Chapter 1

Part I: BMP signaling in vascular diseases

1. BMPs
2. BMP receptors
3. Smad and non-Smad signaling pathways
4. BMP signaling during vessel development
5. BMP signaling pathway in vascular diseases

5.1 Pulmonary arterial hypertension

5.2 Hereditary hemorrhagic telangiectasia

5.3 Atherosclerosis and vascular calcification

5.4 Tumor angiogenesis

6. Conclusions and perspective

Part II: BMP signaling in fibrodysplasia ossificans progressiva (FOP)

Part III: Aims and outline of this thesis

Part I: BMP signaling in vascular diseases

Jie Cai[a], Evangelia Pardali[b], Gonzalo Sánchez-Duffhues[a], Peter ten Dijke[a,*]

[a] *Department of Molecular Cell Biology and Centre for Biomedical Genetics, Leiden University Medical Center, The Netherlands.*

[b] *Department of Cardiology and Angiology, University Hospital Münster, Münster, Germany.*

FEBS Lett. 2012 Jul 4;586(14):1993-2002.

Abstract

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) family that signal via type I and type II serine/threonine kinase receptors and intracellular Smad transcription factors. BMPs are multifunctional regulators of development and tissue homeostasis and they were initially characterized as inducers of bone regeneration. Genetic studies in humans and mice showed that perturbations in BMP signaling lead to various diseases, such as skeletal diseases, vascular diseases and cancer. Mutations in BMP type II receptor and BMP type I receptor/activin receptor-like kinase 1 have been linked to pulmonary arterial hypertension and hereditary hemorrhagic telangiectasia, respectively. BMPs have also been implicated in promoting vascular calcification and tumor angiogenesis. In this review we discuss the role of BMP signaling in vascular diseases and the value of BMP signaling as a vascular disease marker or a therapeutic target.

Key words:

BMP signaling, cardiovascular disease, pulmonary arterial hypertension, hereditary hemorrhagic telangiectasia, vascular calcification, tumor angiogenesis

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) family, which also includes TGF-βs, growth and differentiation factors (GDFs), anti-müllerian hormone (AMH), activins and nodal. BMPs were first identified as potent inducers of ectopic bone formation when implanted subcutaneously in rats (1, 2). Subsequent studies demonstrated that BMPs, as is the case for other TGF-β family members, are multifunctional regulators in development that regulate cell proliferation, differentiation, and apoptosis in different tissues (3, 4). BMPs exert their signals via type I and type II transmembrane serine/threonine kinase receptors. Inside the cell, Smad proteins play an important role in the transduction of the signal from the active receptor complex to the nucleus. Interestingly, misregulated BMP signaling has been shown to be involved in the pathogenesis of skeletal and (cardio) vascular disorders as well as cancer. Despite the recent advances in therapeutic interventions, cardiovascular disease remains the largest health problem worldwide causing morbidity and mortality. This review will focus on the role of BMP signaling in the pathology of vascular diseases and potential clinical applications.

BMPs

Among the 33 members of the TGF-β superfamily, over 20 molecules form the BMP subfamily. The BMP subfamily can be further subdivided into several subgroups, including BMP-2/4, BMP-5/6/7/8, GDF-5/6/7 and BMP-9/10 (4, 5). BMPs are synthesized as large precursor proteins consisting of an amino (N)-terminal signal peptide, a prodomain for folding and secretion, and a bioactive carboxy (C)-terminal mature peptide. BMP precursor proteins are produced in the cytoplasm as dimeric pro-protein complexes, which are cleaved by serine endoproteases (e. g. BMP-4 is cleaved by furin, PC6 and PC7 (6)) to generate N-terminal and C-terminal fragments, of which the latter is capable of binding to its receptor (7). Whereas the secretion of BMPs in a latent inactive form is not common (7), TGF-β is secreted as a latent form in which the N-terminal remnant, also known as latency associated peptide (LAP), sequesters and prevents the bioactive mature part from binding to its receptors. This complex is also associated with the latent TGF-β binding proteins (LTBP). Thus,

proteolytic cleavage of latent TGF-β by different activators is required for the release of the mature, active TGF-β (8).

BMP activity is also regulated by several intracellular and extracellular modulators. A large number of extracellular soluble antagonists bind BMPs and block their interaction with signaling receptors, thus dampening BMP signaling (9). These antagonists can be divided into three subgroups based on their structure similarity: the CAN (Cerberus/DAN) family, twisted gastrulation, chordin and noggin. The CAN family includes gremlin and cerberus, differential screening-selected gene aberrative in neuroblastoma (DAN), protein related to DAN and cerberus (PRDC), coco, uterine sensitization-associated gene-1 (USAG-1) and sclerostin (10). Several additional BMP regulators have been identified, such as cross-veinless 2 [CV2, also referred to as BMP endothelial cell precursor derived regulator (BMPER)], matrix GLA protein (MGP) and neogenin (11-14). MGP is a small, carboxyglutamic acid modified protein, which can bind and inhibit BMP-2 and BMP-4 by direct protein interaction (12, 15, 16). It is highly expressed in kidneys and lungs, where excessive MGP in *MGP*-transgenic mice altered pulmonary BMP-4 distribution and resulted in significant morphological defects in the pulmonary artery tree (17). Neogenin was identified as a receptor for netrins and proteins of the repulsive guidance molecule (RGM) family. The interaction of netrins-neogenin or RGM-neogenin stimulated or repelled neuronal axon guidance depending on the developmental context (18, 19). Recent research suggested that neogenin is a regulator of BMP signaling during chondrogenesis and skeletal development, since there is reduced expression levels of BMP target genes and intracellular BMP signaling mediators in chondrocytes from neogenin mutant mice, and the neogenin-deficient mice is retarded in digit/limb development and endochondral ossification (13). However, others reported that neogenin acts as a repressor of BMP signaling and knockdown of neogenin in C2C12 cells leads to increased BMP-2-induced phosphorylation of Smad1, Smad5, and Smad8 and osteoblast differentiation (14). The expression pattern of BMP antagonists is important for embryonic development, as an aberrant expression pattern can lead to defects in bone, limb and kidney formation (20).

BMP receptors

Like other members of the TGF-β family, BMPs bind to two types of serine-threonine kinase receptors, known as type I and type II receptors (21, 22).

Both receptors share a similar structure and are comprised of a short extracellular domain, a single transmembrane domain and an intracellular domain with serine-threonine kinase activity. The affinity of BMPs for type I receptors is higher than for type II receptors and its affinity is increased by the formation of a heterotetrameric receptor complex (23). The type II receptor kinase is constitutively active in the absence of ligand. BMP type II receptor (BMPR2) has a long C-terminal tail rich in serine and threonine residues (23). Besides BMPR2, BMPs can signal also via the activin type II receptors ACVR2A, and ACVR2B (4, 24), which are expressed in various tissues. Whereas BMPR2 is a specific receptor for BMPs, ACVR2A and ACVR2B also can be used by activins, myostatin and nodal. Based on the structural similarity, BMP type I receptors can be divided into two subgroups: activin receptor-like kinase 3 (ALK3, or BMPR-IA) and ALK6 (BMPR-IB) group, and the ALK1 and ALK2 group. While ALK2 and ALK3/6 are widely expressed in various cell types, ALK1 has a more selective expression pattern being mainly restricted to endothelial cells and few other cell types.

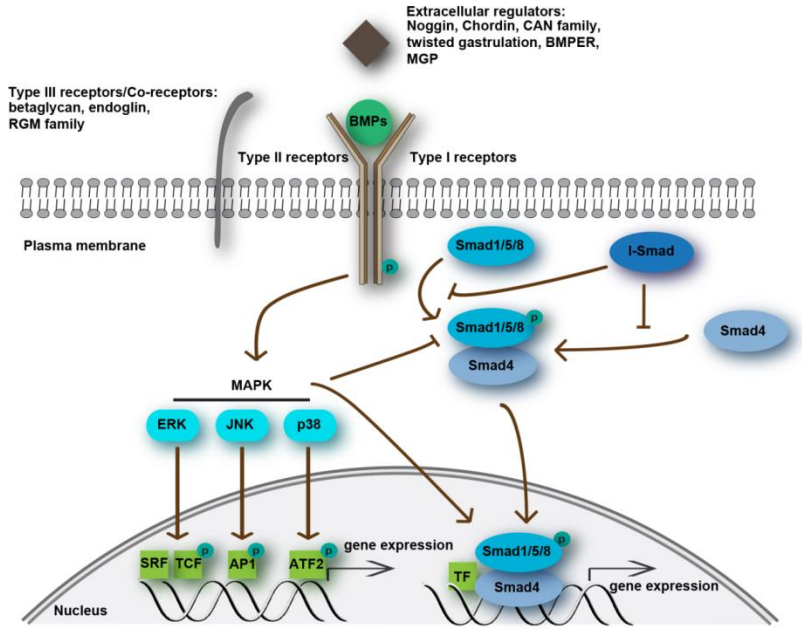

Fig. 1. Schematic overview of the BMP signaling pathway. BMPs interaction with surface receptors induces heteromeric complex formation between specific type II and type I receptors. This activity is regulated by extracellular regulators and type III receptors/Co-receptors. After being activated by type II receptors, the type I receptors phosphorylate Smad1/5/8 (R-Smads) to propagate the signal into the cell. Smad1/5/8 form heteromeric complexes with Smad4 (Co-Smad) and translocate to the nucleus where, by interacting with other transcription factors, they regulate target gene expression (canonical Smad signaling pathway). I-Smads (Smad6/7) inhibit receptor activation of R-Smads. Besides Smad-depend signaling, non-Smad pathways are involved. Activated MAPKs can regulate R-Smad activation by a direct phosphorylation or through their downstream effectors molecules. Activated MAPKs can translocate to the nucleus to phosphorylate a number of transcription factors (TF), such as serum response factor (SRF), ternary complex factor (TCF) family members, activator protein 1 (AP1) complexes and activating transcription factor 2 (ATF2), thereby changing target gene transcription.

