loeys BL, Cooper TK, Myers L, Klein EC, Liu G, Calvi C, Podowski M, Neptune ER, Halushka MK, Bedja D, Gabrielson K, Rifkin DB, Carta L, Ramirez F, Huso DL, Dietz HC. Losartan, an at1 antagonist, prevents aortic aneurysm in a mouse model of marfan syndrome. *Science*. 2006;312:117-121

134. Mikhail G, Chester AH, Gibbs JS, Borland JA, Banner NR, Yacoub MH. Role of vasoactive mediators in primary and secondary pulmonary hypertension. *Am J Cardiol*. 1998;82:254-255

135. Selimovic N, Andersson B, Bergh CH, Sakiniene E, Carlsten H, Rundqvist B. Endothelin-1 across the lung circulation in patients with pulmonary arterial hypertension and influence of epoprostenol infusion. *J Heart Lung Transplant*. 2009;28:808-814

136. Ozaki M, Kawashima S, Yamashita T, Ohashi Y, Rikitake Y, Inoue N, Hirata KI, Hayashi Y, Itoh H, Yokoyama M. Reduced hypoxic pulmonary vascular remodeling by nitric oxide from the endothelium. *Hypertension*. 2001;37:322-327

137. Li H, Chen SJ, Chen YF, Meng QC, Durand J, Oparil S, Elton TS. Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia. *J Appl Physiol*. 1994;77:1451-1459

138. Frasch HF, Marshall C, Marshall BE. Endothelin-1 is elevated in monocrotaline pulmonary hypertension. *Am J Physiol*. 1999;276:L304-310

139. Bideau A, Plauchu H, Brunet G, Robert J. Epidemiological investigation of rendu-osler disease in france: Its geographical distribution and prevalence. *Popul*. 1989;44:3-22

140. Kjeldsen AD, Vase P, Green A. Hereditary haemorrhagic telangiectasia: A population-based study of prevalence and mortality in danish patients. *J Intern Med*. 1999;245:31-39

141. Abdalla SA, Letarte M. Hereditary haemorrhagic telangiectasia: Current views on genetics and mechanisms of disease. *J Med Genet*. 2006;43:97-110

142. Marchuk DA, Srinivasan S, Squire TL, Zawistowski JS. Vascular morphogenesis: Tales of two syndromes. *Hum Mol Genet*. 2003;12 Spec No 1:R97-112

143. Bertolino P, Deckers M, Lebrin F, ten Dijke P. Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. *Chest*. 2005;128:585S-590S

144. Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H. Diagnostic criteria for hereditary hemorrhagic telangiectasia (rendu-osler-weber syndrome). *Am J Med Genet*. 2000;91:66-67

145. Sabba C, Pasculli G, Cirulli A, Gallitelli M, Virgilio G, Resta F, Guastamacchia E, Palasciano G. Hereditary hemorrhagic teleangiectasia (rendu-osler-weber disease). *Minerva Cardioangiol*. 2002;50:221-238

146. Krings T, Chng SM, Ozanne A, Alvarez H, Rodesch G, Lasjaunias PL. Hereditary haemorrhagic telangiectasia in children. Endovascular treatment of neurovascular malformations. Results in 31 patients. *Interv Neuroradiol*. 2005;11:13-23

147. Morgan T, McDonald J, Anderson C, Ismail M, Miller F, Mao R, Madan A, Barnes P, Hudgins L, Manning M. Intracranial hemorrhage in infants and children with hereditary hemorrhagic telangiectasia (osler-weber-rendu syndrome). *Pediatrics*. 2002;109:E12

148. Shovlin CL. Hereditary haemorrhagic telangiectasia: Pathophysiology, diagnosis and treatment. *Blood Rev*. 2010;24:203-219

149. Shah RK, Dhingra JK, Shapshay SM. Hereditary hemorrhagic telangiectasia: A review of 76 cases. *Laryngoscope*. 2002;112:767-773

