

# BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain

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**Abstract** Bone Morphogenetic Protein 9 (BMP9) has been recently found to be the physiological ligand for the activin receptor-like kinase 1 (ALK1), and to be a major circulating vascular quiescence factor. Moreover, a soluble chimeric ALK1 protein (ALK1-Fc) has recently been developed and showed powerful anti-tumor growth and anti-angiogenic effects. However, not much is known concerning BMP9. This prompted us to investigate the

human endogenous sources of this cytokine and to further characterize its circulating form(s) and its function. Analysis of BMP9 expression reveals that BMP9 is produced by hepatocytes and intrahepatic biliary epithelial cells. Gel filtration analysis combined with ELISA and biological assays demonstrate that BMP9 circulates in plasma (1) as an unprocessed inactive form that can be further activated by furin a serine endoprotease, and (2) as a mature and fully active form (composed of the mature form associated with its prodomain). Analysis of BMP9 circulating levels during mouse development demonstrates that BMP9 peaks during the first 3 weeks after birth and then decreases to 2 ng/mL in adulthood. We also show that circulating BMP9 physiologically induces a constitutive Smad1/5/8 phosphorylation in endothelial cells. Taken together, our results argue for the role of BMP9 as a hepatocyte-derived factor, circulating in inactive (40%) and active (60%) forms, the latter constantly activating endothelial cells to maintain them in a resting state.

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## Introduction

Bone Morphogenetic Proteins (BMPs) are members of the TGF- $\beta$  superfamily that were originally characterized by their ability to induce endochondral bone formation. There are at least 20 BMP family members, which are involved in numerous biological processes during embryonic development and adult life. These include pluripotency of embryonic stem cells, dorsoventral patterning of the mesoderm, osteoblastic differentiation, bone homeostasis and, more recently, angiogenesis [12, 23, 40].

BMPs are synthesized as large precursors of about 400–500 amino acids consisting of an N-terminal signal peptide directing secretion, a prodomain for proper folding, and a C-terminal mature peptide [4]. Carboxyterminal mature proteins are known to be proteolytically cleaved upon dimerization from the prodomain at an Arg-X-X-Arg sequence by serine endoproteases (in the case of BMP4 by furin, PC6 and PC7) [27]. Following cleavage, the prodomain remains non-covalently associated with the mature active BMP dimer for most BMPs except for BMP2. While most of these complexes are inactive, some, such as BMP7 or BMP9, have been shown to be active [5, 17]. Secreted BMPs transduce their signal via a combination of transmembrane serine/threonine kinase type I and type II receptors. Three type II receptors [Bone Morphogenetic Receptor type 2 (BMPR2), Activin receptor type 2A (ACTR2A) and type 2B (ACTR2B)], and four type I receptors [Activin receptor-Like Kinase (ALK1, ALK2, ALK3 and ALK6)] are involved in BMP signaling [26, 33]. Following ligand-induced receptor hetero-oligomerization, type I receptors are transphosphorylated and activated by type II receptors. These activated receptor complexes phosphorylate the receptor-regulated Smads (R-Smads: Smad1, Smad5 and Smad8), enabling the formation of a complex with the co-Smad, Smad4. R-Smad/Smad4 complexes translocate into the nucleus and regulate target gene expression by binding to regulatory elements and recruiting transcriptional co-repressor and/or activation complexes on their promoters. BMPs can also stimulate non-Smad signaling pathways [26, 33].

BMP9, also known as Growth and Differentiation Factor 2 (GDF2), has recently been brought to the forefront with the characterization of its binding to ALK1 and therefore its functions in angiogenesis [13, 14, 32]. BMP9 was initially cloned from a mouse liver cDNA library [7], and it was shown in rats to be predominantly expressed in the liver by non-parenchymal cells (i.e., endothelial, Kupffer and stellate cells) [24]. BMP9 was first described as a hematopoietic, hepatogenic, osteogenic and chondrogenic factor [6, 21, 30, 34]. It has also been identified as a regulator of glucose metabolism [8], and as a differentiation factor for cholinergic neurons in the central nervous system [22]. Three years ago, we showed that BMP9 binds with a strong affinity to ALK1, a TGF $\beta$  family type 1 receptor that is preferentially expressed by endothelial cells suggesting a role in angiogenesis [14]. And, indeed, it was further demonstrated by others as well as us that BMP9 is a potent regulator of angiogenesis and vascular tone [13, 14, 32, 36]. We also showed that BMP9 is present in human plasma at a concentration of 2–12 ng/mL, which is above its EC<sub>50</sub>, suggesting that circulating BMP9 could play a physiological role on the endothelium [13, 14]. Interestingly, the soluble form of ALK1 has recently been found to

be a powerful inhibitor of angiogenesis and tumor growth [11, 25] and, as such, a new therapeutic target in cancer. As BMP9 is the only known circulating ALK1 ligand, soluble ALK1 probably works by blocking circulating BMP9. Taken together, these data suggest that circulating BMP9 could play a major role in vascular quiescence, but even so not much is known about BMP9 expression.