A number of BMP co-receptors have been identified. These co-receptors modulate the interactions between BMP ligands and receptors. There are two co-receptors, endoglin and betaglycan, which play important roles in vascular development and disease, although they lack a signaling domain (25). Endoglin and betaglycan can potentiate BMP signaling (26, 27). BMPs can also bind to the decoy receptor BMP and activin membrane-bound inhibitor (BAMBI). BAMBI resembles the type I receptors but lacks an active kinase domain and consequently sequesters ligands from the active receptors and inhibits BMP signaling (28). Family members of RGM, RGMa, RGMb (DRAGON) and RGMc, were shown to be implicated in BMP signaling (29-31). DRAGON was the first RGM family member identified as a BMP co-receptor (30). Cell surface GPI-anchored DRAGON directly binds to BMPs enhancing BMP signaling, but not TGF-β. Moreover, this effect can be reduced by noggin (30). Interestingly, DRAGON interacted directly with all BMP type I receptors as well as BMPR2, ActRII and ActRIIB (30). Furthermore a soluble form of DRAGON fused to Fc (DRAGON-Fc) inhibited BMP signaling *in vitro* (30, 32). It is possible that RGM proteins modulate the ability of cells responding to a low concentration of BMP ligands by altering the sensitivity of BMPR2 to BMP ligands. However, the precise mechanism by which RGM proteins regulate different physiological processes is still not known (33).

Smad and non-Smad signaling pathways

After BMP ligand-induced heteromeric complex formation, the type II receptor kinase phosphorylates the type I receptor. Subsequently, the activated type I receptor initiates intracellular signaling by activating the Smad proteins. Smads can be divided into three groups: receptor-regulated Smads (R-Smads), inhibitory Smads (I-Smads), and a common mediator Smad (i.e. Smad4) (21). Upon type I receptor-mediated phosphorylation/activation of R-Smads, they form heteromeric complexes with Smad4. These heteromeric R-Smad/Smad4 complexes translocate into the nucleus, where they regulate target gene expression by directly binding to Smad-binding elements (SBE), or indirectly through interactions with DNA-binding transcription factors, and by associating with co-activators/co-repressors and histone-modifying factors (34). Inhibitory Smads (I-Smad6 and 7) antagonize BMP and TGF-β receptor-initiated Smad signaling by mediating the degradation of receptors and R-Smads. Smad7 inhibits all TGF-β family members, while Smad6 is more selective towards BMP family members. Smad ubiquitin ligases Smurf1 and Smurf2 are recruited by I-Smads to promote the proteasomal degradation of receptors and Smads (35-37).

Besides canonical BMP receptor/Smad signaling, activated BMP receptors can initiate non-Smad signaling pathways. MAP kinases (ERK, JNK and p38 MAPK), phosphoinositide (PI) 3 kinase/Akt and protein kinase C (PKC) signaling pathways, and Rho-GTPases can also be activated by BMPs and TGF-βs in various cells (38). These non-Smad pathways are also important in creating diversity and fine-tuning of signals generated by the TGF-β family ligands (39, 40). Smad-independent pathways can also be involved in the pathogenesis of vascular diseases, such as in pulmonary arterial hypertension (PAH), which will be discussed later.

BMP signaling during vessel development

The establishment of the vascular system is an important event during embryonic development. Neovascularization involves two mechanisms: first the *de novo* formation of vessels termed vasculogenesis, and second, the sprouting and growth of new vessels from pre-existing ones, known as angiogenesis (41). Angiogenesis is a crucial process, which occurs primarily during embryonic development, and it is almost absent during adulthood besides wound healing,

inflammation and the female reproductive cycle. In healthy tissues, blood vessels are formed by a combination of several mechanisms, such as sprouting angiogenesis, bone-marrow derived and/or vascular-wall-resident endothelial progenitor cells (EPCs) differentiation, and vessel splitting (41). Main players in the process of angiogenesis are the endothelial cells (ECs) as well as smooth muscle cells (SMCs) and pericytes. EC proliferation, migration and tube formation are critical in the process of angiogenesis. Sprouting angiogenesis involves the selection of a leading migrating tip EC that invades the surrounding tissue by extending numerous filopodia. VEGF/VEGFR2 signaling triggers single EC to switch into a tip cell phenotype; these cells thereby express Delta-like 4 (Dll4), a Notch ligand, which instructs neighbor ECs to become so-called stalk cells (42). Stalk cells trail behind the tip cells proliferate and form tubes; stalk cell proliferation ensures elongation of sprouting vessel (43, 44). Ultimately ECs stop proliferating, acquire a quiescent phenotype and become phalanx ECs. Finally, the new formed vessel is stabilized by deposition of basement membrane and recruitment of pericytes/SMCs (45). Interestingly, it has been reported that besides ECs, tumor cells can also contribute to angiogenesis. It has been suggested that cancer cells with stem cell features can dedifferentiate and acquire an EC-like phenotype. These cells can incorporate in the blood vessels and contribute to angiogenesis (41, 46).

The role of BMP signaling in vascular development has been illustrated by studies in knockout animal models (47). Table 1 (see below and references therein) shows a list of mouse knockout models for BMP signaling components, including ligands, receptors and Smads. Genetic deletion or misexpression of different components of BMP signaling leads to embryonic death due to cardiovascular malformations and defects in vascular remodeling. Moreover, proper BMP signaling in both ECs and mural/SMCs has been shown to be required for appropriate vasculogenesis and angiogenesis. Interestingly, deletion of the BMP target genes Id1 and Id3 in mice leads to impaired angiogenesis both in brain and tumor xenografts (48).

It has been reported that BMP-2, -4, -6 and -7 induce angiogenesis, EC proliferation and migration (49, 50). Capillary tube formation is increased upon activation of the BMP signaling pathway by overexpression of BMPs or Id1 (51, 52). In contrast, BMP-9 inhibits basic fibroblast growth factor (bFGF)-stimulated proliferation and migration of bovine aortic endothelial cells (BAECs) and blocks VEGF-induced angiogenesis [36]. BMP-9 has also been

reported to inhibit the migration and growth of human dermal microvascular ECs [37]. Although (high dose) BMP-9 seems to have inhibitory effects on ECs, another report demonstrated that (low dose) BMP-9 induces proliferation of various types of ECs *in vitro* and promoted angiogenesis in matrigel plug assays and human pancreatic cancer xenografts *in vivo* (53). It is likely that BMP-9 has disparate effects on ECs depending on the cellular context and concentration of BMP-9. The effects of BMPs on ECs can be regulated by various BMP antagonists and modulators as well. For example, BMPER is an extracellular matrix protein expressed by ECs, which was shown to modulate BMP-4 activity in a concentration-dependent manner, and to exert proangiogenic effects in vascular ECs (54). Interestingly, *MGP* gene deletion in mice leads to misregulated BMP signaling and as a result in arteriovenous malformation (AVMs) in lungs and kidneys (55). Thus, selective BMP family members can stimulate and/or inhibit angiogenesis. Besides, BMP-induced signaling in ECs response can switch from stimulation to inhibition when co-stimulated with other signals, e. g. Notch (56). As mentioned earlier, Notch was shown to have an important role in stalk cell determination. Recently, Moya *et al.* reported that endothelium-specific inactivation of Smad1/Smad5 in mouse embryos decreased Notch signaling and increased numbers of tip cells. In HUVECs downregulation of Smad1/5 reduced the expression of Notch target genes Hes1 and Hey1, and other stalk cell specific transcripts (57), In addition, Larrivée *et al.* showed that ALK1-dependent SMAD signaling collaborated with Notch signal to induce expression of HEY1 and HEY2 in stalk cells, which would limit the response of stalk cells to VEGF and thus reduce endothelial tip cell formation and sprouting (58).

A lot of research has focused on ECs due to their role in the formation of new vessels. However, research showed that SMCs are also involved in the maturation of the new-formed vessels, as well as in vascular diseases. In addition to their effects on EC function, BMPs were also shown to play key roles in SMC differentiation and function. BMPs have been shown to inhibit the proliferation of vascular SMC while enhancing the differentiation of these cells (59-61). BMP-2 inhibits the proliferation of cultured rat arterial SMCs in the presence of serum and injury-induced intimal hyperplasia in the *in vivo* rat carotid artery balloon injury model by inhibiting SMC proliferation without stimulating extracellular matrix synthesis (61). BMP-7 inhibits primary human aortic SMC proliferation in serum-stimulated conditions, as well as upon

induction with platelet-derived growth factor subunit BB (PDGF-BB) and TGF-β1, and maintains the expression of the vascular SMC phenotype. Furthermore, anti-inflammatory activities have been attributed to BMP-7 suggesting that BMP-7 may play an important role in maintaining vascular integrity (59, 62). BMP-4, however, is expressed by ECs in response to hypoxia and it promotes vascular SMC proliferation (63). It has been demonstrated that vascular SMCs isolated from different parts of the pulmonary vasculature have different proliferation responses to BMP-4. Whereas the proliferation ability of human pulmonary arterial SMCs isolated from proximal pulmonary arteries is inhibited by BMP-4, the proliferation of human pulmonary artery SMCs from peripheral arteries is increased by BMP-4 (64). In summary, similarly to ECs, the effects of BMPs on vascular SMCs depend on the source of cells and the culture condition.

BMP signaling pathway in vascular diseases

The critical role of BMP signaling in vascular function was further corroborated by genetic studies in human (65). Genetic analysis revealed that mutations in genes of the BMP signaling or genes which affect BMP signaling function lead to vascular dysfunction and disease such as hereditary hemorrhagic telangiectasia (HHT) and pulmonary arterial hypertension (PAH), vascular calcification, and tumor angiogenesis. In addition, disturbance of vascular homeostasis due to vascular injury, hypertension or atherosclerosis was shown to affect the expression of BMPs, thereby suggesting a role of BMPs in abnormal vascular responses (65).

