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► **To cite this version:**

Agnès Desroches-castan, Emmanuelle Tillet, Claire Bouvard, Sabine Bailly. BMP9 and BMP10: Two close vascular quiescence partners that stand out. *Developmental Dynamics*, 2021, 10.1002/dvdy.395 . hal-03291998

**HAL Id: hal-03291998**

**<https://hal.science/hal-03291998v1>**

Submitted on 20 Jul 2021

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# BMP9 and BMP10: two close vascular quiescence partners that stand out

Running Head: BMP9 and BMP10 roles in vascular homeostasis

Agnès Desroches-Castan<sup>#</sup>, Emmanuelle Tillet<sup>#</sup>, Claire Bouvard, and Sabine Bailly\*

Laboratory BioSanté, Univ. Grenoble Alpes, INSERM, CEA, F-38000, Grenoble, France.

\*Corresponding author: e-mail: [sabine.bailly@cea.fr](mailto:sabine.bailly@cea.fr)

<sup>#</sup>: co-first authors

## Abstract

Bone morphogenetic proteins (BMPs) are dimeric transforming growth factor  $\beta$  (TGF $\beta$ ) family cytokines that were first described in bone and cartilage formation but have since been shown to be involved in many pleiotropic functions. In human, there are 15 BMP ligands, which initiate their cellular signaling by forming a complex with two copies of type I receptors and two copies of type II receptors, both of which are transmembrane receptors with an intracellular serine/threonine kinase domain. Within this receptor family, ALK1 (Activin receptor-Like Kinase 1), which is a type I receptor mainly expressed on endothelial cells, and BMPRII (BMP Receptor type II), a type II receptor also highly expressed on endothelial cells, have been directly linked to two rare vascular diseases: hereditary haemorrhagic telangiectasia (HHT), and pulmonary arterial hypertension (PAH), respectively. BMP9 (gene name *GDF2*) and BMP10, two close members of the BMP family, are the only known ligands for the ALK1 receptor. This specificity gives them a unique role in physiological and pathological angiogenesis and tissue homeostasis. The aim of this current review is to present an overview of what is known about BMP9 and BMP10 on vascular regulation with a particular emphasis on recent results and the many questions that remain unanswered regarding the roles and specificities between BMP9 and BMP10.

## Introduction

The bone morphogenetic protein (BMP) family of ligands belongs to the large transforming growth factor (TGF- $\beta$ ) family. BMPs, originally identified as osteoinductive components, are now known to play important roles in a wide array of processes from embryonic development to adult organ homeostasis. In this review, we present recent advances and unanswered questions concerning the role of two of them, BMP9 and BMP10, that bind to a receptor mainly expressed on endothelial cells (ALK1 (Activin receptor-Like Kinase 1)), and that have emerged as key regulators of vascular homeostasis<sup>1</sup>. The endothelium is defined as the thin internal cell layer of blood and lymphatic vessels that derived from mesodermal lineage. The endothelium network plays an essential role mainly in providing nutrients and oxygen to organs, regulating antioxidant factors, modulating the response to infection, controlling the vascular tone and the formation of new blood vessels. This last process, named angiogenesis consists of the formation of new blood vessels from the pre-existing ones as a result of angiogenic growth factor induction. In adulthood, the endothelium is quiescent, meaning that the balance between pro- and anti-angiogenic modulators ensures

the stability of blood vessel <sup>2</sup>. However, angiogenesis can be reactivated under either physiological (wound healing, placentation, menstruation) or pathological conditions (cancer, stroke, rheumatoid arthritis, etc). Among the factors controlling vascular development and homeostasis, VEGF (vascular endothelial growth factor) is a key endothelial cell activator. VEGF triggers proliferation toward VEGF gradient, survival and permeability of endothelial cells (for review see <sup>3</sup>). To ensure the stability of the new formed-vessel, a step of vessel maturation is required through quiescent factors control. Among them, BMP9 and BMP10 have been proposed as circulating quiescent factors <sup>4</sup> involved in the regulation of both angiogenesis and lymphangiogenesis <sup>5,6</sup>. Indeed, it was shown that BMP9 and BMP10 bind with high affinity to the receptor complex composed of ALK1 and BMPRII (BMP Receptor Type II), which are two receptors expressed in endothelial cells that will lead to a direct phosphorylation of a specific family of transcription factors called SMADs (Mothers Against Decapentaplegic Homolog) <sup>7</sup>. Of note, mutations on one of these two receptors have been associated with two different rare vascular diseases, namely the Rendu-Osler syndrome also called hereditary haemorrhagic telangiectasia (HHT), and pulmonary arterial hypertension (PAH), bringing BMP9 and BMP10 into the world of vascular diseases. In this review, we provide an update of what is known on their expression, on their binding to the different type I and type II receptors, on the roles of their different circulating forms and their specific and redundant roles in vascular homeostasis, not only in animal models but also in human diseases. We also highlight the main points that we think remain to be explored.

### **BMP9 and BMP10 biosynthesis and expression**

BMP9 and BMP10 are two very close members of the BMP family <sup>8</sup>. BMPs are synthesized as pro-proteins that dimerize via a single disulphide bond within the terminal mature region to form homodimers or heterodimers (Figure 1). These pro-proteins are processed by furin-like pro-protein convertases upon secretion into prodomains and mature growth factor domains <sup>9-11</sup>. It has been shown for several BMP family members including BMP9 and BMP10 that the cleaved prodomain remains non-covalently bound to the mature domain <sup>12,13</sup>. These large complexes will be called hereafter BMP-pd. BMP9 and BMP10 share high sequence similarity in the growth factor domain (64%) yet their prodomains share less than 30% identity.

BMP9 is mainly expressed in the liver <sup>14,15</sup> and its expression site in this organ is restricted to hepatic stellate cells (HSCs) <sup>16-18</sup> (Figure 1). We previously reported BMP9 expression in hepatocytes using anti-BMP9 immunostaining, which led us to a misinterpretation of BMP9 expression in hepatocytes <sup>19</sup>. *BMP9* mRNA expression was also described in the lungs and the brain (septum) albeit at a much lower level than in the liver <sup>19,20</sup>. In contradiction with these data, a group described similar levels of *BMP9* in the liver and lungs <sup>21</sup>. However, the cells expressing BMP9 in the lungs have not been characterized yet.

BMP10 is mostly expressed in the heart, with a spatial-temporal regulation during development from the trabeculated myocardium starting at E8.5 until birth and then restricted to the right atria in the adult mouse <sup>22-26</sup> (Figure 1). *BMP10* cardiac expression has also been shown in human <sup>27</sup>. In addition, we could show that *BMP10* is also expressed in HSCs, like *BMP9*, although at a lower level than in the heart <sup>16</sup>. This result has been confirmed by a transcriptomic analysis of HSCs <sup>18</sup>.

BMP9 and BMP10 expressions have also been studied in zebrafish. Zebrafish express *bmp9* and two duplicate paralogs of BMP10 (*bmp10* and *bmp10-like*, with 64% identity in the active peptide)<sup>28</sup>. *Bmp10* and *bmp10-like* are expressed in the developing heart, starting respectively at 24 hours post fertilization (hpf) and 36 hpf, while *bmp9* is detected in the liver at around 48 hpf<sup>28</sup>. In the adult fish, *bmp9* expression is still restricted to the liver, while *bmp10* is found both in the heart and the liver and *bmp10-like* only in the heart<sup>29</sup>.