The aims of the study were therefore to investigate the cellular source of BMP9 in mouse and human and to characterize the biochemical composition of the circulating forms of BMP9. In the present work, we show that BMP9 is predominantly expressed in the liver, and, in contrast to what had been previously described in rats [24], BMP9 is produced by hepatocytes and biliary epithelial cells in human and mouse liver. We also demonstrate that human and murine BMP9 circulates under both an active form (60%) (mature BMP9 associated with its prodomain) and an inactive form (40%) (unprocessed dimeric pro-BMP9) that can be further activated by recombinant furin. Analysis of BMP9 levels in blood during mouse development showed undetectable levels at E9.5, and a high level around birth and during the first 3 weeks (6 ng/mL), which then decreased and remained at a level of around 2 ng/mL throughout adulthood. We also demonstrate that circulating BMP9 induces a constant activation of Smad1/5/8 phosphorylation in endothelial cells, confirming its role as a major factor involved in the control of vascular quiescence.

## Materials and methods

### Blood and tissue donors

Between December 2006 and July 2007, blood samples (7 mL) were taken from 20 healthy volunteers (8 women, 12 men; mean age, 44  $\pm$  10 years) from whom plasmas (K3E tubes; Becton–Dickinson, Pont de Claix, France) were prepared. Plasma aliquots were frozen at  $-20^{\circ}\text{C}$ . Human liver samples for RNA and immunostainings were obtained from histological normal livers taken from patients suffering of metastatic colorectal cancers. Informed consent was obtained from all donors. The investigation conformed to the principles outlined in the Helsinki declaration.

### Animal care

Blood was taken from OF1 mouse embryos, pups and adults into EDTA-coated tubes (Becton–Dickinson) and plasmas were frozen until BMP9 measurement. Inhalational anesthesia with isoflurane was performed before blood puncture. Eight to ten embryos or pups and 3 adult mice were requested for each developmental stage. Wistar female rats were

anaesthetized with pentobarbital (60 mg/kg). Descending thoracic aorta was then quickly excised and fixed in paraformaldehyde 4%. All animal studies were approved by the institutional guidelines and those formulated by the European Community for the Use of Experimental Animals.

### Reagents

Recombinant human BMP9, recombinant human ALK1 extracellular domain-Fc fusion protein (ALK1ecd) and recombinant furin were purchased from R&D Systems (Abingdon, UK). Anti-BMP9 antibodies were purchased from Biogenesis (1406–1460; AbCys, Paris, France) and from R&D Systems (MAB3209, BAF3209, AF3209, AF3879), anti-phosphoSmad1/5/8 antibody was purchased from Cell Signaling Technology (#9511; Danvers, MA, USA).

### Reporter gene constructs

The reporter plasmid pGL3(BRE)<sub>2</sub>-luc encoding firefly luciferase downstream of a BMP response element [20] was kindly provided to us by Dr P. ten Dijke (Leiden University Medical Center, Leiden, the Netherlands). The pcDNA3-derived expression plasmid pALK1 encoding human ALK1 was kindly provided by Dr C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The pRL-TK-luc plasmid encoding renilla luciferase downstream of the thymidine kinase promoter was purchased from Promega (Madison, WI, USA).

### Cell culture

NIH-3T3 fibroblast cells were maintained in DMEM, 4.5 g/L glucose (Invitrogen, Carlsbad, CA, USA) as previously described [31].