1.1. Pulmonary arterial hypertension

PAH is a disease characterized by elevated pulmonary artery pressure leading to heart failure. Processes underlying PAH include abnormal remodeling of small peripheral vessels in the lung, due to aberrant proliferation and migration of vascular SMCs, ECs and fibroblasts (66). Two types of PAH have been described: sporadic or idiopathic PAH (IPAH) and hereditary or familial PAH (FPAH). Heterozygous germ line mutations in *BMPR2* are found in more than 70% of patients with FPAH and 20% of patients with IPAH (67, 68). Mutations have been found in various regions of *BMPR2*, including the ligand-binding domain, the kinase domain, or the long cytoplasmic tail. Mice expressing a

BMPR2 tail domain mutation in pulmonary SMCs develop vascular lesions similar to PAH (69). Non-sense mutations in the C-terminal tail of *BMPR2* were identified also in some FPAH patients, suggesting that this region might play an important role in BMP signaling (67, 68). Heterozygous and homozygous BMPR2 deletion specifically in pulmonary ECs and pulmonary SMCs mimicked the PAH phenotype (69, 70). Endothelial injury and enhanced inflammatory responses may contribute together with BMPR2 heterozygosity to the development of PAH (71). Interestingly it was shown that disruption of BMPR2 expression in PASMCs leads to reduced BMP-2 and BMP-4 signaling, while signaling by BMP-6 and BMP-7 is enhanced (72). It was shown that reduced BMP/Smad signaling resulted in activation of the p38 MAPK pathway, leading to aberrant PASMC proliferation (64, 73, 74). A recent report suggested that lack of endothelial nitric-oxide synthase (eNOS) due to BMPR2 mutations in pulmonary artery ECs (PAEC) may contribute to the pathogenesis of PAH. BMP-2 and BMP-4 cannot activate eNOS in *BMPR2* knockdown cell lines or in PAEC from *BMPR2* gene mutations patients and inhibition of NOS activity inhibited BMP-2 and BMP-4 stimulated PAEC migration (75).

Mutations in *SMAD8* have also been reported in PAH patients (76). In addition, loss of Smad8 function in mice results in abnormal vascular remodeling and increased vascular inflammation (77). It was demonstrated that *SMAD8* mutation leads to vascular cell proliferation in HPAH, due to decreased expression of specific micro RNAs (miR) miR-21 and miR-27a in pulmonary artery ECs and pulmonary artery SMCs from tissues of PAH patients (78). Additionally, overexpression of Smad8 resulted in increased expression of miRs and reversed the hyper-proliferative phenotype (78). Interestingly, certain HHT2 patients develop PPH-like syndromes, suggesting that ALK1 mutations can also be involved in PPH (79, 80). Moreover $alk1^{+/-}$ mice display increased pulmonary vascular remodeling which may lead to signs of PAH. This was shown to be associated with eNOS-dependent reactive oxygen species (ROS) production and it could be averted by anti-oxidant treatment (81).

Table 1. Deregulated BMP signaling leads to (cardio) vascular abnormalities

Gene	Animal model	Human disease	References
Bmp-2	KO: Embryonic lethal with defect in cardiac development; Het: Susceptible to hypoxic pulmonary hypertension associated with reduced endothelial nitric oxide synthase (eNOS) expression	unknown	(82, 83)
Bmp-4	Het : Less severe hypoxic pulmonary hypertension and vascular smooth muscle cell proliferation, impaired vascular remodeling	unknown	(63)
Bmpr2	Het: Pulmonary hypertension	PAH	(69, 70, 84, 85)
Alk1	KO: Embryonic lethal (E10.5), severe vascular abnormalities; Het: Models HHT type 2; EC conditional KO: Severe vascular malformations mimicking all pathologic features of HHT.	HHT	(86-89)
Alk3	Mesoderm conditional KO: Embryonic lethal (E10.5-E11.5), hemorrhage, impaired vessel remodeling; SMC (embryo): Embryonic lethal (E11) due to vascular and pericardial hemorrhage, impaired vascular remodeling; SMC (adult): Impaired vascular remodeling	unknown	(90-92)
Endoglin	KO: Embryonic lethal (E10.5) due to impaired mature vessel formation; Conditional mutation: AVM	HHT	(93, 94)
Smad1	KO: Embryonic lethal (E9.5) due to defects in allantois formation; with impaired embryonic circulation system	unknown	(95)
Smad4	EC conditional KO: Embryonic lethal (E10.5) due to cardiovascular defects	HHT (with or without JP)	(96-98)
Smad5	KO: Embryonic lethal (E9.5-E11.5) due to cardiac and angiogenesis defects	unknown	(99, 100)
Smad6	KO: Cardiovascular defects, vascular calcification, hypertension	CVM	(101, 102)
Smad7	KO: Embryonic lethal due to cardiovascular defects	unknown	(103)
Smad8	Smad8 mutation mice: Defective pulmonary vascular remodeling	PAH	(77)

Abbreviations: KO, knockout; het, heterozygous; JP, juvenile polyposis; CVM, congenital cardiovascular malformation.

1.2. Hereditary hemorrhagic telangiectasia

Mutations in the ALK1 gene have been reported in some PAH patients (79). ALK1 mediates both TGF-β and BMP-9 signaling in ECs. Interestingly, mutations in ALK1 lead to another vascular disease related to deregulated BMP signaling, HHT. HHT is an autosomal dominant disease and is associated with telangiectases in skin and mucosa, frequent epistaxis, and the presence of AVMs in the lung, liver or brain (104). HHT type 1 (HHT1) results from pathogenic mutations in ENG that lead to haploinsufficiency of endoglin (105), while HHT type 2 (HHT2) is caused by loss of function or dominant negative mutations in ALK1 (106, 107). Interestingly mice heterozygous for *acvrl1* (*alk1*), *tβr1* (*alk*5), *tβr2* and *eng* develop vascular abnormalities highly reminiscent of those described in patients with HHT (25, 50). Several studies have provided evidence that haploinsufficiency of the HHT genes both in ECs and SMCs leads to abnormal EC proliferation and SMC recruitment. As a result, vascular abnormalities and fragile leaky vessels occur, together with the generation of telangiectasias and AVMs (108, 109). In addition, disrupted Notch signaling has been reported to correlate with AVMs (110), and ChIP-seq analyses on human umbilical vein ECs (HUVECs) and pulmonary arterial SMCs pretreated with BMPs have demonstrated JAG1 as a direct target of Smad1/5 (111). Another report showed that human polymorphic variants of tyrosine-protein phosphatase non-receptor type 14 (PTPN14) influences the severity of pulmonary arteriovenous malformation acting via ALK1 and EphrinB2, which suggested that PTPN14 may also be involve in the pathogenesis of HHT (112).

1.3. Atherosclerosis and vascular calcification

Atherosclerosis is a chronic arterial wall disease that is characterized by chronic inflammation and the accumulation of atheromatous lesions in the inner layer of arteries. BMPs have been implicated in atherosclerosis progression by regulating endothelial inflammation and cell differentiation. BMP-2 and -4 have been shown to induce proinflammatory effects in the ECs (113, 114). Besides, inhibiting BMP signaling pathway by MGP resulted in reduced atherosclerotic lesions formation in apolipoprotein (Apo) E knockout mice, while enhanced BMP activity led to increased atherosclerotic lesions formation in Apo E knockout mice (113, 115). Atherosclerosis is the most common cause of aortic

aneurysms, a vascular disease which attributes to misregulation of TGF-β signaling (116, 117). However, Jones *et al.* showed that in 2-week post thoracic aortic aneurysms induction mice, the expression level of BMP signal components and BMP regulators were elevated in mRNA level, indicating that activation of BMP signaling may also be involved in the pathogenesis of aortic aneurysms (118).

One key histological and clinical event of atherosclerosis is vascular calcification, which is known as the abnormal deposition of calcium phosphate salts in blood vessels, myocardium, and cardiac valves. Vascular calcification is a tightly regulated process which leads to differentiation of cells such as SMCs or pericytes into osteoblast-like cells, and the mineralization of the extracellular matrix (119). It is speculated that the course of vascular calcification shares many similarities with that of bone mineralization (120). Pericytes, mesenchymal stem cells, multipotent cells from the adventitia, resident cells in the media or intima and trans-differentiated SMCs, are the possible cells which transdifferentiate into osteoblast-like cells in blood vessels (121-124). It has been suggested that vascular endothelial cells may contribute to osteogenic differentiation (125); ECs can transdifferentiate into mesenchymal stem cells through a process termed endothelial to mesenchymal transition (EndoMT) (126-128). Interestingly, in fibrodysplasia ossificans progressiva (FOP), a disease characterized by overactive osteoblasts and ectopic bone formation and linked to a point mutation in BMP type I receptor ALK2 (129), it was shown that ECs can acquire a progenitor-like phenotype and differentiate into bone forming osteoblastic cells (125).

BMPs expression is increased at vascular calcification sites; in addition BMPs can trigger the differentiation of multipotential cells into the osteogenic lineage. This raises the possibility that BMPs may be involved in the process of vascular calcification (130-134). Indeed it was shown that BMPs can direct osteogenic programming of vascular mesenchymal progenitors of the pericyte lineage (132) and that they can promote expression of osteoblast lineage markers such as alkaline phosphatase in cultured vascular SMCs (115, 119, 120, 135-137). Cheng *et al.* showed that BMP-2 and the osteoblast homeoprotein Msx2 were expressed during the osteogenic process in the aorta of diabetic patients. The BMP-2-Msx2 signaling pathway may enhance vascular calcification by promoting the differentiation of myofibroblasts into the osteogenic lineage (138). In addition BMP-2 enhances the expression of Runx2,

a core transcription factor that is known to regulate osteoblast and chondrocyte differentiation and promote vascular SMCs calcification by increasing oxidative stress and endoplasmic reticulum (ER) stress in human coronary artery SMCs. Interestingly, the inhibition of oxidant stress or ER stress reversed this gene expression pattern and mineralization process (139). Moreover, recent research showed that BMPs are involved in vascular calcification in low-density lipoprotein (LDL) receptor-deficient (LDLR-/-) mice. Blockade of BMP type I receptor function by using either the small molecule inhibitor LDN-193189 or ALK3-Fc in LDLR-/- mice inhibited high-fat diet-induced vascular inflammation as well as osteogenic activity and calcification, thus suggesting BMP inhibition as a potential treatment for vascular calcification.

BMP signaling antagonists have been also implicated in vascular calcification. Research suggested that MGP might influence vascular calcification by modulating the effect of BMP-2. In C3H10T1/2 cells, MGP overexpression inhibited BMP-2 induced osteogenic and chondrogenic differentiation, whereas lack of MGP enhanced these differentiation processes (140). Notably, it was shown that transgenic expression of MGP in $ApoE^{-/-}$ mice results in diminished Smad1/5/8 signaling and reduced inflammation, lesion formation, and calcification after fat feeding (115). On the other hand MGP deficient $ApoE^{-/-}$ mice displayed enhanced Smad1/5/8 signaling and extensive medial calcification (115). However, recent research showed that MGP can inhibit calcification in a BMP-2 independent manner in intact vessels and lack of GlaMGP (carboxylated MGP) was not the reason for medial calcification in rat renal failure model (141).