Not much is known about the regulation of *BMP9* expression. Whole-mount X-gal staining of *Bmp9*<sup>+/-</sup> embryos revealed that the onset of liver-specific expression is around E9.75-10 and increases until birth<sup>30</sup>. In mice, *Bmp9* mRNA expression is shown to decrease with age, both in the liver and lungs<sup>21</sup>. To our knowledge, no one has studied the regulation of *BMP9* expression in HSCs. What has been shown in pathological conditions, is that acute liver injury caused by partial hepatectomy or by the injection of carbon tetrachloride (CCL4) or lipopolysaccharide (LPS) results in transient downregulation of *Bmp9* mRNA<sup>17,31</sup>. Liver *Bmp9* mRNA and protein levels are also decreased in high fat diet mice and obese *db/db* mice<sup>32</sup>. *Bmp10* expression in mice starts earlier (E8.5) than *Bmp9*<sup>30</sup>. Due to its major role in heart development, the regulation of *Bmp10* expression in the heart has been studied more extensively. *Bmp10* expression in the heart is upregulated by the transcription factor myocardin<sup>33</sup>. In parallel, depletion of the cardiac homeobox gene *Nkx2-5* has also been shown to induce aberrant *Bmp10* expression at the trabeculae, supporting a *Nkx2-5*-*BMP10* axis of regulation in the heart<sup>34</sup>. Moreover, the Iroquois homeobox (*Irx*) homeodomain transcription factors repress *Bmp10* expression in the endocardium<sup>35</sup>. In pathological circumstances, hypertension, obtained by high-salt diet in rats, and hyperglycemia induce *Bmp10* expression<sup>27,36</sup>. In a mouse model of *Notch* depletion, it was shown that *NOTCH* maintains *Bmp10* expression in the heart, which is necessary for cardiomyocyte proliferation<sup>37</sup>. In zebrafish, deleting the T-box transcription factors *tbx3b* and *tbx2a* represses *bmp10* expression<sup>38</sup>. Together, these data demonstrate that conditions affecting heart size will modify heart *BMP10* expression, supporting a key role of *BMP10* in heart homeostasis. On the other hand, nothing is known concerning the regulation of *Bmp10* expression in liver HSCs. Thus, so far, no key direct regulators of *BMP9* and *BMP10* have been identified in HSCs, and this represents one challenging research field for the future.

## **Circulating forms of BMP9 & BMP10 and regulation of their activity**

### ***Homo and heterodimers circulate in blood***

*BMP9* and *BMP10* can be both detected in human and mouse blood circulation under biologically active concentration<sup>4,19,39,40</sup> (Figure 1). It was first thought that this circulating biological activity was due to *BMP9*, as we could show that a neutralizing anti-*BMP9* antibody blocked serum-induced *SMAD1/5/9* phosphorylation<sup>4</sup>. However, using more specific neutralizing anti-*BMP9* and anti-*BMP10* antibodies, it was shown that both *BMP9* and *BMP10* display some activity in human serum<sup>30</sup>. The presence of active circulating *BMP9* and *BMP10* was further supported by the loss of activity of both *Bmp9*-Knockout (KO) and *Bmp10*-Knockdown (KD) mice plasma<sup>5,16</sup>. In fact, very surprisingly, plasma from *Bmp9*-KO or *Bmp10*-KD mice were unable to activate the *SMAD1/5/9* pathway in endothelial cells and we could demonstrate that the circulating biologically active form was due to a *BMP9*-*10* heterodimeric form<sup>16</sup>. Through biochemical approaches, we could demonstrate that *BMP9* and *BMP10* are indeed able to heterodimerize by disulphide bonding, thus increasing

the diversity of the possible circulating ligands with both homodimeric and heterodimeric proteins<sup>16</sup>.

Where do these circulating forms come from? Circulating BMP9 probably comes from the liver where it is mostly produced. The heterodimeric BMP9-10 is also likely produced by the liver as hepatic stellate cells are the only cells that express both BMP9 and BMP10. However, for circulating BMP10, it remains to be determined whether it is secreted from the heart, the liver or both and whether heart BMP10 and liver BMP10 are both involved in vascular regulation.

The presence of active BMP9 and BMP10 in the circulation supports that, in addition to their local autocrine and paracrine activities, BMP9 and BMP10 are also endocrine factors that circulate throughout the body and are able to reach all endothelial cells, where they bind to their high affinity receptor ALK1 expressed specifically on endothelial cells.

### ***BMP9 & BMP10 latent and mature forms in plasma: a real challenge to unravel bioavailability***

BMP9 and BMP10 circulate mainly in association with their prodomain which is either cleaved leading to the BMP-pd active complex or still unprocessed leading to an inactive form<sup>16,19,41</sup>. As a result, BMP9 circulating forms exceed 100 kDa<sup>19</sup>. BMP processing usually occur intracellularly in the Golgi apparatus. We were able to show that the addition of furin could further activate human plasma<sup>19</sup>, supporting the hypothesis that the unprocessed form could be activated after secretion. However, how and when this uncleaved pool can be activated *in vivo* is still a highly relevant question<sup>11</sup>. Nevertheless, determining the ratio of active versus latent form and the ratio of homodimeric versus heterodimeric BMP9 and BMP10 is a real challenge since the mature active form detected in blood probably represents only a portion of the available BMPs.

Many groups have developed BMP9 and BMP10 ELISAs (Enzyme-Linked Immuno-Sorbent Assay) and commercial ones are also now available although with differences among them concerning specificities<sup>16,19,30,41</sup>. The choice of antibody pairs is crucial since they can discriminate mature and latent forms. Indeed, the use of capture and detecting antibodies specific to the mature form can displace the cleaved prodomain from the BMP-pd complex<sup>41</sup>. Therefore, this type of ELISA measures mature BMP9, regardless of its association with its prodomain but does not recognize unprocessed BMP9 precursor<sup>41</sup>. On the other hand, the use of a couple of anti-prodomain BMP9/anti-mature BMP9 for the respective capture and detection antibody was shown to detect essentially the unprocessed forms<sup>41</sup>.

Classical BMP9 and BMP10 ELISA using antibodies against mature peptides probably also detect the heterodimeric form as circulating BMP9 immunoreactivity is decreased in *Bmp10*-KO animals and vice versa<sup>16</sup>. To measure specifically the BMP9-BMP10 heterodimeric form, we have developed a crossed ELISA using a capture anti-mature BMP9 antibody and an anti-mature BMP10 detection antibody<sup>16</sup>. Together, these ELISAs demonstrate the presence of both homodimeric BMP9 and BMP10 and heterodimeric BMP9-10 circulating forms in blood circulation.

Another way to measure BMP9 and BMP10 circulating activities is to use a cell-based assay that exploits their high affinity for the receptor ALK1<sup>7</sup>. A biological assay was developed by cloning the BMP responsive element (BRE) derived from the promoter ID1 (Inhibitor of DNA

Binding 1) <sup>42</sup> five prime to the firefly luciferase in cells expressing ALK1 either constitutively (endothelial cells) or after ALK1 transfection (3T3 fibroblasts) <sup>7</sup>. Using a low concentration of plasma or serum, only BMPs binding with high affinity to ALK1 would activate this reporter, but caution is warranted with doses above 1% serum/plasma as other BMPs with lower affinities can be present at high concentrations and possibly activate this BRE luciferase assay. Additionally, even when starting with a low plasma/serum concentration, circulating inhibitors or transporters binding to these two BMPs might interfere and make their quantification difficult. A BMP Bioassay was also developed by stably transfecting the BRE-Luc construct in the C2C12 mouse myoblast cell line in order to measure circulating BMPs <sup>39</sup>.

Altogether, BMP9-10 levels can be assessed either via specific ELISA or according to their biological activities. However, we would like to clarify that we are currently unable to accurately measure all the different BMP9 and 10 forms (latent versus mature, active versus inactive) with our current knowledge and tools, and that we should be careful when interpreting our data.

### ***Regulation of the activity***

The association of BMP9 and BMP10 to its prodomain is considered to influence both localization and activities of BMPs. Interestingly, the structure of mature BMP9 complexed to its prodomain (BMP9-pd) indicates that BMP9-pd can adopt both cross-armed conformation, that could hamper its activity, and open-armed conformation <sup>43</sup>. Cross-armed structure does not seem to occur when BMP9 is recombinantly expressed, whereby BMP9-pd and BMP10-pd can activate endothelial cells to the same extent as the mature form. However, it has been suggested that this cross-armed structure could occur *in vivo*, by binding to the extracellular matrix and thus locally control BMP activity <sup>43</sup>. Indeed, secreted BMPs can function as circulating factors, but they can also be trapped in the extracellular matrix. In the case of BMP10, but not for BMP9, the prodomain has been shown to form stable complexes with extracellular matrix proteins fibrillin-1 and-2, that could be good candidates to trap and regulate BMP10 activity <sup>13</sup>.