### Real-time polymerase chain reaction analysis

Total RNA from 20 human tissues was purchased from Clontech (Human total RNA master panel II, batch 1001243A). Total RNAs from the human liver and from the different liver cell subtypes were also obtained from two histological normal biopsies taken from colorectal cancer patients after extraction using TRIzol reagent (Invitrogen). Reverse transcription was performed on 1 µg total RNA with Superscript II-RnaseH reverse transcriptase (Invitrogen, Cergy Pontoise, France). We also used cDNA from human hepatocytes, human hepatic stellate cells, human hepatic sinusoidal endothelial cells and human intrahepatic biliary epithelial cells bought from ScienCell Research Laboratories (Carlsbad, CA, USA). BMP9 quantitative RT-PCR was performed with the Light Cycler-

FastStart Master plus SYBR Green I (Roche Diagnostics, Meylan, France) on the Light Cycler apparatus (Roche Diagnostics) using the following primers: 5'-GACGTCC GATAAGTCGACTACGC-3' and 5'-AAGATGTGCTTCT GGAAGGGGAA-3'. The results were normalized to HPRT mRNA expression level using the following primers: 5'-TTG AGC ACA CAG AGG GCT ACA ATG-3' and 5'-ATG GAC AGG ACT GAA CGT CTT GCT-3' or to RPL13a mRNA expression level using the following primers: 5'-TTC CTT GCT CCC AGC TTC CTA TGT-3' and 5'-TTA ATT CCT CAT GCG TTG CCT GCC-3'.

### Immunostaining and image acquisition

Formalin-fixed and paraffin-embedded human and mouse liver sections and rat aorta cross-sections were dewaxed in xylene, rehydrated through graded alcohols to water and then subjected to heat-induced epitope retrieval in sodium citrate buffer in a microwave. The liver sections were stained overnight with anti-BMP9 antibodies (Biogenesis, dilution 1:500, or MAB3209, R&D Systems, dilution 1:50) in Dako Antibody Diluent (Dako, Glostrup, Denmark). The rat aortic cross-sections were stained overnight with anti-PhosphoSmad1/5/8 antibody (Cell signaling, dilution 1:100) in 5% goat serum. For liver sections using the anti-BMP9 from Biogenesis, immunoreactivity was visualized using EnVision + system horseradish peroxidase kit (Dako) following the manufacturer's instructions. For liver sections using the anti-BMP9 from R&D Systems and aortic cross-sections, immunoreactivity was visualized using ABC reagent and DAB reagent (Vecstatain ABC kit; Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with haematoxylin and mounted in Fluor Save Reagent (Calbiochem) before microscopy. The slides were observed using a Zeiss AxioPlan light microscope (Zeiss, Oberkochen, Germany). Images were captured with a computer-supported AxioCam camera and analyzed using Axiovision 4.7 software (Zeiss).

### Size exclusion chromatography

Amount of 250 µL of plasma or 1 ng of recombinant human BMP9 were run in a final volume of 500 µL (PBS; flow rate: 0.5 mL/min; detector: UV-280 nm) through a Superdex-200 HR 10/30 connected to Fast Liquid Chromatography system (BioLogic; Biorad, Hercules, CA, USA), and 50 fractions of 500 µL were collected. The column was calibrated by successive injections of the following molecular mass markers [Dextran blue (2 GDa); Thyroglobulin (669 kDa); Ferritin (443 kDa); Immunoglobulin (160 kDa); Bovine serum albumin (68 kDa); Carbonic anhydrase (29 kDa); and acetone (0.1 kDa)] all purchased from Sigma-Aldrich (St. Louis, MO, USA).

### ALK1-BRE-luciferase assay

NIH-3T3 cells (70,000 cells/well in 24-well plates) were transfected in Opti-MEM (Invitrogen) using lipofectamine (Invitrogen) with 0.2 µg of pGL3(BRE)<sub>2</sub>-luc, 0.02 µg of pRL-TK-luc and 0.01 µg of pALK1. Four hours after transfection, cells were treated for 15 h with human recombinant BMP9, or diluted plasma (0.5%), or plasma fractions. Neutralizing anti-BMP9 antibodies (1 µg/mL, AF3209; R&D Systems) were incubated with plasma or fractions when indicated. A standard curve (2.5–40 pg/mL) of recombinant human BMP9 (R&D Systems) was performed. Firefly and renilla luciferase activities were measured sequentially with the Dual-Luciferase reporter assay (Promega). Results are expressed as ratios of firefly luciferase activity over renilla luciferase activity.