As mentioned earlier the inhibitory Smad6 interferes specifically with the BMP pathway. Interestingly, perturbation of Smad6 expression was found to be associated with calcification of the aortic valve. In human aortic valve (AV), high levels of BMP antagonists (noggin and CV-2/BMPER) and Smad6 were detected in the ventricular endothelium, while low levels of such inhibitors were found in the fibrosa endothelium. This uneven distribution was shown to be responsible for the side-dependent calcification of human AVs (142). In addition, mutations in the *Smad6* gene were found to predispose to congenital cardiovascular malformation. The capacity of Smad6 to inhibit BMP-induced osteogenic differentiation was significantly decreased by a C484F mutation in Smad6 (102). Thus, BMPs may be important in the pathology of vascular calcification, even though definitive evidence supporting this is still lacking.

1.4. Tumor angiogenesis

Tumor growth beyond 2-3 mm in size makes diffusion insufficient to supply tumor cells with oxygen and nutrients and for the removal of the waste products (143). Angiogenesis, i.e. the formation of new blood vessels from pre-existing ones, is then needed for the tumors to grow. In addition, blood vessels provide the main route for metastatic spread (143). Several inhibitors of angiogenesis, such as bevacizumab (monoclonal antibody targeting VEGF) and sorafenib and sunitinib (tyrosine kinase inhibitors) have been used for the treatment of solid tumors (144, 145).

BMPs have been found misexpressed in gastric, ovarian, prostate, pancreatic breast, lung and colon tumors (146-152). BMP-2 and BMP-4 were shown to favor angiogenesis by stimulating the secretion of pro-angiogenic growth factors, such as VEGF (52, 153). In the case of lung cancer, BMP-2 is highly expressed in the majority of patient-derived lung carcinomas (154) and recombinant BMP-2 potently increases the size and number of blood vessels in tumors formed by A549 cells in nude mice (155). Moreover, either recombinant noggin or an anti-BMP-2 antibody could inhibit the activity of BMP-2, resulting in a significant reduction in tumor growth (154). Besides BMP-2, other BMPs have also been reported to be involved in tumor angiogenesis. Rothhammer *et al.* showed that BMP-2 and BMP-4 are highly expressed in malignant melanomas, and they promoted cell invasion and migration of microvascular endothelial cells. Moreover, ECs have a reduced tube formation capacity when BMPs activities were inhibited (156). BMP antagonist chordin has been reported to inhibit *in vitro* BMP-4 induced tube formation in malignant melanoma cells (156).

ALK1, a type I receptor for TGF-β, BMP-9 and BMP-10 have received a lot of attention recently as an anti-angiogenesis target. A recent study indicated that ALK1 is widely expressed on prostate, skin, thyroid, kidney, ovary, lung, pancreas, and liver tumor blood vessels (157, 158). ALK1 is mainly expressed in developing arterial endothelial cells and is greatly reduced in adult arteries. However, ALK1 expression can be induced during tumor angiogenesis (158, 159). It has been suggested that ALK1 signaling and function in ECs may depend on multiple proangiogenic factors (including VEGF and bFGF), and BMP-9-induced (tumor) angiogenesis can be specifically inhibited by an ALK1 antibody (anti-ALK1) (157). Besides, anti-ALK1 can decrease tumor growth and angiogenesis when combined with VEGF receptor inhibitor in

human/mouse chimera tumor model (157). Other research described that a soluble chimeric protein (ALK1-Fc) which serves as BMP-9 (and -10) ligand trap, can inhibit (tumor) angiogenesis by interfering with ALK1 signaling both *in vitro* and *in vivo* (158, 160). Therefore, targeting ALK1 may effectively inhibit tumor angiogenesis and it is therefore a promising therapeutic strategy for cancer patients.

Endoglin plays a crucial role in EC function. Studies in mice revealed that tumor growth and angiogenesis is reduced in endoglin-haploinsufficient mice (161). In addition endoglin neutralizing antibodies have been used for vascular targeting and it was shown that they can inhibit both endothelial cell proliferation and tumor growth in mouse cancer models (25). It is known that a soluble form of endoglin (sol Eng) contributes to the pathogenesis of preeclampsia (25). Research showed that a fusion protein, which combined the endoglin extracellular domain (ECD) and immunoglobulin Fc domain, can significantly reduce VEGF induced angiogenesis *in vitro* and *ex vivo* (162), presumably by specifically binding to pro-angiogenic BMP-9 with a high affinity. These results suggest that endoglin-Fc may be used as a potential anti-angiogenesis therapeutic agent (163). Since the process of angiogenesis is tightly regulated by BMPs, a further understanding of their molecular mechanisms will provide opportunities for better diagnosis and development of new therapies targeting angiogenesis, tumor growth, and metastatic spread of disease.

Conclusions and perspective

BMP signaling plays a crucial role in cardiovascular homeostasis and disease. Genetic studies in mice indicate that components of BMP signaling are involved in EC and SMC interactions, EC function and angiogenesis. The knowledge regarding the role of BMP signaling in vascular diseases and cancer has mainly come from mouse models and clinical investigations. However, definitive evidences from functional studies in human tissues are still rare. Genetic mouse model studies showed that BMP function might depend on cell type and environment, but the availability of human tissues and the limited life span of patient-derived somatic cells limit the development of this research area. The use of induced pluripotent stem cells (iPSCs) technology could help to overcome these limitations (164-166). Generated iPSCs from human skin fibroblasts, keratinocytes, adipose stem cells and lymphocytes (167-169), can be

differentiated into various cell types (170), including ECs and SMCs (171). It is possible to utilize this new technology to generate ECs and SMCs from patients with vascular disorders (and from healthy volunteers) in order to investigate the pathology of vascular diseases and perhaps transplant cells to cure patients (172), or perform screens to identify small chemical compounds to rescue disease phenotypes. Of interest, the BMP receptor antagonist dorsomorphin and its more selective derivative LDN-193189 have recently been reported to inhibit BMP signaling (173, 174). Yu *et al.* found that dorsomorphin selectively inhibited the BMP type I receptors ALK2, ALK3 and ALK6 and blocked BMP-mediated SMAD1/5/8 phosphorylation (173). In addition, an optimized compound (LDN-193189 or DM-3189) with higher activity and specificity for BMP type I receptors has been developed from a structure-activity relationship study of dorsomorphin (174). The ongoing development of small molecule inhibitors/activators of BMP signaling will offer new opportunities for manipulating BMP signaling in therapeutic means. This will benefit future therapy of BMP related diseases caused by insufficient BMP signaling, such as PAH and overactive BMP signaling, such as tumor angiogenesis and FOP (175).

Acknowledgements

We are grateful to Miriam de Boeck, Jose Maring and Beerend P. Hierck for critical reading the manuscript and provided valuable comments and suggestions. This work was supported by LeDucq foundation, the Netherlands Organization for Scientific Research and the Centre for Biomedical Genetics, KNAW and the ‘Innovative Medizinische Forschung’ (IMF).

Part II: BMP signaling in fibrodysplasia ossificans progressiva (FOP)

During embryonic development there are two mechanisms for creating bone tissues: endochondral ossification and intramembranous ossification (176). Bone undergoes constant remodeling by osteoclasts that degrade and by osteoblasts that form bone. This dynamic process is highly regulated by many regulators, especially by BMPs. For instance, BMP2 and BMP4 induce bone

and cartilage formation by stimulating osteoblast and chondrocyte differentiation.

The knowledge about this critical role of the BMP signaling pathway in bone and cartilage formation was initially mainly obtained from transgenic animal models. Overexpression of the negative BMP regulator Noggin in transgenic mice resulted in severe defects in cartilaginous components (177). Knockout of *Bmp2* in chondrocytes showed defects in chondrocyte phenotypes (178), while overexpression of *Bmp4* in the skeleton leaded to an increase of cartilage production and enhanced chondrocyte differentiation in mice (177). In addition, BMP receptors regulate bone formation (179). Furthermore, the downstream Smad pathway is involved in bone development; for instance, osteoblast-specific Smad1 knockout mice showed impaired osteoblast proliferation and differentiation (180). In addition, multiple human diseases with skeletal defects have been linked to mutations in BMP signaling components.

Clarifying the role that the BMP signaling pathway plays in bone and cartilage formation helps us to understand the pathologies of BMP related bone diseases. Fibrodysplasia ossificans progressiva (FOP) is a rare disease known by its progressive heterotopic ossification (HO) in soft tissues, caused by gain-of-function mutations in ALK2 (181). In the first decade of life, most FOP patients develop painful and highly inflammatory soft tissue swellings, which transform the soft tissues into bone through endochondral ossification processes (182, 183). Most FOP patients have an R206H mutation in the GS domain of ALK2. The R206H mutation was shown to interferes with the binding of the negative regulator FKBP12 to ALK2 and leads to the leakage of BMP signaling in the absence of BMP ligands (184). The prevalence of FOP is about 1 in 2 million. FOP patients appear normal at birth apart from malformation of the great toe (182).

There is currently no cure for FOP. Surgical removal of the ectopic bone tissue is risky as the surgical trauma might induce the formation of new heterotopic bone. The recurrent mutations in ALK2 may provide a specific target to prevent HO in FOP patients. LDN-193189, a BMP type I receptor kinase inhibitor, was reported to reduce ectopic ossification in transgenic mice carrying an inducible constitutively active $ALK2^{Q207D}$ gene (185). However, although LDN-193189 is a potent inhibitor of BMP signaling at higher dosages, it inhibits TGF-β signaling as well. The newer ALK2 inhibitor LDN-212854

showed selective inhibition towards the ALK2 receptor and had comparable inhibitory effects *in vivo* as LDN-193189 (186). Strategies to block ALK2 activity by genetic tools, including antisense therapy and RNA interference were also reported (187-189). Identification of new therapeutic tools for FOP could also be useful for other situations, for instance, it may help to cure of nongenetic forms of HO which occur after deep burning or hip arthroplasty.