The regulation of BMP10 activity is further emphasized by the increase of immunoreactivity and activity found in plasma after purification steps through an anti-BMP10 column <sup>16</sup>, suggesting that this treatment releases a BMP10 fraction that was not detected in the crude plasma. Such an increase in BMP activity upon purification from tissues has been reported a long time ago for other BMPs <sup>44</sup> and has been proposed to result from the release of inhibitors that could maintain BMPs in an inactive conformations <sup>43</sup>. BMP9 and BMP10 do not bind to the natural BMP antagonist noggin <sup>4,7,45</sup> which is probably one of the reasons for the potent effect of BMP9 versus other BMPs in bone formation <sup>46</sup>. Another modulator, Crossveinless 2 (CV2), also called BMPER (Bone Morphogenetic Protein-binding Endothelial Regulator), has been shown to bind BMP9 and BMP10 and it was proposed to inhibit the SMAD pathway <sup>47,48</sup>. However, this result is still debated as a more recent study showed that CV2 did not inhibit BMP9 signaling <sup>49</sup>. Therefore, there is still no clue for a possible antagonist *in vivo* that could hide a “reservoir” of BMP9 or BMP10.

### **BMP9 and BMP10 receptors and signaling**

#### ***Receptors and affinities***

BMPs, which are mostly homodimeric molecules, bind to a signaling receptor complex composed of two type I and two type II receptors. There are seven type I receptors within the TGF $\beta$  family and five type two receptors. BMPs can bind to the type I receptors ALK1, ALK2, ALK3 or ALK6 and the type II receptors BMPRII, ActRIIA (Activin Receptor IIA) and ActRIIB<sup>8</sup>.

BMP9 and BMP10 bind to the type I receptor ALK1 with sub-nanomolar affinities ( $EC_{50}$  of around 50 pg/mL<sup>-1</sup>), which are much higher than those of other BMPs for their cognate type I receptor ALK2, ALK3, and ALK6 ( $EC_{50}$  of around 50 ng/mL<sup>-1</sup>)<sup>7,12,41,50,51</sup>. Mature BMP9 and BMP9-pd bind with the same affinity to ALK1<sup>43,49</sup>. BMP9 also binds to ALK2 albeit with a much lower affinity and BMP9-pd binds to ALK2 with an even lower affinity compared to mature BMP9<sup>43,49</sup>. On the contrary, BMP10 does not bind to ALK2 and has been proposed to bind to ALK3 and ALK6<sup>52</sup>. However, in another work, it was shown, using surface plasmon resonance (SPR), that BMP10 could bind ALK6 but not ALK3<sup>53</sup>. Therefore, a better characterization of the binding and activation of ALK3 or ALK6 by BMP10 at both the biochemical and the cellular level is needed. Both BMP10 and BMP10-pd can activate cells expressing ALK1 (mostly endothelial cells) but the C2C12 muscle cell line, which does not express ALK1, is activated by mature BMP10 but not by BMP10-pd<sup>54,55</sup> supporting that BMP10 prodomain could differentially control BMP10 activity depending on the type I receptor.

Concerning the type II receptors, BMP9 seems to display a significant discrimination in type II receptor binding, whereas BMP10 does not<sup>50,56</sup>. Townson *et al.*<sup>50</sup> found that BMP9 binds with a similar affinity to ALK1 and ActRIIB, and a lower affinity to BMPRII and even lower to ActRIIA in accordance with Kuo *et al.*<sup>51</sup>. On the other hand, BMP10 had a similar affinity for the three type II receptors<sup>50,56</sup>. On the contrary, the group of Kienast *et al.*<sup>41</sup> found that BMP9 binds with a much higher affinity to ALK1 than to the type II receptors. BMPRII is highly expressed in endothelial cells (ECs) in comparison to ActRIIA and ActRIIB levels. It was shown that BMP9 co-immunoprecipitates with ALK1 and BMPRII in BAECs (bovine aortic ECs) and HUVECs (human umbilical vein ECs), but also with ActRIIB and ActRIIA in HUVECs although at a much lower level<sup>57</sup>, correlating with their expression levels. Binding of BMP9 to ALK2 is greatly facilitated when BMPRII or ACTRIIA/B receptors are co-expressed, suggesting that, in that case, binding to the type II receptors precedes binding to the type I receptor<sup>57</sup>.

BMP9 and BMP10 also directly bind with high affinity to the co-receptor endoglin that is strongly expressed in endothelial cells<sup>58,59</sup>. Mutations of this co-receptor are found in HHT patients<sup>60</sup>. Although endoglin is not required for BMP9/BMP10 activation of ALK1, it enhances signaling output as measured by pSMAD1/5/9<sup>7,61</sup>. Binding of BMP9 to the type II receptors displaces the prodomain and cell surface endoglin to form a signaling complex<sup>62</sup>. In addition, soluble endoglin can be found in the circulation and was long thought to act as a ligand trap for circulating BMP9 or BMP10<sup>58</sup>. However, it has been recently shown that a single chain soluble ENG/BMP9 complex is present in the circulation and that this complex retains a full signaling activity. It has therefore been proposed that monomeric soluble endoglin does not act as a ligand trap for BMP9 but on the contrary might preferentially direct BMP9 signaling via cell-surface endoglin<sup>62</sup>.

The current working model is that BMP9 or BMP10 can bind either to ALK1 or the type II receptors and then recruit the type I or type II receptor<sup>63</sup>.

### *BMP9 & BMP10 intracellular signaling*

Upon BMP9 or BMP10 binding, the type II receptor will phosphorylate the type I receptor, which will then phosphorylate SMAD1/5/9 within 5 min for a period depending on the concentration of BMP9 or BMP10. This period ranges from few hours with low doses (0.1 ng/mL) reaching up to 24h with higher doses (10 ng/mL) <sup>7,64</sup>. The complex BMP9/ALK1 is rapidly endocytosed and can then either be recycled to the membrane or degraded by proteolysis <sup>65</sup>. It has also been shown that in some endothelial cells, BMP9 can induce SMAD2 phosphorylation although at a much lower level than SMAD1/5/9 phosphorylation <sup>66</sup>. Endothelial cells express both ALK1 and ALK2, and their relative RNA levels varies depending on the endothelial cell origin. However, it is worth noting that only siRNA against ALK1 inhibits BMP9 SMAD1/5/9 signaling in endothelial cells suggesting that ALK2 is not directly involved in Smad1/5/9 signaling in response to BMP9. The presence of mixed type I receptor complexes (ALK1/ALK5 and ALK2/ALK3) leading to “lateral” SMAD activation has been identified <sup>67,68</sup>. We could thus hypothesise the existence of mixed ALK1/ALK2 complexes present on endothelial cells leading to specific signaling pathways. Individually silencing *BMPR2* or *ACTR2A*, the two main type II receptors present on endothelial cells, does not on their own inhibit pSMAD1/5/9 signaling, but the simultaneous deletion of the two receptors is required to inhibit pSMAD1/5/9 in human pulmonary artery ECs and umbilical artery ECs <sup>7,66</sup>. However, the relative contribution of the type II receptors seems to vary when looking at other responses; indeed, loss of *BMPR2* had a dramatic effect upon IL-8 (Interleukin-8) or E-selectin expression, conversely reduction of *ActRII* had a greater impact on SMAD2 phosphorylation <sup>66</sup>.

In addition to canonical Smad signaling, several kinases have been implicated downstream of BMP9 and BMP10 in endothelial cell such as JNK (c-Jun N-terminal kinase), p38, phosphatidylinositol-3-kinase (PI3K) (for review <sup>69</sup>). There are also an important crosstalks between the BMP9 and BMP10 and the Notch signaling pathways <sup>70,71</sup> and the Hippo pathway has also been shown to interact with BMP9 signaling <sup>72</sup>.