### Enzyme-linked immunosorbent assay (ELISA)

A 96-well microplate was coated with the capture antibody MAB3209 (dilution 1:500; R&D Systems) for the BMP9 ELISA or the capture antibody AF3879 (dilution 1:200; R&D Systems) for the pro-BMP9 ELISA and incubated overnight at room temperature. After blocking for 90 min with 5% BSA in PBS-Tween 20 0.5% and 4 washes (PBS-Tween 20 0.05%), samples were added (100 µL/well) in duplicate for overnight incubation at room temperature. A standard curve (10–640 pg/mL) using twofold serial dilutions of recombinant human BMP9 (R&D Systems) in PBS-Triton 0.5% was performed for the BMP9 ELISA. The washing step was repeated and the detection antibody BAF3209 (dilution 1:500; R&D Systems) was added (100 µL/well) and incubated for 2 h at room temperature. After four washes, incubation with Streptavidin-horseradish peroxidase for 30 min was followed by four washes and the enzymatic activity was detected with Substrate Solution (mixture 1:1; R&D Systems) for 1 h in the dark. The reaction was stopped by adding 100 µL of 2 N H<sub>2</sub>SO<sub>4</sub>. Plates were read at 450 and 620 nm wavelengths on a microplate reader (Multiskan EX; Thermo Labsystems). For the pro-BMP9 ELISA, data are presented as optical densities, as recombinant pro-BMP9 was not available as a standard. The following BMPs (BMP10, BMP2, BMP4, BMP5, BMP7, BMP8b, BMP13), prepared at 50 ng/mL, were assayed in the BMP9 ELISA and exhibited no cross-reactivity or interference.

### Albumin removal

Albumin removal and albumin elution were realized using Vivapure Anti-HSA resin following the manufacturer's instructions (Sartorius Biotech, Aubagne, France). Three microliter of plasma were diluted in binding buffer and

added to a Vivaclear spin column containing Vivapure affinity resin for 15 min. The columns were then centrifuged to recover the albumin-depleted sample. Albumin was eluted from the column in 0.1 M Glycine/HCl pH 2.5. These solutions were then gel-filtered on Sephadex G50 column equilibrated in DMEM 4.5 g/L glucose. BMP9 activity was determined using the ALK1-BRE-luciferase assay as indicated above.

### Furin treatment

Plasmas or plasmatic fractions were incubated for 2 h at 30°C in 100 mM Tris, 1 mM CaCl<sub>2</sub>, pH 9.0 in the presence or absence (control) of 0.6 µg/mL of recombinant human furin (1503-SE; R&D Systems). BMP9 concentrations were determined using the ALK1-BRE-luciferase assay and/or the BMP9 ELISA, as indicated above.

### Statistics

Data analysis and statistical tests were performed using unpaired Student's *t* test.

## Results

### Sites of BMP9 expression

Analysis of BMP9 mRNA expression in 20 different human tissues (Total RNA Master panel from Clontech) using HPRT mRNA levels for normalization revealed that BMP9 is mostly expressed in the liver, and also, although at a much lower level, in the brain and the lungs (more than 100-fold less) (Table 1; only 9 tissues are shown, the others did not express BMP9 mRNA). This high level of BMP9 mRNA was confirmed in two other livers obtained from histological normal biopsies taken from colorectal cancer patients (data not shown). Analysis of BMP9 mRNA expression in the different liver cell-types (hepatocytes, hepatic stellate cells, hepatic endothelial cells, and intra-hepatic biliary epithelial cells (all obtained from ScienCell) demonstrated that BMP9 is expressed by hepatocytes and biliary epithelial cells (Table 2). Similar results were obtained from the analysis of isolated cells derived from two histological normal biopsies taken from colorectal cancer patients (data not shown).

To confirm the hepatocyte and biliary epithelial cell BMP9 expression at the protein level, we performed immunohistochemical staining on human liver sections obtained from normal biopsies taken from colorectal cancer patients. We tested several anti-BMP9 antibodies commercially available and two antibodies gave specific BMP9 staining which could be competed by an excess of

**Table 1** BMP9 mRNA expression in nine human tissues

Human tissues	BMP9mRNA/HPRTmRNA <sup>a</sup>
Liver	0.275748
Lung	0.000693
Brain	0.000590
Skeletal muscle	0.000237
Bone marrow	0.000081
Spleen	0.000080
Prostate	0.000052
Small intestine	0.000035
Spinal cord	0.000024

<sup>a</sup> Quantitative RT-PCR analysis of BMP9 mRNA expression in nine human tissues obtained from Clontech performed in duplicates. Results are shown as the mean BMP9 mRNA level normalized to HPRT mRNA levels