Part III: Aims and outline of this thesis

BMP signaling has been implicated in an enormous plethora of biological activities during embryonic development and in adult tissue homeostasis. Disruption of the BMP signaling pathway has been linked to various human diseases. In this thesis, two BMP related genetic diseases, FOP and PAH are studied. The main purpose of this thesis is to clarify the (dys)-regulation of BMP signaling in the disease context which may help to develop novel therapeutic approaches for these diseases. Furthermore, research on rare diseases like FOP might provide basic knowledge that can be used for the treatment of more common diseases, such as osteoporosis and non-junction fractures.

The first part of this thesis is predominantly about BMP signaling in FOP. A human iPSC model for FOP is introduced in **chapter 2**. Previous research on FOP was mainly conducted in murine cell lines; a human cell system was therefore expected to be more suitable for preclinical FOP studies. FOP iPSCs could recreate the disease phenotypes by differentiating into FOP bone-forming progenitors, ECs and pericytes. The approach to rescue the osteoblast differentiation phenotypes in FOP iPSCs derived cells might be used for drug development for FOP.

This thesis also presents a novel therapeutic approach for FOP. BMP receptor ALK2 antisense-oligonucleotide (AON)-mediated exon skipping was introduced in ECs and other cell types. The AON targeting the wild-type exon of ALK2 was found to downregulate *Alk2* expression and represses BMP6-induced osteoblast differentiation (**chapter 3**).

The second part of this thesis analyses rescue of the insufficient BMP signaling in PAH by the US food and drug administration (FDA) approved drug FK506. Combined targeting of the BMP signaling pathway and inhibition of local inflammation could improve treatment of PAH. FK506 can induce

BMPR2 signaling both by acting as an inhibitor of the phosphatase calcineurin and by inhibiting the binding of the BMP signaling inhibitor FKBP12 to the BMP receptor. Importantly, FK506 can rescue the dysfunctional EC signaling and gene regulation in experimental PAH animal models to prevent and reverse PAH **(chapter 4)**.

In **chapter 5**, we demonstrate that soluble endoglin regulates BMP9 signaling through TGFβR2 and/or BMPR2. This regulation of BMP9 signaling by soluble endoglin provides another layer of regulation of TGF-β signaling pathway in ECs. It may also alter the inflammatory responses of ECs in different cellular contexts.

Finally, the main findings reported in this thesis are summarized and discussed in **chapter 6.**

References

1. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, and Wang EA. Novel regulators of bone formation: molecular clones and activities. *Science.* 1988;242(4885):1528-34.
2. Urist MR. Bone: formation by autoinduction. *Science.* 1965;150(3698):893-9.
3. Massague J, and Chen YG. Controlling TGF-beta signaling. *Genes Dev.* 2000;14(6):627-44.
4. Miyazono K, Kamiya Y, and Morikawa M. Bone morphogenetic protein receptors and signal transduction. *J Biochem.* 2010;147(1):35-51.
5. Kawabata M, Imamura T, and Miyazono K. Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev.* 1998;9(1):49-61.
6. Nelsen SM, and Christian JL. Site-specific cleavage of BMP4 by furin, PC6, and PC7. *J Biol Chem.* 2009;284(40):27157-66.
7. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, and Nohe A. Bone morphogenetic proteins: a critical review. *Cellular signalling.* 2011;23(4):609-20.
8. Annes JP, Munger JS, and Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci.* 2003;116(Pt 2):217-24.
9. Canalis E, Economides AN, and Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr Rev.* 2003;24(2):218-35.
10. Avsian-Kretchmer O, and Hsueh AJ. Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein antagonists. *Mol Endocrinol.* 2004;18(1):1-12.
11. Moser M, Binder O, Wu Y, Aitsebaomo J, Ren R, Bode C, Bautch VL, Conlon FL, and Patterson C. BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation. *Mol Cell Biol.* 2003;23(16):5664-79.
12. Zebboudj AF, Imura M, and Bostrom K. Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2. *J Biol Chem.* 2002;277(6):4388-94.

13. Zhou Z, Xie J, Lee D, Liu Y, Jung J, Zhou L, Xiong S, Mei L, and Xiong WC. Neogenin regulation of BMP-induced canonical Smad signaling and endochondral bone formation. *Dev Cell.* 2010;19(1):90-102.
14. Hagihara M, Endo M, Hata K, Higuchi C, Takaoka K, Yoshikawa H, and Yamashita T. Neogenin, a receptor for bone morphogenetic proteins. *J Biol Chem.* 2011;286(7):5157-65.
15. Wallin R, Cain D, Hutson SM, Sane DC, and Loeser R. Modulation of the binding of matrix Gla protein (MGP) to bone morphogenetic protein-2 (BMP-2). *Thromb Haemost.* 2000;84(6):1039-44.
16. Yao Y, Shahbazian A, and Bostrom KI. Proline and gamma-carboxylated glutamate residues in matrix Gla protein are critical for binding of bone morphogenetic protein-4. *Circ Res.* 2008;102(9):1065-74.
17. Yao Y, Nowak S, Yochelis A, Garfinkel A, and Bostrom KI. Matrix GLA protein, an inhibitory morphogen in pulmonary vascular development. *J Biol Chem.* 2007;282(41):30131-42.
18. Cole SJ, Bradford D, and Cooper HM. Neogenin: A multi-functional receptor regulating diverse developmental processes. *Int J Biochem Cell Biol.* 2007;39(9):1569-75.
19. De Vries M, and Cooper HM. Emerging roles for neogenin and its ligands in CNS development. *J Neurochem.* 2008;106(4):1483-92.
20. Walsh DW, Godson C, Brazil DP, and Martin F. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. *Trends Cell Biol.* 2010;20(5):244-56.
21. Heldin CH, Miyazono K, and ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature.* 1997;390(6659):465-71.
22. Derynck R, and Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature.* 2003;425(6958):577-84.
23. Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, and Miyazono K. Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proc Natl Acad Sci U S A.* 1995;92(17):7632-6.
24. Moustakas A, and Heldin CH. The regulation of TGFbeta signal transduction. *Development.* 2009;136(22):3699-714.
25. ten Dijke P, Goumans MJ, and Pardali E. Endoglin in angiogenesis and vascular diseases. *Angiogenesis.* 2008;11(1):79-89.
26. Kirkbride KC, Townsend TA, Bruinsma MW, Barnett JV, and Blobe GC. Bone morphogenetic proteins signal through the transforming growth factor-beta type III receptor. *J Biol Chem.* 2008;283(12):7628-37.
27. David L, Mallet C, Mazerbourg S, Feige JJ, and Bailly S. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood.* 2007;109(5):1953-61.
28. Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massague J, and Niehrs C. Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature.* 1999;401(6752):480-5.
29. Babitt JL, Zhang Y, Samad TA, Xia Y, Tang J, Campagna JA, Schneyer AL, Woolf CJ, and Lin HY. Repulsive guidance molecule (RGMa), a DRAGON homologue, is a bone morphogenetic protein co-receptor. *J Biol Chem.* 2005;280(33):29820-7.

30. Samad TA, Rebbapragada A, Bell E, Zhang Y, Sidis Y, Jeong SJ, Campagna JA, Perusini S, Fabrizio DA, Schneyer AL, et al. DRAGON, a bone morphogenetic protein co-receptor. *J Biol Chem.* 2005;280(14):14122-9.
31. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet.* 2006;38(5):531-9.
32. Andriopoulos B, Jr., Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, et al. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet.* 2009;41(4):482-7.
33. Corradini E, Babitt JL, and Lin HY. The RGM/DRAGON family of BMP co-receptors. *Cytokine Growth Factor Rev.* 2009;20(5-6):389-98.
34. Ross S, and Hill CS. How the Smads regulate transcription. *Int J Biochem Cell Biol.* 2008;40(3):383-408.
35. Zhu H, Kavsak P, Abdollah S, Wrana JL, and Thomsen GH. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature.* 1999;400(6745):687-93.
36. Ogunjimi AA, Briant DJ, Pece-Barbara N, Le Roy C, Di Guglielmo GM, Kavsak P, Rasmussen RK, Seet BT, Sicheri F, and Wrana JL. Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. *Mol Cell.* 2005;19(3):297-308.
37. Murakami G, Watabe T, Takaoka K, Miyazono K, and Imamura T. Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. *Mol Biol Cell.* 2003;14(7):2809-17.
38. Zhang YE. Non-Smad pathways in TGF-beta signaling. *Cell Res.* 2009;19(1):128-39.
39. Massague J, and Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J.* 2000;19(8):1745-54.
40. Mulder KM. Role of Ras and Mapks in TGFbeta signaling. *Cytokine Growth Factor Rev.* 2000;11(1-2):23-35.
41. Carmeliet P, and Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature.* 2011;473(7347):298-307.
42. Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature.* 2007;445(7129):776-80.
43. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol.* 2003;161(6):1163-77.
44. Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, and Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev.* 2002;16(20):2684-98.
45. Conway EM, Collen D, and Carmeliet P. Molecular mechanisms of blood vessel growth. *Cardiovasc Res.* 2001;49(3):507-21.
46. Pardali E, van der Schaft DW, Wiercinska E, Gorter A, Hogendoorn PC, Griffioen AW, and ten Dijke P. Critical role of endoglin in tumor cell plasticity of Ewing sarcoma and melanoma. *Oncogene.* 2011;30(3):334-45.