In addition, blood flow contributes to the regulation of BMP9 and BMP10 signal transduction. In cultured ECs, flow potentiates BMP9-induced ALK1 signaling <sup>73,74</sup>. It was shown that this enhanced sensitivity to BMP9 was due to a cooperation with the primary cilia <sup>75</sup>. Because BMP9 and BMP10 are secreted into the blood stream, the role of blood flow in ALK1 signaling is particularly relevant. In the zebrafish model, BMP9-BMP10-ALK1 axis has been shown to mediate a blood flow-based signal that controls directional cell migration and thus vessel caliber and arteriovenous shunts <sup>63</sup>. It is interesting to note that many BMP9/BMP10 regulated genes (*smad6*, *smad7*, *endothelin-1*) have been previously implicated as mechanoresponsive genes <sup>76,77</sup>.

### *Regulation of cell signaling*

Interestingly, it has recently been proposed that BMPRII could counteract cell signaling by behaving as an inhibitory receptor. Indeed, in myeloma cells and HEPG2 liver carcinoma cells, BMP9 activity was potentiated by knockdown of *BMPR2* <sup>78</sup>. This was not the case for BMP10 that does not bind to ALK2. BMPRII could thus preclude ALK2 from forming active receptor complexes with *ActRIIA* and *ActRIIB* <sup>78</sup>. The notion of BMPRII acting as a negative gatekeeper has also been described in an endothelial cell line (EAhy926), but in these cells,



the knockdown of *BMPR2* increased the ALK2/3-mediated BMP6 activation and not the BMP9 response that signals through ALK1<sup>68</sup>. Strikingly, Theilmann et al.<sup>64</sup> recently showed that *BMPR2* loss in HPAECs (Human Pulmonary Arterial Endothelial Cells) did not enhance the level but changed the kinetics of BMP9-mediated SMAD1/5/9 phosphorylation. Consequently, they observed that BMP9-induced prolonged *ID1* expression correlates with a higher proliferation rate in *BMPR2*-deficient cells<sup>64</sup>. Such results should be studied with the utmost attention as they could highlight essential mechanisms in the development of PAH. Indeed, PAH is associated with *BMPR2* mutations<sup>79</sup> and so far no *ActR2A/B* mutations have been described in PAH, supporting that BMPRII achieves a unique function in EC that cannot be replaced by another type II receptor (see below the mutation paragraph for discussion).

Another interesting notion is the competition between ligands or receptors (for review see<sup>80</sup>). For example, activin A and inhibin A have been shown to compete for BMP9 binding in HepG2 and myeloma cells that express ALK2 but not ALK1<sup>56,81,82</sup>. It was also recently proposed by the group of P. Knaus that BMP9 could serve as an antagonist for TGF $\beta$  by competing with TGF $\beta$  for binding ALK1 and ALK2 in a mixed-heteromeric receptor complex<sup>68</sup>.

### Target genes

Although BMP9-pd and BMP10-pd are the circulating forms, most of the studies performed *in vitro* employed mature BMP9 and BMP10. It was recently shown that mature BMP9 and BMP10 and BMP9-pd or BMP10-pd induce similar *ID1* expression in PAECs supporting that *in vitro*, in a given endothelial cell type, the mature and complexed forms are functionally equivalent ALK1 ligands<sup>5,49</sup>. In the same work, a comparison of the genes regulated by BMP9-pd and BMP10-pd was performed on PAECs and no significant difference in gene expression was found, suggesting that BMP9 and BMP10 are equivalent ligands *in vitro* on endothelial cells<sup>49</sup>. This seems also the case for the heterodimeric BMP9-10 form, at least *in vitro*, in regard to SMAD1/5/9 phosphorylation in endothelial cells<sup>16</sup>. Many BMP9 and BMP10 transcriptional targets have been identified both *in vitro*, after stimulation of endothelial cells and *in vivo* by deleting or blocking BMP9 and/or BMP10. Recently, several microarrays or RNAseq analyses have also been performed. The best targets identified are the transcriptional repressors (*ID1*, *ID2*, *ID3*), the inhibitory SMADs (*SMAD6* and *7*), the BMP receptors (*ENG* and *BMPR2*), and the canonical NOTCH targets (induced by RBPJ/NICD (Recombination Signal Binding Protein For Immunoglobulin Kappa J Region/ Notch intercellular domain) such as *HEY1* and *HEY2*<sup>7,49,83,84</sup>). In addition, several angiogenic factors (downregulation of *VEGFR1* (*VEGF Receptor 1*) and up-regulation of *VEGFR2* and *ANGPT2* (*Angiopoietin2*)), lymphatic maturation factors (*FOXC2* (Forkhead Box C2), *LYVE-1* (Lymphatic Vessel Endothelial Hyaluronan Receptor 1))<sup>6</sup>, regulators of endothelial to mesenchymal transition (EndMT) (*SNAI1* (Snail Family Transcriptional Repressor 1), *SNAI2*, *ZEB2* (Zinc Finger E-Box Binding Homeobox 2), *TWIST1* (Twist Family BHLH Transcription Factor 1))<sup>85,86</sup>, and vasoconstriction regulators (upregulation of endothelin and adrenomedullin and downregulation of apelin)<sup>49,87-89</sup> have been shown to be regulated by BMP9 and/or BMP10. Chemokines, interleukins, and cell surface adhesion molecules (upregulation of *IL6*, *CXCL8* (*C-X-C Motif Chemokine Ligand 8*, also known as IL-8), *SELE* (selectin-E) and down-regulation of *CCL2* (*C-C Motif Chemokine Ligand 2*) that affect monocyte/macrophages recruitment and endothelial permeability were also identified to be regulated by these two BMPs<sup>90,91</sup>.

## **BMP9 and BMP10 functions in the cardiovascular system: specific and redundant roles**

### ***In vitro/ex vivo:***

BMP9 and BMP10 (although less studied) have been mostly described as inhibitors of endothelial cell proliferation, migration, tube formation, sponge angiogenesis and metatarsal outgrowth (For review <sup>1,92</sup>). BMP9 has thus been proposed as a vascular quiescence factor <sup>2,4</sup> (Figure 1). However, it is important to note that in certain endothelial cells (derived from embryonic stem cells) BMP9 has also been described with pro-angiogenic functions <sup>93,94</sup>. Additionally, BMP9 has been shown to prevent vascular permeability by limiting VEGFR2 signaling and VE-cadherin internalization and by promoting occludin expression <sup>95</sup>. *In vitro*, BMP9 protects human pulmonary artery EC against apoptosis and excessive permeability induced by TNF $\alpha$  (Tumor Necrosis Factor alpha), LPS or thrombin <sup>83</sup>. BMP10 has been recently involved in the differentiation of human pluripotent stem cell-derived cardiovascular progenitors into the endocardial lineage by sustaining *Nkx2-5* expression<sup>96</sup>.

### ***In vivo: What do we learn from in vivo animal models (Table 1)***

*In vivo* BMP9 and BMP10 tend to have both similar and specific functions depending on their expression site, expression kinetic, localization and target cells expressing specific combination of receptors.