**Table 2** BMP9 mRNA expression in the different cell subtypes of human liver

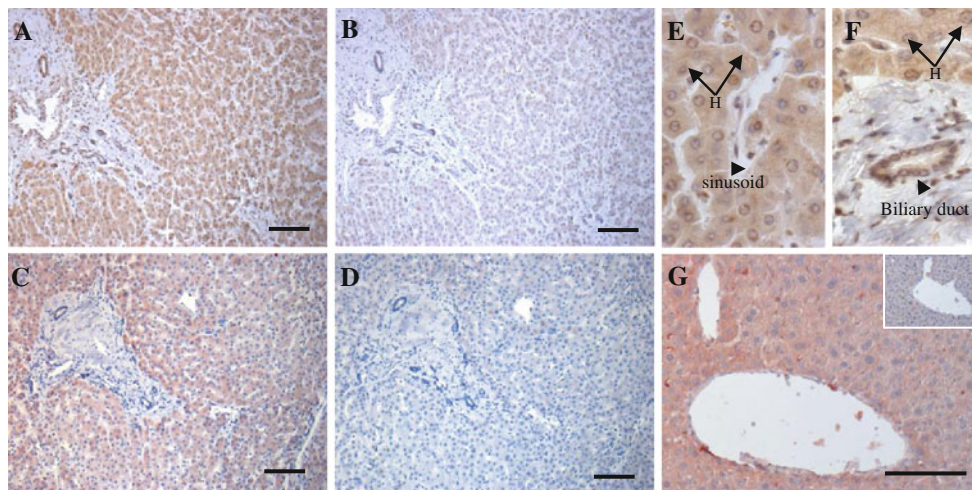
	BMP9mRNA/HPRTmRNA <sup>a</sup>
Intrahepatic biliary epithelial cells	0.038218
Hepatocytes	0.005638
Hepatic stellate cells	0.000343
Hepatic sinusoidal endothelial cells	0.000229

<sup>a</sup> Quantitative RT-PCR analysis of BMP9 expression levels in hepatocytes, hepatic stellate cells, hepatic sinusoidal endothelial cells and intrahepatic biliary epithelial cells obtained from ScienCell performed in duplicates. Results are shown as the mean BMP9 mRNA level normalized to HPRT mRNA levels

recombinant human BMP9 protein (Fig. 1a, c vs. b, d, respectively). Figure 1 shows BMP9 staining in hepatocytes (a, c, e, and f at a higher magnification) and biliary epithelial cells (f) while the blood vessels (sinusoids, large veins and arteries) and the mesenchyme are negative (Fig. 1a, c, and e at a higher magnification). A similar pattern was obtained in mouse liver sections (Fig. 1g, inset corresponds to control IgG).

#### Biochemical characterization of BMP9 circulating forms

BMP9 is synthesized as a precursor protein (pre-pro-BMP9) composed of 429 amino acids (aa) that includes a 22 aa signal peptide, a 297 aa prodomain (33 kDa) and a 110 aa mature protein (12.5 kDa). It is then cleaved by serine endoproteases, leading to a short dimeric mature form of 25 kDa, and the prodomain which can remain non-covalently associated with the mature short form, giving a size of about 100 kDa (Fig. 2a). We first estimated the molecular mass of plasmatic BMP9 through gel permeation chromatography. For this, we applied human plasma onto a Superdex 200 column. After filtration, the BMP9 activity of each fraction was measured using the ALK1-BRE-luciferase assay. BMP9 activity was detected in fractions 33–35 and peaked in fraction 34, corresponding to an approximate molecular mass of  $100 \pm 20$  kDa (Fig. 2b). When we applied recombinant mature BMP9 to this same column, its activity was present in fractions 38–41 and peaked in fraction 40, corresponding to an approximate



**Fig. 1** Origin of BMP9 expression. Immunostaining for BMP9 in human (a–f) and mouse (g, inset corresponds to rabbit control immunoglobulins) liver sections. Human and mouse tissue sections were stained with anti-BMP9 antibodies from R&D Systems (a, b, e, f, g) or from Biogenesis (c, d). The specificity of the staining was checked by addition of an excess of recombinant human BMP9 (1  $\mu$ g/mL) (b, d). Note the strong immunostaining in hepatocytes and