47. Goumans MJ, and Mummery C. Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *Int J Dev Biol.* 2000;44(3):253-65.
48. Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, et al. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature.* 1999;401(6754):670-7.
49. David L, Feige JJ, and Bailly S. Emerging role of bone morphogenetic proteins in angiogenesis. *Cytokine Growth Factor Rev.* 2009;20(3):203-12.
50. Pardali E, and Ten Dijke P. TGFbeta signaling and cardiovascular diseases. *Int J Biol Sci.* 2012;8(2):195-213.
51. Valdimarsdottir G, Goumans MJ, Rosendahl A, Brugman M, Itoh S, Lebrin F, Sideras P, and ten Dijke P. Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. *Circulation.* 2002;106(17):2263-70.
52. Deckers MM, van Bezooijen RL, van der Horst G, Hoogendam J, van Der Bent C, Papapoulos SE, and Lowik CW. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology.* 2002;143(4):1545-53.
53. Suzuki Y, Ohga N, Morishita Y, Hida K, Miyazono K, and Watabe T. BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. *Journal of cell science.* 2010;123(Pt 10):1684-92.
54. Heinke J, Wehofsits L, Zhou Q, Zoeller C, Baar KM, Helbing T, Laib A, Augustin H, Bode C, Patterson C, et al. BMPER is an endothelial cell regulator and controls bone morphogenetic protein-4-dependent angiogenesis. *Circ Res.* 2008;103(8):804-12.
55. Yao Y, Jumabay M, Wang A, and Bostrom KI. Matrix Gla protein deficiency causes arteriovenous malformations in mice. *J Clin Invest.* 2011;121(8):2993-3004.
56. Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP, Hamamori Y, Kedes L, Kato M, and ten Dijke Pt P. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *EMBO J.* 2004;23(3):541-51.
57. Moya IM, Umans L, Maas E, Pereira PN, Beets K, Francis A, Sents W, Robertson EJ, Mummery CL, Huylebroeck D, et al. Stalk cell phenotype depends on integration of notch and smad1/5 signaling cascades. *Dev Cell.* 2012;22(3):501-14.
58. Larrivee B, Prahst C, Gordon E, Del Toro R, Mathivet T, Duarte A, Simons M, and Eichmann A. ALK1 Signaling Inhibits Angiogenesis by Cooperating with the Notch Pathway. *Dev Cell.* 2012;22(3):489-500.
59. Dorai H, Vukicevic S, and Sampath TK. Bone morphogenetic protein-7 (osteogenic protein-1) inhibits smooth muscle cell proliferation and stimulates the expression of markers that are characteristic of SMC phenotype in vitro. *J Cell Physiol.* 2000;184(1):37-45.
60. Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK, and Trembath RC. Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation.* 2001;104(7):790-5.
61. Nakaoka T, Gonda K, Ogita T, Otawara-Hamamoto Y, Okabe F, Kira Y, Harii K, Miyazono K, Takuwa Y, and Fujita T. Inhibition of rat vascular smooth muscle

proliferation in vitro and in vivo by bone morphogenetic protein-2. *J Clin Invest.* 1997;100(11):2824-32.
62. Dorai H, and Sampath TK. Bone morphogenetic protein-7 modulates genes that maintain the vascular smooth muscle cell phenotype in culture. *J Bone Joint Surg Am.* 2001;83-A Suppl 1(Pt 1):S70-8.
63. Frank DB, Abtahi A, Yamaguchi DJ, Manning S, Shyr Y, Pozzi A, Baldwin HS, Johnson JE, and de Caestecker MP. Bone morphogenetic protein 4 promotes pulmonary vascular remodeling in hypoxic pulmonary hypertension. *Circ Res.* 2005;97(5):496-504.
64. Yang X, Long L, Southwood M, Rudarakanchana N, Upton PD, Jeffery TK, Atkinson C, Chen H, Trembath RC, and Morrell NW. Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circ Res.* 2005;96(10):1053-63.
65. Lowery JW, and de Caestecker MP. BMP signaling in vascular development and disease. *Cytokine Growth Factor Rev.* 2010;21(4):287-98.
66. Farber HW, and Loscalzo J. Pulmonary arterial hypertension. *N Engl J Med.* 2004;351(16):1655-65.
67. Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, 3rd, Loyd JE, Nichols WC, and Trembath RC. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet.* 2000;26(1):81-4.
68. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet.* 2000;67(3):737-44.
69. West J, Harral J, Lane K, Deng Y, Ickes B, Crona D, Albu S, Stewart D, and Fagan K. Mice expressing BMPR2R899X transgene in smooth muscle develop pulmonary vascular lesions. *Am J Physiol Lung Cell Mol Physiol.* 2008;295(5):L744-55.
70. Hong KH, Lee YJ, Lee E, Park SO, Han C, Beppu H, Li E, Raizada MK, Bloch KD, and Oh SP. Genetic ablation of the BMPR2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension. *Circulation.* 2008;118(7):722-30.
71. Song Y, Coleman L, Shi J, Beppu H, Sato K, Walsh K, Loscalzo J, and Zhang YY. Inflammation, endothelial injury, and persistent pulmonary hypertension in heterozygous BMPR2-mutant mice. *Am J Physiol Heart Circ Physiol.* 2008;295(2):H677-90.
72. Yu PB, Beppu H, Kawai N, Li E, and Bloch KD. Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. *The Journal of biological chemistry.* 2005;280(26):24443-50.
73. Dewachter L, Adnot S, Guignabert C, Tu L, Marcos E, Fadel E, Humbert M, Dartevelle P, Simonneau G, Naeije R, et al. Bone morphogenetic protein signalling in heritable versus idiopathic pulmonary hypertension. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology.* 2009;34(5):1100-10.
74. Rudarakanchana N, Flanagan JA, Chen H, Upton PD, Machado R, Patel D, Trembath RC, and Morrell NW. Functional analysis of bone morphogenetic protein

type II receptor mutations underlying primary pulmonary hypertension. *Human molecular genetics.* 2002;11(13):1517-25.
75. Gangopahyay A, Oran M, Bauer EM, Wertz JW, Comhair SA, Erzurum SC, and Bauer PM. Bone morphogenetic protein receptor II is a novel mediator of endothelial nitric-oxide synthase activation. *J Biol Chem.* 2011;286(38):33134-40.
76. Shintani M, Yagi H, Nakayama T, Saji T, and Matsuoka R. A new nonsense mutation of SMAD8 associated with pulmonary arterial hypertension. *Journal of medical genetics.* 2009;46(5):331-7.
77. Huang Z, Wang D, Ihida-Stansbury K, Jones PL, and Martin JF. Defective pulmonary vascular remodeling in Smad8 mutant mice. *Hum Mol Genet.* 2009;18(15):2791-801.
78. Drake KM, Zygmunt D, Mavrakis L, Harbor P, Wang L, Comhair SA, Erzurum SC, and Aldred MA. Altered MicroRNA processing in heritable pulmonary arterial hypertension: an important role for Smad-8. *Am J Respir Crit Care Med.* 2011;184(12):1400-8.
79. Fujiwara M, Yagi H, Matsuoka R, Akimoto K, Furutani M, Imamura S, Uehara R, Nakayama T, Takao A, Nakazawa M, et al. Implications of mutations of activin receptor-like kinase 1 gene (ALK1) in addition to bone morphogenetic protein receptor II gene (BMPR2) in children with pulmonary arterial hypertension. *Circ J.* 2008;72(1):127-33.
80. Trembath RC. Mutations in the TGF-beta type 1 receptor, ALK1, in combined primary pulmonary hypertension and hereditary haemorrhagic telangiectasia, implies pathway specificity. *J Heart Lung Transplant.* 2001;20(2):175.
81. Jerkic M, Kabir MG, Davies A, Yu LX, McIntyre BA, Husain NW, Enomoto M, Sotov V, Husain M, Henkelman M, et al. Pulmonary hypertension in adult Alk1 heterozygous mice due to oxidative stress. *Cardiovascular research.* 2011;92(3):375-84.
82. Zhang H, and Bradley A. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development.* 1996;122(10):2977-86.
83. Anderson L, Lowery JW, Frank DB, Novitskaya T, Jones M, Mortlock DP, Chandler RL, and de Caestecker MP. Bmp2 and Bmp4 exert opposing effects in hypoxic pulmonary hypertension. *Am J Physiol Regul Integr Comp Physiol.* 2010;298(3):R833-42.
84. Beppu H, Ichinose F, Kawai N, Jones RC, Yu PB, Zapol WM, Miyazono K, Li E, and Bloch KD. BMPR-II heterozygous mice have mild pulmonary hypertension and an impaired pulmonary vascular remodeling response to prolonged hypoxia. *Am J Physiol Lung Cell Mol Physiol.* 2004;287(6):L1241-7.
85. Song Y, Jones JE, Beppu H, Keaney JF, Jr., Loscalzo J, and Zhang YY. Increased susceptibility to pulmonary hypertension in heterozygous BMPR2-mutant mice. *Circulation.* 2005;112(4):553-62.
86. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, et al. 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(6):2626-31.
87. Urness LD, Sorensen LK, and Li DY. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet.* 2000;26(3):328-31.

88. Srinivasan S, Hanes MA, Dickens T, Porteous ME, Oh SP, Hale LP, and Marchuk DA. A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. *Hum Mol Genet.* 2003;12(5):473-82.
89. Park SO, Lee YJ, Seki T, Hong KH, Fliess N, Jiang Z, Park A, Wu X, Kaartinen V, Roman BL, et al. ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2. *Blood.* 2008;111(2):633-42.
90. El-Bizri N, Wang L, Merklinger SL, Guignabert C, Desai T, Urashima T, Sheikh AY, Knutsen RH, Mecham RP, Mishina Y, et al. Smooth muscle protein 22alpha-mediated patchy deletion of Bmpr1a impairs cardiac contractility but protects against pulmonary vascular remodeling. *Circ Res.* 2008;102(3):380-8.
91. El-Bizri N, Guignabert C, Wang L, Cheng A, Stankunas K, Chang CP, Mishina Y, and Rabinovitch M. SM22alpha-targeted deletion of bone morphogenetic protein receptor 1A in mice impairs cardiac and vascular development, and influences organogenesis. *Development.* 2008;135(17):2981-91.
92. Park C, Lavine K, Mishina Y, Deng CX, Ornitz DM, and Choi K. Bone morphogenetic protein receptor 1A signaling is dispensable for hematopoietic development but essential for vessel and atrioventricular endocardial cushion formation. *Development.* 2006;133(17):3473-84.
93. Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, et al. Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Developmental biology.* 2000;217(1):42-53.
94. Mahmoud M, Allinson KR, Zhai Z, Oakenfull R, Ghandi P, Adams RH, Fruttiger M, and Arthur HM. Pathogenesis of arteriovenous malformations in the absence of endoglin. *Circ Res.* 2010;106(8):1425-33.
95. Lechleider RJ, Ryan JL, Garrett L, Eng C, Deng C, Wynshaw-Boris A, and Roberts AB. Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. *Dev Biol.* 2001;240(1):157-67.
96. Gallione C, Aylsworth AS, Beis J, Berk T, Bernhardt B, Clark RD, Clericuzio C, Danesino C, Drautz J, Fahl J, et al. Overlapping spectra of SMAD4 mutations in juvenile polyposis (JP) and JP-HHT syndrome. *Am J Med Genet A.* 2010;152A(2):333-9.
97. Gallione CJ, Richards JA, Letteboer TG, Rushlow D, Prigoda NL, Leedom TP, Ganguly A, Castells A, Ploos van Amstel JK, Westermann CJ, et al. SMAD4 mutations found in unselected HHT patients. *J Med Genet.* 2006;43(10):793-7.
98. Lan Y, Liu B, Yao H, Li F, Weng T, Yang G, Li W, Cheng X, Mao N, and Yang X. Essential role of endothelial Smad4 in vascular remodeling and integrity. *Mol Cell Biol.* 2007;27(21):7683-92.
99. Yang X, Castilla LH, Xu X, Li C, Gotay J, Weinstein M, Liu PP, and Deng CX. Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development.* 1999;126(8):1571-80.
100. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, and Zwijsen A. Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development.* 1999;126(8):1631-42.
101. Galvin KM, Donovan MJ, Lynch CA, Meyer RI, Paul RJ, Lorenz JN, Fairchild-Huntress V, Dixon KL, Dunmore JH, Gimbrone MA, Jr., et al. A role for