#### ***Loss of Bmp9:***

*Bmp9* knockout (KO) mice are viable and fertile and in the C57BL/6 strain exhibit only mild phenotypes <sup>5,30,97</sup>. Interestingly, while blood vessels did not display any detectable alterations, the lymphatic vessels were significantly affected <sup>6,97</sup>. Mesenteric collecting vessels are larger and present a reduced number of lymphatic valves in *Bmp9*-KO versus wild-type mice supporting a specific role for BMP9 in lymphatic maturation <sup>6</sup>. The loss of *Bmp9* was rather modest as we could not observe lymph edema but still it decreased lymphatic drainage in adult mice. More recently, a *Bmp9*-KO mouse was generated by CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology in the C57BL/6 strain. No obvious vascular malformations could be observed in these mice, however the smooth muscle layer of the lung vessels showed thinning and discontinuity <sup>98</sup>. These *Bmp9*-KO mice also showed a marked fat accumulation and liver steatosis which had not been described previously <sup>32</sup>. Interestingly, we identified a strain-dependent effect for *Bmp9* deletion with a stronger phenotype in the 129/Ola background versus C57BL/6 and BALB/c background <sup>99</sup> as previously shown in *Eng*-KO mice <sup>100,101</sup>. The reason for this strain specificity is not yet understood but clearly supports the notion of genetic modifiers in BMP9 signaling. This aspect is highly relevant for HHT disease whose clinical signs are very variable even within the same family supporting a role for modifier genes in HHT <sup>102</sup>. The analysis of the *Bmp9*-KO phenotype in the 129/Ola strain allowed to demonstrate a paracrine role of BMP9 in liver that acts directly on sinusoidal endothelial cell (LSEC) fenestration via regulation of *PLVAP* (*Plasmalemma vesicle-associated protein*) and the transcription factor *GATA-4* (*GATA Binding Protein 4*) and that affects liver homeostasis <sup>103</sup>. It is however important to note that the exact role of BMP9 on liver fibrosis remains debated as other groups have found that *Bmp9*-deficient mice present a reduced severity of CCL4-induced liver fibrosis <sup>17,104</sup>. The role of BMP9 has also been studied in zebrafish, where it was shown that zebrafish *bmp9* mutants display no overt phenotype, and the animals survive until

adulthood<sup>29</sup>. However, in a study from another group, it was shown that *bmp9*<sup>-/-</sup> zebrafish exhibited structural anomalies in the caudal vein plexus<sup>98</sup>.

#### **Loss of *Bmp10*:**

*Bmp10*-KO mice die in utero between embryonic day E9.5 and E10.5 and exhibit failed cardiac trabeculation supporting a specific key role for BMP10 in heart formation<sup>25</sup>. The role of BMP10 has since been reexamined more carefully and it was found that *Bmp10* deletion also induces defects in early vascular development with AVMs reminiscent of the vascular phenotype resulting from the loss of *Acvrl1*<sup>30,105,106</sup>. In zebrafish, concomitant knockdown of the duplicate paralogs, *bmp10* and *bmp10-like*, results in embryonic lethal cranial AVMs indistinguishable from *Alk1* mutants<sup>28</sup>. Furthermore, in the juvenile-to-adult period, *bmp10* zebrafish mutants exhibit vessel enlargements, supporting a unique role for *bmp10* after birth<sup>29</sup>.

In mouse embryo, *Bmp10* is detected earlier (E8.5) than *Bmp9* (E9.75–E10)<sup>30</sup>. This temporal difference could explain why BMP10 serves as a requisite endogenous ALK1 ligand during early embryonic vascular development. In accordance with this hypothesis, insertion of *Bmp9* into the *Bmp10* locus in mice (*Bmp10*<sup>9/9</sup>-KI), which allows *Bmp9* to be expressed in the endogenous spatiotemporal pattern of *Bmp10*, prevented embryonic AVMs<sup>30</sup>. However, trabeculation defects could not be rescued, presumably because of a unique requirement for BMP10 in inducing cardiomyocyte regulation independent of ALK1<sup>30</sup>.

More recently, mice with inducible or tissue-specific deletion of *Bmp10* have been generated. It was shown that adult *Bmp10* knockdown in the C57BL/6 background was not lethal and showed no obvious vascular phenotype<sup>107,108</sup>. More recently, a mouse model specifically deleted for *Bmp10* in the right atrium was generated (*Bmp10*<sup>ANF</sup>)<sup>109</sup>. These mice overcame embryonic cardiac lethality and were viable with no obvious phenotype, suggesting that cardiac BMP10 is not involved in postnatal vascular remodeling.

#### **Loss of both *Bmp9* and *Bmp10*:**

In order to address the role of BMP9 and BMP10, in absence of an inducible *Bmp10*-KD mouse that could bypass the heart defect, neutralizing anti-BMP10 antibodies were used. Although injection of anti-BMP10 antibody at post-natal stage did not affect retinal vascularization in WT animals, injection in *Bmp9*-KO animals induced retinal hypervascularization<sup>5,30</sup> and prevented the closure of the ductus arteriosus<sup>85</sup>. Similarly, co-injection of neutralizing anti-BMP9 and anti-BMP10 antibodies in WT animals induced increased retinal vascular density and arteriovenous shunts<sup>30,110,111</sup>. BMP9 and BMP10 activity can also be blocked by injecting a soluble form of the receptor ALK1 (ALK1-Fc) that can act as a ligand-trap. Postnatal ALK1-Fc injection in mice results in defective lymphatic development<sup>112</sup>. It also led to retinal hypervascularization and the appearance of AVMs<sup>70</sup>.

Recently, mice deleted for both *Bmp9* and *Bmp10* have been generated. *Bmp10*<sup>ANF</sup> mice crossed with *Bmp9*-KO mice developed a strong vascular phenotype at 8 weeks of age, with a decrease in vascular tone and diminution of the VSM (Vascular Smooth Muscle) layer contractility<sup>109</sup>. Unexpectedly, the authors showed that deletion of *Acvrl1* in VSMCs (Vascular Smooth Muscle Cells) recapitulated the pulmonary *Bmp9/Bmp10* double deletion phenotype, supporting a role for ALK1 in VSMCs. In this work, the role of endothelial ALK1 was not studied and thus its role in endothelial cells versus vascular smooth muscle cells remains to be further studied. In our hands, the global knockdown (KD) of *Bmp10* in adult *Bmp9*-KO mice leads to a phenotype that is partially in accordance with this work, characterized by vessel dilation, a decrease in blood pressure, and a larger heart<sup>108</sup>.

Together these results indicate that blockade of *Bmp10* at post-natal stage in *Bmp9-KO* mice leads to the death of these pups while loss of *Bmp10* in adult *Bmp9-KO* mice is viable although these mice present many vascular problems. It is also interesting to note, that BMP10<sup>ANF</sup> mice crossed with *Bmp9-KO* mice, which will lead to a rapid loss of BMP10 after birth as BMP10 is mainly expressed by the right atria at this stage, leads to viable mice underscoring a role for other sources of BMP10 (such as the liver) at post-natal stage.

#### **Loss of BMP9 and /or BMP10 in pathological contexts:**

The role of BMP9 and BMP10 has also been addressed in pathological contexts. Loss of *Bmp9* using different approaches (neutralizing anti-BMP9 antibodies, *Bmp9-KO* mice or the soluble ALK1 receptor (ALK1-Fc)) has been investigated in several mouse and rat pulmonary hypertension (PH) preclinical models and showed that blocking BMP9 partially protected these animals from PH<sup>89</sup>. On the contrary, another group, showed that ALK1-Fc induced severe PH in mice treated with hypoxia<sup>113</sup>. On the other hand, *Bmp10-KD* in adult mice do not seem to be protected from PH when challenged<sup>108</sup> supporting a specific role for BMP9 in lung remodeling in PH. These apparently contradictory results are further discussed below and have been very accurately discussed in the editorial from Ormiston et al<sup>114</sup>.

The role of BMP9 and BMP10 has also been addressed in the context of tumor angiogenesis. We found that tumor growth (E0771 mammary orthotopic xenografts) was increased in adult *Bmp9-KO* and *Bmp9-KO;Bmp10-KD* mice but not in *Bmp10-KD* mice, and that this augmentation in tumor size could be partially attributed to an activation of tumor angiogenesis supporting a specific role for BMP9 in tumor angiogenesis<sup>107</sup>. In line with this result, it was shown that BMP9 overexpression, in addition to a decreased tumor size, promotes the normalization of tumor blood vessels in the Lewis Lung Carcinoma model<sup>115</sup>.