150. Karapantzos I, Tsimpiris N, Goulis DG, Van Hoecke H, Van Cauwenberge P, Danielides V. Management of epistaxis in hereditary hemorrhagic telangiectasia by nd:Yag laser and quality of life assessment using the hr-qol questionnaire. *Eur Arch Otorhinolaryngol*. 2005;262:830-833

151. Geisthoff UW, Fiorella ML, Fiorella R. Treatment of recurrent epistaxis in hht. *Curr Pharm Des*. 2006;12:1237-1242

152. Shovlin CL, Jackson JE, Bamford KB, Jenkins IH, Benjamin AR, Ramadan H, Kulinskaya E. Primary determinants of ischaemic stroke/brain abscess risks are independent of severity of pulmonary arteriovenous malformations in hereditary haemorrhagic telangiectasia. *Thorax*. 2008;63:259-266

153. Soderman M, Andersson T, Karlsson B, Wallace MC, Edner G. Management of patients with brain arteriovenous malformations. *Eur J Radiol*. 2003;46:195-205

154. Faughnan ME, Palda VA, Garcia-Tsao G, Geisthoff UW, McDonald J, Proctor DD, Spears J, Brown DH, Buscarini E, Chesnutt MS, Cottin V, Ganguly A, Gossage JR, Guttmacher AE, Hyland RH, Kennedy SJ, Korzenik J, Mager JJ, Ozanne AP, Piccirillo JF, Picus D, Plauchu H, Porteous ME, Pyeritz RE, Ross DA, Sabba C, Swanson K, Terry P, Wallace MC, Westermann CJ, White RI, Young LH, Zarrabeitia R. International guidelines for the diagnosis and management of hereditary haemorrhagic telangiectasia. *J Med Genet*. 2011;48:73-87

155. Yaniv E, Preis M, Hadar T, Shvero J, Haddad M. Antiestrogen therapy for hereditary hemorrhagic telangiectasia: A double-blind placebo-controlled clinical trial. *Laryngoscope*. 2009;119:284-288

156. Morales-Angulo C, Perez del Molino A, Zarrabeitia R, Fernandez A, Sanz-Rodriguez F, Botella LM. [treatment of epistaxes in hereditary haemorrhagic telangiectasia (rendu-osler-weber disease) with tranexamic acid]. *Acta Otorrinolaringol Esp*. 2007;58:129-132

157. Shovlin CL, Sulaiman NL, Govani FS, Jackson JE, Begbie ME. Elevated factor viii in hereditary haemorrhagic telangiectasia (hht): Association with venous thromboembolism. *Thromb Haemost*. 2007;98:1031-1039

158. Lastella P, Sabba C, Lenato GM, Resta N, Lattanzi W, Gallitelli M, Cirulli A, Guanti G. Endoglin gene mutations and polymorphisms in italian patients with hereditary haemorrhagic telangiectasia. *Clin Genet*. 2003;63:536-540

159. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, et al. Endoglin, a tgf-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet*. 1994;8:345-351

160. McDonald MT, Papenberg KA, Ghosh S, Glatfelter AA, Biesecker BB, Helmbold EA, Markel DS, Zolotor A, McKinnon WC, Vanderstoep JL, et al. A disease locus for hereditary haemorrhagic telangiectasia maps to chromosome 9q33-34. *Nat Genet*. 1994;6:197-204

161. Johnson DW, Berg JN, Gallione CJ, McAllister KA, Warner JP, Helmbold EA, Markel DS, Jackson CE, Porteous ME, Marchuk DA. A second locus for hereditary hemorrhagic telangiectasia maps to chromosome 12. *Genome Res*. 1995;5:21-28

162. Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet*. 1996;13:189-195

163. Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in madh4 (smad4). *Lancet*. 2004;363:852-859

164. Govani FS, Shovlin CL. Fine mapping of the hereditary haemorrhagic telangiectasia (hht)3 locus on chromosome 5 excludes ve-cadherin-2, sprouty4 and other interval genes. *J Angiogenes Res*. 2010;2:15

165. Bayrak-Toydemir P, McDonald J, Akarsu N, Toydemir RM, Calderon F, Tuncali T, Tang W, Miller F, Mao R. A fourth locus for hereditary hemorrhagic telangiectasia maps to chromosome 7. *Am J Med Genet A*. 2006;140:2155-2162

166. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A*. 2000;97:2626-2631

167. Urness LD, Sorensen LK, Li DY. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet*. 2000;26:328-331

168. Srinivasan S, Hanes MA, Dickens T, Porteous ME, Oh SP, Hale LP, Marchuk DA. A mouse model for hereditary hemorrhagic telangiectasia (hht) type 2. *Hum Mol Genet*. 2003;12:473-482

169. Park SO, Wankhede M, Lee YJ, Choi EJ, Fliess N, Choe SW, Oh SH, Walter G, Raizada MK, Sorg BS, Oh SP. Real-time imaging of de novo arteriovenous malformation in a mouse model of hereditary hemorrhagic telangiectasia. *J Clin Invest*. 2009;119:3487-3496

170. Velnar T, Bailey T, Smrkolj V. The wound healing process: An overview of the cellular and molecular mechanisms. *J Int Med Res*. 2009;37:1528-1542

171. Chen Y, Pawlikowska L, Yao JS, Shen F, Zhai W, Achrol AS, Lawton MT, Kwok PY, Yang GY, Young WL. Interleukin-6 involvement in brain arteriovenous malformations. *Ann Neurol*. 2006;59:72-80

172. Chen Y, Fan Y, Poon KY, Achrol AS, Lawton MT, Zhu Y, McCulloch CE, Hashimoto T, Lee C, Barbaro NM, Bollen AW, Yang GY, Young WL. Mmp-9 expression is associated with leukocytic but not endothelial markers in brain arteriovenous malformations. *Front Biosci*. 2006;11:3121-3128

173. Chen Y, Zhu W, Bollen AW, Lawton MT, Barbaro NM, Dowd CF, Hashimoto T, Yang GY, Young WL. Evidence of inflammatory cell involvement in brain arteriovenous malformations. *Neurosurgery*. 2008;62:1340-1349; discussion 1349-1350

174. Sadick H, Naim R, Sadick M, Hormann K, Riedel F. Plasma level and tissue expression of angiogenic factors in patients with hereditary hemorrhagic telangiectasia. *Int J Mol Med*. 2005;15:591-596

175. Sadick H, Naim R, Gossler U, Hormann K, Riedel F. Angiogenesis in hereditary hemorrhagic telangiectasia: Vegf165 plasma concentration in correlation to the vegf expression and microvessel density. *Int J Mol Med*. 2005;15:15-19

176. Lebrin F, Srun S, Raymond K, Martin S, van den Brink S, Freitas C, Breant C, Mathivet T, Larrivee B, Thomas JL, Arthur HM, Westermann CJ, Disch F, Mager JJ, Snijder RJ, Eichmann A, Mummery CL. Thalidomide stimulates vessel maturation and reduces epistaxis in individuals with hereditary hemorrhagic telangiectasia. *Nat Med*. 2010;16:420-428

177. Mitchell A, Adams LA, MacQuillan G, Tibballs J, vanden Driesen R, Delriviere L. Bevacizumab reverses need for liver transplantation in hereditary hemorrhagic telangiectasia. *Liver Transpl*. 2008;14:210-213

178. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. Vegf contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J*. 1999;18:3964-3972

179. Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Luthi U, Barberis A, Benjamin LE, Makinen T, Nobes CD, Adams RH. Ephrin-b2 controls vegf-induced angiogenesis and lymphangiogenesis. *Nature*. 2010;465:483-486

180. Kerbel RS. Tumor angiogenesis. *N Engl J Med*. 2008;358:2039-2049

181. Mattsby-Baltzer I, Jakobsson A, Sorbo J, Norrby K. Endotoxin is angiogenic. *Int J Exp Pathol*. 1994;75:191-196

182. Harmey JH, Bucana CD, Lu W, Byrne AM, McDonnell S, Lynch C, Bouchier-Hayes D, Dong Z. Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. *Int J Cancer*. 2002;101:415-422

183. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science*. 1992;258:1798-1801