smad6 in development and homeostasis of the cardiovascular system. *Nat Genet.* 2000;24(2):171-4.
102. Tan HL, Glen E, Topf A, Hall D, O'Sullivan JJ, Sneddon L, Wren C, Avery P, Lewis RJ, Ten Dijke P, et al. Non-synonymous variants in the SMAD6 gene predispose to congenital cardiovascular malformation. *Hum Mutat.* 2012.
103. Chen Q, Chen H, Zheng D, Kuang C, Fang H, Zou B, Zhu W, Bu G, Jin T, Wang Z, et al. Smad7 is required for the development and function of the heart. *The Journal of biological chemistry.* 2009;284(1):292-300.
104. McDonald J, Bayrak-Toydemir P, and Pyeritz RE. Hereditary hemorrhagic telangiectasia: an overview of diagnosis, management, and pathogenesis. *Genet Med.* 2011;13(7):607-16.
105. 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. *Nature genetics.* 1994;8(4):345-51.
106. Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, et al. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet.* 1996;13(2):189-95.
107. Gu Y, Jin P, Zhang L, Zhao X, Gao X, Ning Y, Meng A, and Chen YG. Functional analysis of mutations in the kinase domain of the TGF-beta receptor ALK1 reveals different mechanisms for induction of hereditary hemorrhagic telangiectasia. *Blood.* 2006;107(5):1951-4.
108. Abdalla SA, and Letarte M. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. *J Med Genet.* 2006;43(2):97-110.
109. Ricard N, Bidart M, Mallet C, Lesca G, Giraud S, Prudent R, Feige JJ, and Bailly S. Functional analysis of the BMP9 response of ALK1 mutants from HHT2 patients: a diagnostic tool for novel ACVRL1 mutations. *Blood.* 2010;116(9):1604-12.
110. Gridley T. Notch signaling in vascular development and physiology. *Development.* 2007;134(15):2709-18.
111. Morikawa M, Koinuma D, Tsutsumi S, Vasilaki E, Kanki Y, Heldin CH, Aburatani H, and Miyazono K. ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. *Nucleic Acids Res.* 2011;39(20):8712-27.
112. Benzinou M, Clermont FF, Letteboer TG, Kim JH, Espejel S, Harradine KA, Arbelaez J, Luu MT, Roy R, Quigley D, et al. Mouse and human strategies identify PTPN14 as a modifier of angiogenesis and hereditary haemorrhagic telangiectasia. *Nat Commun.* 2012;3(616.
113. Nakagawa Y, Ikeda K, Akakabe Y, Koide M, Uraoka M, Yutaka KT, Kurimoto-Nakano R, Takahashi T, Matoba S, Yamada H, et al. Paracrine osteogenic signals via bone morphogenetic protein-2 accelerate the atherosclerotic intimal calcification in vivo. *Arterioscler Thromb Vasc Biol.* 2010;30(10):1908-15.
114. Pachori AS, Custer L, Hansen D, Clapp S, Kemppa E, and Klingensmith J. Bone morphogenetic protein 4 mediates myocardial ischemic injury through JNK-dependent signaling pathway. *J Mol Cell Cardiol.* 2010;48(6):1255-65.
115. Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ, and Bostrom KI. Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. *Circ Res.* 2010;107(4):485-94.

116. Pannu H, Fadulu VT, Chang J, Lafont A, Hasham SN, Sparks E, Giampietro PF, Zaleski C, Estrera AL, Safi HJ, et al. Mutations in transforming growth factor-beta receptor type II cause familial thoracic aortic aneurysms and dissections. *Circulation.* 2005;112(4):513-20.
117. Loeys BL, Schwarze U, Holm T, Callewaert BL, Thomas GH, Pannu H, De Backer JF, Oswald GL, Symoens S, Manouvrier S, et al. Aneurysm syndromes caused by mutations in the TGF-beta receptor. *N Engl J Med.* 2006;355(8):788-98.
118. Jones JA, Barbour JR, Stroud RE, Bouges S, Stephens SL, Spinale FG, and Ikonomidis JS. Altered transforming growth factor-beta signaling in a murine model of thoracic aortic aneurysm. *J Vasc Res.* 2008;45(6):457-68.
119. Johnson RC, Leopold JA, and Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. *Circ Res.* 2006;99(10):1044-59.
120. Hruska KA, Mathew S, and Saab G. Bone morphogenetic proteins in vascular calcification. *Circ Res.* 2005;97(2):105-14.
121. Speer MY, Yang HY, Brabb T, Leaf E, Look A, Lin WL, Frutkin A, Dichek D, and Giachelli CM. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. *Circ Res.* 2009;104(6):733-41.
122. Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, Kawakami Y, and Ikeda Y. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. *J Leukoc Biol.* 2003;74(5):833-45.
123. Collett G, Wood A, Alexander MY, Varnum BC, Boot-Handford RP, Ohanian V, Ohanian J, Fridell YW, and Canfield AE. Receptor tyrosine kinase Axl modulates the osteogenic differentiation of pericytes. *Circ Res.* 2003;92(10):1123-9.
124. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, and Kaneda Y. Bone marrow-derived osteoblast progenitor cells in circulating blood contribute to ectopic bone formation in mice. *Biochem Biophys Res Commun.* 2007;354(2):453-8.
125. Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, and Olsen BR. Conversion of vascular endothelial cells into multipotent stem-like cells. *Nature medicine.* 2010;16(12):1400-6.
126. Lipton BH, Bensch KG, and Karasek MA. Histamine-modulated transdifferentiation of dermal microvascular endothelial cells. *Exp Cell Res.* 1992;199(2):279-91.
127. Azhar M, Runyan RB, Gard C, Sanford LP, Miller ML, Andringa A, Pawlowski S, Rajan S, and Doetschman T. Ligand-specific function of transforming growth factor beta in epithelial-mesenchymal transition in heart development. *Dev Dyn.* 2009;238(2):431-42.
128. Romero LI, Zhang DN, Herron GS, and Karasek MA. Interleukin-1 induces major phenotypic changes in human skin microvascular endothelial cells. *J Cell Physiol.* 1997;173(1):84-92.
129. Shore EM, and Kaplan FS. Insights from a rare genetic disorder of extra-skeletal bone formation, fibrodysplasia ossificans progressiva (FOP). *Bone.* 2008;43(3):427-33.
130. Schluesener HJ, and Meyermann R. Immunolocalization of BMP-6, a novel TGF-beta-related cytokine, in normal and atherosclerotic smooth muscle cells. *Atherosclerosis.* 1995;113(2):153-6.

131. Yao Y, Watson AD, Ji S, and Bostrom KI. Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix Gla protein. *Circ Res.* 2009;105(6):575-84.
132. Bostrom K, Watson KE, Horn S, Wortham C, Herman IM, and Demer LL. Bone morphogenetic protein expression in human atherosclerotic lesions. *J Clin Invest.* 1993;91(4):1800-9.
133. Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, Tordoir JH, Spronk HM, Vermeer C, and Daemen MJ. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol.* 2001;21(12):1998-2003.
134. Griethe W, Schmitt R, Jurgensen JS, Bachmann S, Eckardt KU, and Schindler R. Bone morphogenic protein-4 expression in vascular lesions of calciphylaxis. *J Nephrol.* 2003;16(5):728-32.
135. Hayashi K, Nakamura S, Nishida W, and Sobue K. Bone morphogenetic protein-induced MSX1 and MSX2 inhibit myocardin-dependent smooth muscle gene transcription. *Molecular and cellular biology.* 2006;26(24):9456-70.
136. Shioi A, Katagi M, Okuno Y, Mori K, Jono S, Koyama H, and Nishizawa Y. Induction of bone-type alkaline phosphatase in human vascular smooth muscle cells: roles of tumor necrosis factor-alpha and oncostatin M derived from macrophages. *Circulation research.* 2002;91(1):9-16.
137. Tintut Y, Abedin M, Cho J, Choe A, Lim J, and Demer LL. Regulation of RANKL-induced osteoclastic differentiation by vascular cells. *Journal of molecular and cellular cardiology.* 2005;39(2):389-93.
138. Cheng SL, Shao JS, Charlton-Kachigian N, Loewy AP, and Towler DA. MSX2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors. *J Biol Chem.* 2003;278(46):45969-77.
139. Liberman M, Johnson RC, Handy DE, Loscalzo J, and Leopold JA. Bone morphogenetic protein-2 activates NADPH oxidase to increase endoplasmic reticulum stress and human coronary artery smooth muscle cell calcification. *Biochem Biophys Res Commun.* 2011;413(3):436-41.
140. Bostrom K, Tsao D, Shen S, Wang Y, and Demer LL. Matrix GLA protein modulates differentiation induced by bone morphogenetic protein-2 in C3H10T1/2 cells. *J Biol Chem.* 2001;276(17):14044-52.
141. Lomashvili KA, Wang X, Wallin R, and O'Neill WC. Matrix Gla protein metabolism in vascular smooth muscle and role in uremic vascular calcification. *J Biol Chem.* 2011;286(33):28715-22.
142. Ankeny RF, Thourani VH, Weiss D, Vega JD, Taylor WR, Nerem RM, and Jo H. Preferential activation of SMAD1/5/8 on the fibrosa endothelium in calcified human aortic valves--association with low BMP antagonists and SMAD6. *PLoS One.* 2011;6(6):e20969.
143. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med.* 1971;285(21):1182-6.
144. Hayes DF. Bevacizumab treatment for solid tumors: boon or bust? *JAMA.* 2011;305(5):506-8.
145. Gotink KJ, and Verheul HM. Anti-angiogenic tyrosine kinase inhibitors: what is their mechanism of action? *Angiogenesis.* 2010;13(1):1-14.