Together, these mouse models show a clear specific role for BMP10 in heart formation and cardiac remodeling via a direct role of BMP10 on cardiomyocytes that is probably not due to ALK1 signaling (Figure 1). On the other hand, deletion of *Bmp9* seems to be sufficient to affect lymphatic maturation<sup>6</sup>, supporting that BMP9 and BMP10 do not play redundant roles for lymphatic regulation. However, the role of BMP10 has never been studied on lymphatic maturation, so we cannot exclude at this stage a role for BMP10 or BMP9-10 heterodimer on lymphatic maturation. Similarly, we can say that BMP9 plays a specific role on liver homeostasis, revealed under a specific mouse strain (129/Ola), by preventing LSEC capillarization<sup>103</sup>. However, the role of BMP10 has not been addressed leaving the door open for a potential role for the BMP9-10 heterodimeric form in liver homeostasis. On the other hand, what seems to be clear is an earlier role for BMP10 in embryonic angiogenesis, in line with BMP10 earlier expression. After birth, BMP9 and BMP10 clearly play redundant roles in postnatal angiogenesis (retinal vascularization and closure of the ductus arteriosus<sup>5,30,85</sup>) but also in adult vascular homeostasis (under physiological conditions) as only the double *Bmp9/Bmp10* deletion leads to a vascular phenotype<sup>108,109</sup>. Due to technical issues, the role of BMP9 has been addressed via a complete and constitutive KO while the role of BMP10 has been addressed via either neutralizing antibodies, or tamoxifen inducible knockdown in adult or tissue-specific deletion in the right atria in order to bypass cardiac embryonic lethality. In zebrafish, no transcriptional adaptation or genetic compensation was found for *bmp9* or *bmp10-like* expression or in *bmp10* mutants<sup>29</sup>. Nonetheless, we cannot

completely exclude at this stage, that the absence of phenotype in *Bmp9*-KO mice or in *Bmp10*-KD is not due to a genetic compensation <sup>116</sup>.

It has not yet been clearly demonstrated that BMP9 and BMP10 effects on vascular homeostasis are all mediated by the receptor ALK1. It is also important to keep in mind that loss of *Acvr11*, even in adult mice, leads to a very rapid death of the animals (in 10 days) <sup>117</sup> that does not occur by the loss of its two main ligands, BMP9 and BMP10, supporting a role for ALK1 that is either ligand-independent or depending on another unknown ligand. Another possible explanation for this discrepancy is that *Bmp10* deletion by inducible knockdown could be incomplete.

BMP9 and BMP10 play a key role in embryonic and postnatal angiogenesis and lymphangiogenesis and in vessel quiescence allowing organ homeostasis both locally in an autocrine (hepatic stellate cells) <sup>17</sup> and paracrine manner (liver) <sup>103</sup> and at distance via circulation in an endocrine manner (lungs) <sup>89</sup> (Figure 1). - the only factors involved in the control of vascular quiescence and it is clear that they interplay with many other vascular signaling pathways<sup>2</sup>. BMP9 has been shown to dampen VEGF-mediated activation of AKT (Serine/Threonine Kinase 1) and ERK (Extracellular signal-regulated kinase) and to increase PTEN (phosphatase and tensin homolog) activity <sup>69-71</sup>. There is also an important crosstalk between BMP9 and BMP10 and the Notch signaling pathway <sup>70,71</sup>. Indeed, in a retinal model of vascular development, BMP9 rescued hypersprouting induced by Notch inhibition<sup>70</sup>, while inhibition of Notch signaling lead to a decrease of BMP9 signaling and conversely <sup>71</sup>.

The absence of BMP9 and BMP10 will lead to the formation of AVMs in the retina and intestine of young mice in accordance to HHT. Their loss will also lead to blood and lymphatic vessel dilations <sup>6,103,109</sup>. Accordingly, BMP9 and BMP10 have been involved as direct regulators of vasoconstriction; BMP9 and BMP10 induce vessel vasoconstriction in the CAM (Chick Chorioallantoic Membrane) assay <sup>4</sup> and regulate several genes associated to vasoconstriction (*apelin*, *endothelin-1* and *adrenomedullin*) <sup>49,89</sup>. BMP9 also regulates vascular permeability, endothelial barrier function and inflammation. Indeed, delivery of BMP9 by adenoviral vectors significantly decreased the loss of retinal barrier function in diabetic mice <sup>95</sup>. In line with this result, BMP9 regulates VEGFR2 signaling and thus VE-cadherin endocytosis and occludin expression (see paragraph *in vitro* function) <sup>95</sup>. Subacute neutralization of endogenous BMP9 leads to lung vascular injury, including enhanced endothelial permeability and neutrophil extravasation <sup>31</sup>. Inflammation is also observed in *Bmp9*-KO mice in the 129/Ola background in association with liver fibrosis <sup>103</sup> and in lungs of adult *Bmp9*-KO; *Bmp10*-KD <sup>108</sup>. It could thus be inferred from these results that BMP9 and BMP10 play a role in inflammation either directly or through vessel permeability and/or injury.

Many points that have not been addressed yet remain to be answered:

- What is the role of liver BMP10 versus cardiac BMP10 in vascular remodelling?
- What is the role of the circulating BMP9-10 heterodimer, which is responsible for most of the BMP9/BMP10 circulating activity?
- In the 129/Ola strain, the deletion of BMP9 is sufficient to observe a strong phenotype on endothelial capillarization and liver homeostasis. What are the genetic modifiers present in 129/Ola versus C57BL/6 mice? Could BMP10 deletion also have a strain-dependent effect (C57BL/6 versus 129/Ola)? Could the BMP9-10 heterodimer play different roles in different mouse strains?

### ***In vivo*: What do we learn from human diseases?**

Interestingly, the first knowledges that we have learned from BMP9 and BMP10 roles in the endothelium come from human data. Indeed, in 1994 and 1996 mutations in two orphan receptors (*ACVRL1* encoding ALK1 and *ENG* encoding endoglin) were linked to the rare disease HHT<sup>60,119</sup> and in 2000, mutations in *BMPR2* were associated with another rare disease PAH<sup>79</sup>. Although these two diseases involved the same signaling complex, which is expressed in endothelial cells, these two diseases are quite different from each other.

HHT is an autosomal dominant genetic disorder that affects 1 in 5000 to 8000 individuals worldwide<sup>120</sup>. It is characterized by several vascular defects such as small vascular malformations called telangiectasia that appear as red spots on the surface of the skin or of mucosa and AVMs in internal organs (Lungs, liver, brain). Telangiectasia in the lining of the nose can cause recurrent and mild to severe nosebleed (epistaxis) and intestinal telangiectasia can cause gastrointestinal bleeding. As a consequence of this bleeding, some patients become anaemic. AVM in internal organs can be asymptomatic but depending on the localization and severity, various complications may occur. Hepatic AVMs are found in 70% of the patients, which can increase cardiac output. Pulmonary AVMs are found in 15-45% of the patients and can increase the risk of stroke. Cerebral AVM are less frequent (10-23% of the patients), but can have severe consequences such as brain haemorrhage and seizure. Therapeutic options include surgery (for some AVMs) and anti-VEGF treatment, which successfully reduces epistaxis and cardiac output in patients with severe liver involvement<sup>121</sup> (for review<sup>122</sup>). Although HHT is caused by *ACVRL1* or *ENG* mutations in 90% of the cases, a much smaller proportion of HHT cases (<2%) is caused by *SMAD4* mutations; However, these patients have different clinical features and their disorder is called juvenile polyposis-HHT. In addition, *BMP9/GDF2* mutations have been described in 3 patients with HHT-like symptoms in 2013<sup>123</sup> and confirmed in another patient two years later<sup>124</sup>, but since then only two *BMP9* mutations have been described in HHT patients, the latest being an homozygous mutation<sup>125,126</sup> and thus the involvement of *BMP9* mutations in HHT is still debated.