184. Oosting S, Nagengast W, de Vries E. More on bevacizumab in hereditary hemorrhagic telangiectasia. *N Engl J Med*. 2009;361:931; author reply 931-932

185. Flieger D, Hainke S, Fischbach W. Dramatic improvement in hereditary hemorrhagic telangiectasia after treatment with the vascular endothelial growth factor (vegf) antagonist bevacizumab. *Ann Hematol*. 2006;85:631-632

186. Retornaz F, Rinaldi Y, Duvoux C. More on bevacizumab in hereditary hemorrhagic telangiectasia. *N Engl J Med*. 2009;361:931; author reply 931-932

187. Simonds J, Miller F, Mandel J, Davidson TM. The effect of bevacizumab (avastin) treatment on epistaxis in hereditary hemorrhagic telangiectasia. *Laryngoscope*. 2009;119:988-992

188. Bose P, Holter JL, Selby GB. Bevacizumab in hereditary hemorrhagic telangiectasia. *N Engl J Med*. 2009;360:2143-2144

189. Davidson TM, Olitsky SE, Wei JL. Hereditary hemorrhagic telangiectasia/avastin. *Laryngoscope*. 2010;120:432-435

190. Brinkerhoff BT, Poetker DM, Choong NW. Long-term therapy with bevacizumab in hereditary hemorrhagic telangiectasia. *N Engl J Med*. 2011;364:688-689

191. Buscarini E, Manfredi G, Zambelli A. Bevacizumab to treat complicated liver vascular malformations in hereditary hemorrhagic telangiectasia: A word of caution. *Liver Transpl*. 2008;14:1685-1686; author reply 1687-1688

192. Chen St, Karnezis T, Davidson TM. Safety of intranasal bevacizumab (avastin) treatment in patients with hereditary hemorrhagic telangiectasia-associated epistaxis. *Laryngoscope*. 2011;121:644-646

193. Karnezis TT, Davidson TM. Efficacy of intranasal bevacizumab (avastin) treatment in patients with hereditary hemorrhagic telangiectasia-associated epistaxis. *Laryngoscope*. 2011;121:636-638

194. Plauchu H, de Chadarevian JP, Bideau A, Robert JM. Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. *Am J Med Genet*. 1989;32:291-297

195. Kim H, Hysi PG, Pawlikowska L, Poon A, Burchard EG, Zaroff JG, Sidney S, Ko NU, Achrol AS, Lawton MT, McCulloch CE, Kwok PY, Young WL. Common variants in interleukin-1-beta gene are associated with intracranial hemorrhage and susceptibility to brain arteriovenous malformation. *Cerebrovasc Dis*. 2009;27:176-182

196. Achrol AS, Pawlikowska L, McCulloch CE, Poon KY, Ha C, Zaroff JG, Johnston SC, Lee C, Lawton MT, Sidney S, Marchuk DA, Kwok PY, Young WL. Tumor necrosis factor-alpha-238g>a promoter polymorphism is associated with increased risk of new hemorrhage in the natural course of patients with brain arteriovenous malformations. *Stroke*. 2006;37:231-234

197. Pawlikowska L, Poon KY, Achrol AS, McCulloch CE, Ha C, Lum K, Zaroff JG, Ko NU, Johnston SC, Sidney S, Marchuk DA, Lawton MT, Kwok PY, Young WL. Apolipoprotein e epsilon 2 is associated with new hemorrhage risk in brain arteriovenous malformations. *Neurosurgery*. 2006;58:838-843; discussion 838-843

198. Torsney E, Charlton R, Diamond AG, Burn J, Soames JV, Arthur HM. Mouse model for hereditary hemorrhagic telangiectasia has a generalized vascular abnormality. *Circulation*. 2003;107:1653-1657

199. Martich GD, Boujoukos AJ, Suffredini AF. Response of man to endotoxin. *Immunobiology*. 1993;187:403-416

200. Scallon BJ, Moore MA, Trinh H, Knight DM, Ghrayeb J. Chimeric anti-tnf-alpha monoclonal antibody ca2 binds recombinant transmembrane tnf-alpha and activates immune effector functions. *Cytokine*. 1995;7:251-259