146. Hatakeyama S, Ohara-Nemoto Y, Kyakumoto S, and Satoh M. Expression of bone morphogenetic protein in human adenocarcinoma cell line. *Biochem Biophys Res Commun.* 1993;190(3):695-701.
147. Hatakeyama S, Gao YH, Ohara-Nemoto Y, Kataoka H, and Satoh M. Expression of bone morphogenetic proteins of human neoplastic epithelial cells. *Biochem Mol Biol Int.* 1997;42(3):497-505.
148. Ide H, Yoshida T, Matsumoto N, Aoki K, Osada Y, Sugimura T, and Terada M. Growth regulation of human prostate cancer cells by bone morphogenetic protein-2. *Cancer Res.* 1997;57(22):5022-7.
149. Kiyozuka Y, Nakagawa H, Senzaki H, Uemura Y, Adachi S, Teramoto Y, Matsuyama T, Bessho K, and Tsubura A. Bone morphogenetic protein-2 and type IV collagen expression in psammoma body forming ovarian cancer. *Anticancer Res.* 2001;21(3B):1723-30.
150. Kleeff J, Maruyama H, Ishiwata T, Sawhney H, Friess H, Buchler MW, and Korc M. Bone morphogenetic protein 2 exerts diverse effects on cell growth in vitro and is expressed in human pancreatic cancer in vivo. *Gastroenterology.* 1999;116(5):1202-16.
151. Deng H, Makizumi R, Ravikumar TS, Dong H, Yang W, and Yang WL. Bone morphogenetic protein-4 is overexpressed in colonic adenocarcinomas and promotes migration and invasion of HCT116 cells. *Exp Cell Res.* 2007;313(5):1033-44.
152. Bieniasz M, Oszajca K, Eusebio M, Kordiak J, Bartkowiak J, and Szemraj J. The positive correlation between gene expression of the two angiogenic factors: VEGF and BMP-2 in lung cancer patients. *Lung Cancer.* 2009;66(3):319-26.
153. Kozawa O, Matsuno H, and Uematsu T. Involvement of p70 S6 kinase in bone morphogenetic protein signaling: vascular endothelial growth factor synthesis by bone morphogenetic protein-4 in osteoblasts. *J Cell Biochem.* 2001;81(3):430-6.
154. Langenfeld EM, Calvano SE, Abou-Nukta F, Lowry SF, Amenta P, and Langenfeld J. The mature bone morphogenetic protein-2 is aberrantly expressed in non-small cell lung carcinomas and stimulates tumor growth of A549 cells. *Carcinogenesis.* 2003;24(9):1445-54.
155. Langenfeld EM, and Langenfeld J. Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors. *Mol Cancer Res.* 2004;2(3):141-9.
156. Rothhammer T, Bataille F, Spruss T, Eissner G, and Bosserhoff AK. Functional implication of BMP4 expression on angiogenesis in malignant melanoma. *Oncogene.* 2007;26(28):4158-70.
157. Hu-Lowe DD, Chen E, Zhang L, Watson KD, Mancuso P, Lappin P, Wickman G, Chen JH, Wang J, Jiang X, et al. Targeting activin receptor-like kinase 1 inhibits angiogenesis and tumorigenesis through a mechanism of action complementary to anti-VEGF therapies. *Cancer Res.* 2011;71(4):1362-73.
158. Cunha SI, Pardali E, Thorikay M, Anderberg C, Hawinkels L, Goumans MJ, Seehra J, Heldin CH, ten Dijke P, and Pietras K. Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *The Journal of experimental medicine.* 2010;207(1):85-100.
159. Seki T, Yun J, and Oh SP. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ Res.* 2003;93(7):682-9.

160. Mitchell D, Pobre EG, Mulivor AW, Grinberg AV, Castonguay R, Monnell TE, Solban N, Ucran JA, Pearsall RS, Underwood KW, et al. ALK1-Fc inhibits multiple mediators of angiogenesis and suppresses tumor growth. *Mol Cancer Ther.* 2010;9(2):379-88.
161. Duwel A, Eleno N, Jerkic M, Arevalo M, Bolanos JP, Bernabeu C, and Lopez-Novoa JM. Reduced tumor growth and angiogenesis in endoglin-haploinsufficient mice. *Tumour Biol.* 2007;28(1):1-8.
162. Hawinkels LJ, Kuiper P, Wiercinska E, Verspaget HW, Liu Z, Pardali E, Sier CF, and ten Dijke P. Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. *Cancer research.* 2010;70(10):4141-50.
163. Castonguay R, Werner ED, Matthews RG, Presman E, Mulivor AW, Solban N, Sako D, Pearsall RS, Underwood KW, Seehra J, et al. Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. *J Biol Chem.* 2011;286(34):30034-46.
164. Takahashi K, and Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126(4):663-76.
165. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007;318(5858):1917-20.
166. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, and Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131(5):861-72.
167. Sun N, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F, Hu S, Cherry AM, Robbins RC, Longaker MT, et al. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci U S A.* 2009;106(37):15720-5.
168. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bilic J, Pekarik V, Tiscornia G, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol.* 2008;26(11):1276-84.
169. Hanna J, Markoulaki S, Schorderet P, Carey BW, Beard C, Wernig M, Creyghton MP, Steine EJ, Cassady JP, Foreman R, et al. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell.* 2008;133(2):250-64.
170. Saha K, and Jaenisch R. Technical challenges in using human induced pluripotent stem cells to model disease. *Cell Stem Cell.* 2009;5(6):584-95.
171. Zhang J, Lian Q, Zhu G, Zhou F, Sui L, Tan C, Mutalif RA, Navasankari R, Zhang Y, Tse HF, et al. A human iPSC model of Hutchinson Gilford Progeria reveals vascular smooth muscle and mesenchymal stem cell defects. *Cell Stem Cell.* 2011;8(1):31-45.
172. Ikonomou L, Hemnes AR, Bilousova G, Hamid R, Loyd JE, Hatzopoulos AK, Kotton DN, Majka SM, and Austin ED. Programmatic change: lung disease research in the era of induced pluripotency. *Am J Physiol Lung Cell Mol Physiol.* 2011;301(6):L830-5.
173. Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, Lin HY, Bloch KD, and Peterson RT. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol.* 2008;4(1):33-41.

174. Cuny GD, Yu PB, Laha JK, Xing X, Liu JF, Lai CS, Deng DY, Sachidanandan C, Bloch KD, and Peterson RT. Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorg Med Chem Lett.* 2008;18(15):4388-92.
175. Hong CC, and Yu PB. Applications of small molecule BMP inhibitors in physiology and disease. *Cytokine Growth Factor Rev.* 2009;20(5-6):409-18.
176. Olsen BR, Reginato AM, and Wang W. Bone development. *Annual review of cell and developmental biology.* 2000;16(191-220.
177. Tsumaki N, Nakase T, Miyaji T, Kakiuchi M, Kimura T, Ochi T, and Yoshikawa H. Bone morphogenetic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2002;17(5):898-906.
178. Shu B, Zhang M, Xie R, Wang M, Jin H, Hou W, Tang D, Harris SE, Mishina Y, O'Keefe RJ, et al. BMP2, but not BMP4, is crucial for chondrocyte proliferation and maturation during endochondral bone development. *Journal of cell science.* 2011;124(Pt 20):3428-40.
179. Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR, and Chen D. Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. *The Journal of cell biology.* 2002;157(6):1049-60.
180. Wang M, Jin H, Tang D, Huang S, Zuscik MJ, and Chen D. Smad1 plays an essential role in bone development and postnatal bone formation. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2011;19(6):751-62.
181. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. *Nature genetics.* 2006;38(5):525-7.
182. Kaplan FS, Le Merrer M, Glaser DL, Pignolo RJ, Goldsby RE, Kitterman JA, Groppe J, and Shore EM. Fibrodysplasia ossificans progressiva. *Best practice & research Clinical rheumatology.* 2008;22(1):191-205.
183. Kaplan FS, Pignolo RJ, and Shore EM. From mysteries to medicines: drug development for fibrodysplasia ossificans progressive. *Expert opinion on orphan drugs.* 2013;1(8):637-49.
184. Groppe JC, Wu J, Shore EM, and Kaplan FS. In vitro analyses of the dysregulated R206H ALK2 kinase-FKBP12 interaction associated with heterotopic ossification in FOP. *Cells, tissues, organs.* 2011;194(2-4):291-5.
185. Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, et al. BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nature medicine.* 2008;14(12):1363-9.
186. Mohedas AH, Xing X, Armstrong KA, Bullock AN, Cuny GD, and Yu PB. Development of an ALK2-biased BMP type I receptor kinase inhibitor. *ACS chemical biology.* 2013;8(6):1291-302.
187. Shi S, Cai J, de Gorter DJ, Sanchez-Duffhues G, Kemaladewi DU, Hoogaars WM, Aartsma-Rus A, t Hoen PA, and ten Dijke P. Antisense-oligonucleotide mediated exon skipping in activin-receptor-like kinase 2: inhibiting the receptor that is overactive in fibrodysplasia ossificans progressiva. *PloS one.* 2013;8(7):e69096.

188. Takahashi M, Katagiri T, Furuya H, and Hohjoh H. Disease-causing allele-specific silencing against the ALK2 mutants, R206H and G356D, in fibrodysplasia ossificans progressiva. *Gene therapy*. 2012;19(7):781-5.
189. Kaplan J, Kaplan FS, and Shore EM. Restoration of normal BMP signaling levels and osteogenic differentiation in FOP mesenchymal progenitor cells by mutant allele-specific targeting. *Gene therapy*. 2012;19(7):786-90.