Pulmonary hypertension (PH) is an incurable condition with a high mortality rate is defined by a resting mean pulmonary arterial pressure > 20 mmHg in association with normal pulmonary arterial wedge pressure ( $\leq$  15 mmHg) and pulmonary vascular resistance (> 3 Wood unit) measured by right heart catheterization<sup>127</sup>. There are more than 30 types of pulmonary hypertension that have been classified by the WHO (World Health Organization) into 5 groups<sup>127</sup>. Among them, PAH is a rare disease with an estimated prevalence ranging from 10 to 52 cases per million<sup>128</sup>. PAH is characterized by a pulmonary vascular remodelling with an hypertrophy of the media, adventitial fibrosis, thrombotic lesions, plexiform lesions and perivascular infiltration of inflammatory cells<sup>129</sup>. Although the pathogenic mechanisms are not well understood, vasoconstriction, proliferation, thrombosis and inflammation in the lung microcirculation seem to drive the progression of the disease. The resulting increase in pulmonary vascular resistance leads to right ventricular overload and eventually right heart failure and death. In 2000, genetic analysis of families with heritable PAH identified heterozygous germline mutations in *BMPR2*<sup>79</sup>. Further investigation showed that *BMPR2* is the most frequently mutated gene as *BMPR2* mutations are found in 80% of familial PAH. However, the penetrance is incomplete and the mechanism through which these mutations increase the risk of developing PAH is still unclear. Since then, other less frequent mutations

have been identified within this signaling pathway (in *ACVRL1*, *ENG*, *SMAD9*, *BMP9/GDF2* and *BMP10*)<sup>130</sup>. The first *BMP9/GDF2* mutation in PAH was published in 2016 as a case report of a homozygous nonsense *BMP9* mutation in a child with PAH, while the parents with the same heterozygous mutation had no PAH symptoms<sup>131</sup>. Since then many heterozygous mutations in *BMP9/GDF2* have been found associated with PAH<sup>130,132–135</sup>. A truncating mutation and a predicted loss of function variant were also found in *BMP10* in two severely affected PAH patients<sup>130</sup>. Some *BMP9* mutations have recently been associated with impaired processing and secretion and reduced activity, while other *BMP9* mutations had no functional consequences<sup>132–134</sup>. Biochemical and functional characterizations of *BMP10* mutations have not been reported yet. However, due to the role of BMP10 in heart morphogenesis, it is possible that these mutations could cause cardiac problems that could increase the risk of developing pulmonary hypertension. Indeed, one of the rare patients with *BMP10* mutation presents features of congenital heart disease<sup>130</sup>. As BMP9 and BMP10 are dimeric proteins, it is important to keep in mind that heterozygote mutations in *BMP9/GDF2* or *BMP10* could have a dominant negative effect on BMP9 or BMP10 homo- and heterodimers.

Taken together, these results support a stronger implication for *BMP9* mutations in PAH versus HHT although the rationale for this is not yet understood.

Noteworthy, in 2020, a homozygous variant in *BMP9* was associated with lymphatic dysplasia with hydrothorax and immune hydrops fetalis<sup>136</sup>.

Although therapeutic strategies targeting BMP9/10-ALK1 pathway have been proposed, as discussed below, it is not clear whether this pathway should be activated or inhibited, especially in PAH. Nevertheless, these mutations highlight the importance of this pathway in vascular homeostasis.

#### **BMP9 and BMP10 as biomarkers:**

##### ***Modification of BMP9 and BMP10 circulating levels in pathologies***

Many recent publications show that circulating BMP9 and BMP10 levels are significantly affected in different pathologies, but these results have to be treated cautiously since as discussed in the paragraph “Latent and mature form in plasma: a real challenge to unravel bioavailability”, it remains challenging to measure accurate circulating levels of BMP9 and BMP10. Due to the recent identification of *BMP9* and *BMP10* mutations in PAH, several groups have measured the circulating levels of these two BMPs in this disease. It has been described that *BMP9* mutation carriers had low plasma levels of BMP9 measured by ELISA or BRE activity<sup>133,134</sup>. Interestingly, the level of circulating BMP10 was also lower in PAH patients carrying *BMP9* mutations, which could be the consequence of a lower amount of circulating BMP9-10 heterodimers. It was also shown that BMP9 median plasma levels were significantly reduced in patients with idiopathic PAH<sup>134</sup> but this was not confirmed in a larger analysis<sup>133</sup>. Circulating BMP10 levels were not significantly different between controls and PAH patients except in female PAH patients that showed a significantly lower level<sup>133</sup>. It is interesting to note that, in this work, circulating BMP9 and BMP10 were higher in females than in males. On the other hand, significantly decreased circulating concentration of BMP9 and BMP10 were found in patients with porto-pulmonary hypertension or hepatopulmonary

syndrome<sup>113,137</sup>, two liver diseases that can cause pulmonary vascular complications. These results are of interest since the liver is the site of expression of BMP9 but also BMP10 as well as BMP9-10 heterodimers. Higher BMP10 circulating levels were also detected via a proteomic approach in the first week of life of preterm infants with bronchopulmonary dysplasia and pulmonary hypertension<sup>138</sup>.

BMP9 and BMP10 circulating levels have also been measured in liver diseases. Decreased levels of BMP9 in cirrhosis, NASH and alcoholic cirrhosis versus non-alcoholic cirrhosis have been described while circulating BMP10 levels decreased only in NASH (Non-Alcoholic SteatoHepatitis)<sup>139</sup>. In another study, patients with cirrhosis had low levels of BMP9 and BMP10 but increased levels of circulating endoglin<sup>140</sup>. However, in hepatic pathologies it remains unclear whether the decrease in BMP9 or 10 is a cause or a consequence of the disease. Indeed, during liver fibrosis or cirrhosis, LSECs lose some specific features, such as their fenestration and hepatic stellate cells are concomitantly activated involving phenotypic and biochemical changes, which may lead to a decrease in BMP9 and BMP10 biosynthesis. BMP9 circulating levels have also been shown to be significantly lower in patients with vascular malformations, hypertension and coronary heart disease<sup>98,141,142</sup>. Elevated circulating BMP10 has also been shown to predict atrial fibrillation ablation<sup>143</sup>. Circulating BMP9 has also been measured in a small cohort (n=10) of patients with systemic inflammatory response syndrome (SIRS) and sepsis, and was found to be significantly reduced compared to healthy controls<sup>31</sup>.

### **BMP9 and BMP10 in therapies**

BMP9 and BMP10 treatments have been proposed in several diseases. First of all, although not the topic of this review, BMP9, due to its potent role in bone formation, has been proposed in bone healing treatments<sup>144</sup>. However, its use in bone generation might be limited because of its major role in angiogenesis and potent vasoconstrictor side effects. BMP10 could also be interesting but in that case one has to consider its role on cardiomyocytes. A possibility would be to develop modified BMPs that would only bind to a specific receptor. In line with this idea, a BMP9 mutant (D366E) was designed so that it would not bind to ALK2 and could thus be used as a specific activator of ALK1<sup>49</sup>.

Still, with these limitations in mind, the beneficial effects of injecting recombinant BMP9 have been demonstrated in rat preclinical models of PAH<sup>83</sup>. This result is in accordance with a recent work that found that ALK1-Fc exacerbated hypoxia-induced pulmonary hypertension in mice<sup>113</sup> but in contrast to our work as we found that blocking BMP9 by different means (KO, anti-BMP9 antibodies or ALK1-Fc) partially protected against experimental pulmonary hypertension in mice and rats<sup>89</sup>. In this work, we proposed that these animals were protected because of a loss of *Bmp9*-induced vasoconstriction. In another preclinical study, it was shown that BMP9 treatment of rat pups with hyperoxia-induced experimental bronchopulmonary dysplasia reduced alveolar enlargement, lung fibrosis and prevented inflammation but did not attenuate vascular remodelling and right ventricular hypertrophy<sup>145</sup>. Interestingly, it was recently shown that BMP9 induced aberrant EndMT in PAH pulmonary endothelial cells but not in healthy PAECs<sup>86</sup>. Similarly, a BMPRII-dependent shift in the endothelial proliferative response to BMP9 was reported, whereby BMP9 inhibited the proliferation of healthy donors endothelial cells but enhanced the proliferation of endothelial cells from *BMPR2* mutation bearing PAH patients<sup>64</sup>. At first



glance, these results seem contradictory, however different strategies have been used (adding exogenous BMP9 versus loss or inhibition of BMP9) that could contribute to different interpretations which has been thoroughly discussed in the editorial from Ormiston *et al.*<sup>114</sup>. Together, these data indicate an important role for BMP9 in PAH pathogenesis and underscore that further investigations for the therapeutic use of BMP agonists in PAH are needed before going to PAH patients.