201. Wong M, Ziring D, Korin Y, Desai S, Kim S, Lin J, Gjertson D, Braun J, Reed E, Singh RR. Tnfalpha blockade in human diseases: Mechanisms and future directions. *Clin Immunol*. 2008;126:121-136

202. Botero TM, Mantellini MG, Song W, Hanks CT, Nor JE. Effect of lipopolysaccharides on vascular endothelial growth factor expression in mouse pulp cells and macrophages. *Eur J Oral Sci*. 2003;111:228-234

203. Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N. Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. *Nature*. 1987;329:630-632

204. Pandey A, Shao H, Marks RM, Polverini PJ, Dixit VM. Role of b61, the ligand for the eck receptor tyrosine kinase, in tnf-alpha-induced angiogenesis. *Science*. 1995;268:567-569

205. Sabba C, Gallitelli M, Palasciano G. Efficacy of unusually high doses of tranexamic acid for the treatment of epistaxis in hereditary hemorrhagic telangiectasia. *N Engl J Med*. 2001;345:926

206. de Gussem EM, Snijder RJ, Disch FJ, Zanen P, Westermann CJ, Mager JJ. The effect of n-acetylcysteine on epistaxis and quality of life in patients with hht: A pilot study. *Rhinology*. 2009;47:85-88

207. Albinana V, Bernabeu-Herrero ME, Zarrabeitia R, Bernabeu C, Botella LM. Estrogen therapy for hereditary haemorrhagic telangiectasia (hht): Effects of raloxifene, on endoglin and alk1 expression in endothelial cells. *Thromb Haemost*. 2010;103:525-534

208. Huang S, Tang B, Usoskin D, Lechleider RJ, Jamin SP, Li C, Anzano MA, Ebendal T, Deng C, Roberts AB. Conditional knockout of the smad1 gene. *Genesis*. 2002;32:76-79

209. Lednicky JA, Butel JS. Tissue culture adaptation of natural isolates of simian virus 40: Changes occur in viral regulatory region but not in carboxy-terminal domain of large t-antigen. *J Gen Virol*. 1997;78 (Pt 7):1697-1705

210. Stewart AR, Lednicky JA, Butel JS. Sequence analyses of human tumor-associated sv40 dnas and sv40 viral isolates from monkeys and humans. *J Neurovirol*. 1998;4:182-193

211. Acharya AP, Clare-Salzler MJ, Keselowsky BG. A high-throughput microparticle microarray platform for dendritic cell-targeting vaccines. *Biomaterials*. 2009;30:4168-4177

212. Choe SW, Acharya AP, Keselowsky BG, Sorg BS. Intravital microscopy imaging of macrophage localization to immunogenic particles and co-localized tissue oxygen saturation. *Acta Biomater*. 2010;6:3491-3498

213. Thomas M, Docx C, Holmes AM, Beach S, Duggan N, England K, Leblanc C, Lebret C, Schindler F, Raza F, Walker C, Crosby A, Davies RJ, Morrell NW, Budd DC. Activin-like kinase 5 (alk5) mediates abnormal proliferation of vascular smooth muscle cells from patients with familial pulmonary arterial hypertension and is involved in the progression of experimental pulmonary arterial hypertension induced by monocrotaline. *Am J Pathol*. 2009;174:380-389

BIOGRAPHICAL SKETCH

Chul Han was born in Daejon, South Korea in 1975. He is the oldest of two sons born to Jeong-gun Han and Duk-ae Lee. Chul attended Korea University from 1994-2003 where he studied molecular biology and biochemistry and earned a Bachelor of Science in genetic engineering in 2001 and a Master of Science in molecular medical science in 2003. He served in the air force for two and half years (1998-2000). After graduation, Chul worked as a research scientist in the Samsung medical center (2003-2006). Then he moved to Gainesville, Florida and joined the Interdisciplinary Program in Biomedical Sciences at the University of Florida College of Medicine. Chul did his graduate work in Dr. S. Paul Oh's laboratory in the Department of Functional Genomics and Physiology and completed his Ph.D. dissertation in August 2011.