BMP9 treatment has also been proposed in preclinical models of oxygen-induced retinal neovascularization and left ventricular heart failure<sup>146,147</sup>. Finally, BMP9 was shown to induce ischemic neovascularization when endothelial progenitor cells had been exposed to BMP9 stimulation *in vitro* before injection<sup>148</sup>.

In conclusion, we are probably still far from clinical assays using recombinant BMP9 or BMP10. We will need to better understand the molecular insight of this signaling pathway to provide essential information for improving the specificity and the efficacy of therapies that target this endothelial signaling pathway. The future might come with development of small peptides derived from these BMPs that could selectively and specifically enhance ALK1-mediated BMP9 signaling as recently proposed<sup>149</sup>.

#### Concluding remarks:

BMP9 and BMP10 are two cytokines exhibiting pleiotropic functions in an autocrine, paracrine and endocrine manner (Figure 1). While the role of these growth factors is now relatively well accepted concerning the maintenance of vascular quiescence, their use in the clinic, in particular in the context of PAH, is still strongly debated. This highlights the need for a better understanding of the fine regulation of BMP9 and BMP10. At present, few modulators of BMP9 and BMP10 expression have been identified. Another important point to highlight is the different possible associations of receptors that can generate context-dependent responses in tissue and cells and that may explain the discrepancy between studies. One of the main questions that remains unanswered concerns the role of the circulating forms and their origin. If it appears obvious that heterodimer and BMP9 come from the liver, however, BMP10 can come from both heart and liver. It is also not known, whether the source of BMP10, liver or heart, could have an impact on the function.

This review focuses on the role of BMP9 and BMP10 on vascular homeostasis and diseases linked to the ALK1 signaling pathway. However, it is clear that it is not the only BMP pathway involved in vascular regulation as elegantly demonstrated by the loss of function of *Alk2* and *Alk3* and that a lot remains to be learned through the interactions of these receptors<sup>150</sup>. The current working hypothesis is that BMP2 and BMP6 are pro-angiogenic ligands and signal through ALK2 and ALK3, whereas BMP9 and BMP10 are homeostatic ligands that signal through the receptor ALK1<sup>151</sup>. Unknowns are how BMPs, which all signal via SMAD1/5/9 apply different phenotypic outputs to similar canonical BMP signaling receptors and what is the role of the non-canonical BMP signaling pathways. Another point that we have not addressed is the BMP9/BMP10 response in venous, versus arterial, versus capillaries endothelial cells and within capillaries from different vascular beds. The recent data obtained from single cell RNAseq analyses will clearly help us to identify these vascular specificities<sup>152,153</sup> and will in the future give us important clues to better understand the vascular functions of BMP9 and BMP10.

Altogether, there has been recent substantial work on BMP9 and BMP10 that leads to a better understanding of their roles in vascular homeostasis and vascular diseases but that also leads to numerous open questions, along with exciting avenues of future research aiming to develop new therapies for vascular diseases.

Legend to Figure 1:

**Figure: biosynthesis and role of BMP9, BMP10, BMP9-10 in vascular homeostasis.** BMP9 and BMP10 are synthesized as pro-proteins that dimerize via single disulphide bond within the terminal mature region to form homo or heterodimers. These pro-proteins are then processed by convertases into prodomains and mature growth factor domains. The cleaved prodomain remains non-covalently bound to the mature domain to form a complex called BMP-pd. After birth, BMP10 homodimer is mostly expressed by cardiomyocytes of the right atria of the heart under the activation of myocardin. BMP10 is a modulator of Nkx2-5 expression and in return contributes to heart homeostasis. BMP9 and BMP10 homodimers, and BMP9-10 heterodimer are produced by hepatic stellate cells in the liver. BMP9 exerts a paracrine role by regulating liver sinusoidal endothelial cell fenestration through PLVAP and GATA-4 regulation and an autocrine activity on hepatic stellate cells. All forms may be found in the systemic blood circulation. Both unprocessed and BMPs-pd forms can be found in the systemic blood circulation. BMP9-pd, BMP10-pd and BMP9-10-pd bind to a signaling complex composed of two type 1 receptors (ALK1) and two type 2 receptors (BMPRII, ActRIIA or ActRIIB) expressed on endothelial cells where they will exert endocrine functions to maintain vascular quiescence and lymphatic vessel maturation. Absence of BMP9 and/or BMP10 will lead to several vascular dysfunctions among which arteriovenous malformation, vessel dilation, inflammation and vascular permeability.

**Table 1: phenotypes of BMP9 and 10 deletion in mouse models**

**Table: phenotypes of *Bmp9* and *Bmp10* deletion in mouse models**

Function Animal model	Heart	Lymphatic	Liver	Retina	Ductus arteriosus	AVMS other than retina	Blood pressure	Lungs PAH	Tumor angiogenesis
<i>Bmp9</i> -KO	No effect <sup>110</sup>	Enlarged vessels <sup>6,99</sup> Valves, drainage <sup>6</sup>	Fibrosis <sup>17,101,105</sup> Fenestration <sup>105</sup> Obesity <sup>12</sup>	No effect <sup>5</sup>	No effect <sup>87</sup>	NR	NR	Partial protection <sup>91</sup>	Increase <sup>109</sup>
Anti-BMP9	NR	NR	NR	Hyper-vascularization <sup>73</sup> No effect <sup>30</sup>	NR	NR	NR	NR	NR
<i>Bmp10</i> -KO	Heart development <sup>25</sup>	NR	NR	NR	NR	Yolk sac <sup>30</sup>	NR	NR	NR
<i>Bmp10</i> -iKD adult	NR	NR	NR	NR	NR	NR	NR	No effect <sup>110</sup>	No effect <sup>109</sup>
Anti-BMP10 post-natal	NR	NR	NR	No effect <sup>5,30</sup>	No effect <sup>87</sup>	NR	NR	NR	NR
ALK1-Fc	NR	Defective development Valves drainage <sup>113</sup>	NR	Hyper-vascularization <sup>73,30</sup> AVM <sup>72,73</sup>	NR	NR	NR	NR	NR
<i>Bmp9</i> -KO+anti-BMP10 post-natal	NR	NR	NR	Hyper-vascularization <sup>5,30</sup>	Impaired closure <sup>87</sup>	NR	NR	NR	NR
<i>Bmp9</i> -KO; <i>Bmp10</i> -iKD adult	Larger heart <sup>110</sup>	NR	NR	NR	NR	Yes, organ not identified <sup>110</sup>	Decrease <sup>110</sup>	Partial protection <sup>110</sup>	Increase <sup>109</sup>
<i>Bmp9</i> -KO; <i>Bmp10</i> <sup>ΔH</sup>	NR	NR	NR	NR	NR	NR	Decrease <sup>111</sup>	NR	NR
Anti-BMP9 and anti-BMP10 antibodies postnatal	NR	NR	NR	Hyper-vascularization <sup>112,30</sup> AVM <sup>112,72</sup>	NR	Intestines <sup>72</sup>	NR	NR	NR
CONCLUSIONS	Specific to BMP10 (embryonic stage)	BMP9 or BMP9-10		Redundant functions, not due to BMP9-10 heterodimer			Specific to BMP9		

KO: knockout; KD: Knockdown induced by tamoxifen injection; BMP9-10: heterodimers of BMP9 and BMP10; NR: Not Reported

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#### Acknowledgements:

The authors thank Nicolas Ricard and Tala Al Tabosh for reading the manuscript. The team is supported by the French National Agency for Research (ANR) (ANR-17-CE14-0006, ANR-20-CE14-0002-02) and by the National Institute for Health and Medical Research (INSERM), the University of Grenoble, by the CEA (commissariat à l'Énergie Atomique et aux énergies Alternatives, DRF/IRIG/DS), the Fondation pour la Recherche Médicale (EQU202003010188), H2020-msca-ITN-2018 (V.A.Cure-814316), the Association Maladie de Rendu-Osler (AMRO/HHT France). This project received funding from GRAL, a program from the Chemistry Biology Health (CBH) Graduate School of University Grenoble Alpes (ANR-17-EURE-0003)

#### Conflict of interests:

The authors declare no conflicts of interests.

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