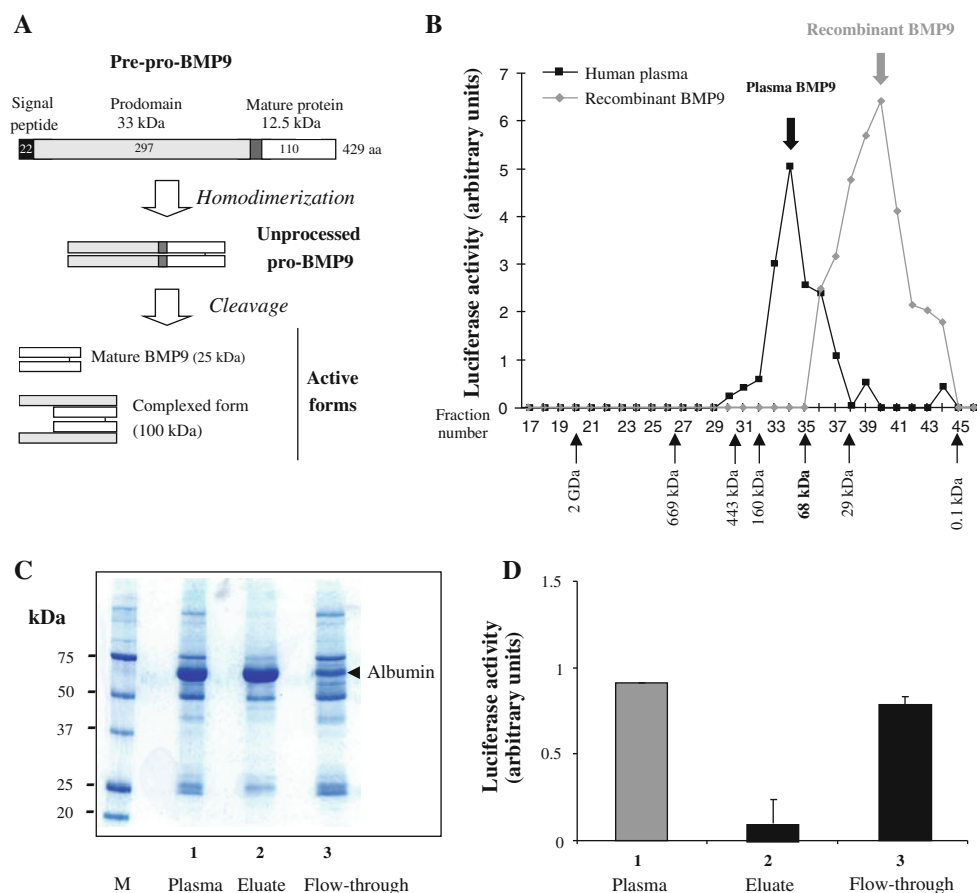
biliary ducts. In e, enlargement of a blood sinusoid [arrowhead indicates absence of staining in liver endothelial cells and arrows indicate staining in hepatocytes (H)]. In f, enlargement of a biliary duct [arrowhead indicates intrahepatic biliary epithelial cells and arrows indicate staining in hepatocytes (H)]. Slides were counterstained with haematoxylin. Scale bars 100  $\mu$ m

molecular mass of  $25 \pm 5$  kDa. Taken together, these results demonstrated that BMP9 is not circulating as a short mature form of 25 kDa and suggested that BMP9 is associated with other proteins.

As the peak of elution of albumin is centered on fraction 35, i.e., very close to the peak of BMP9 activity, we wondered whether albumin could be associated with BMP9. For this, plasma was depleted of albumin using a Vivapure anti-albumin resin that retained most of the albumin present in the samples, as shown when comparing lane 3 (flow-through) with lane 2 (eluate) and lane 1 (plasma) of Fig. 2c. We then analyzed the BMP9 activity of these fractions. There was no BMP9 activity in the albumin fraction (lane 2) while BMP9 activity was still present in the fraction devoid of albumin (lane 3),

demonstrating that BMP9 is not associated with albumin (Fig. 2d).

The observed molecular mass of the circulating form of BMP9 is around 100 kDa, which could correspond to the complexed BMP9 form [prodomain ( $2 \times 35$  kDa) + mature peptide ( $2 \times 12.5$  kDa)]. To test this hypothesis, we developed an ELISA that can recognize the propeptide complexed to the mature peptide using an anti-human BMP9 propeptide antibody as a capture antibody and a biotinylated polyclonal anti-human mature BMP9 protein as a detection antibody (pro-BMP9 ELISA). Using this ELISA, a peak of immunoreactivity was detectable in the same fractions that were positive in the ALK1-BRE-luciferase assay (33–35), supporting the hypothesis that mature BMP9 circulates as a large complex (the mature

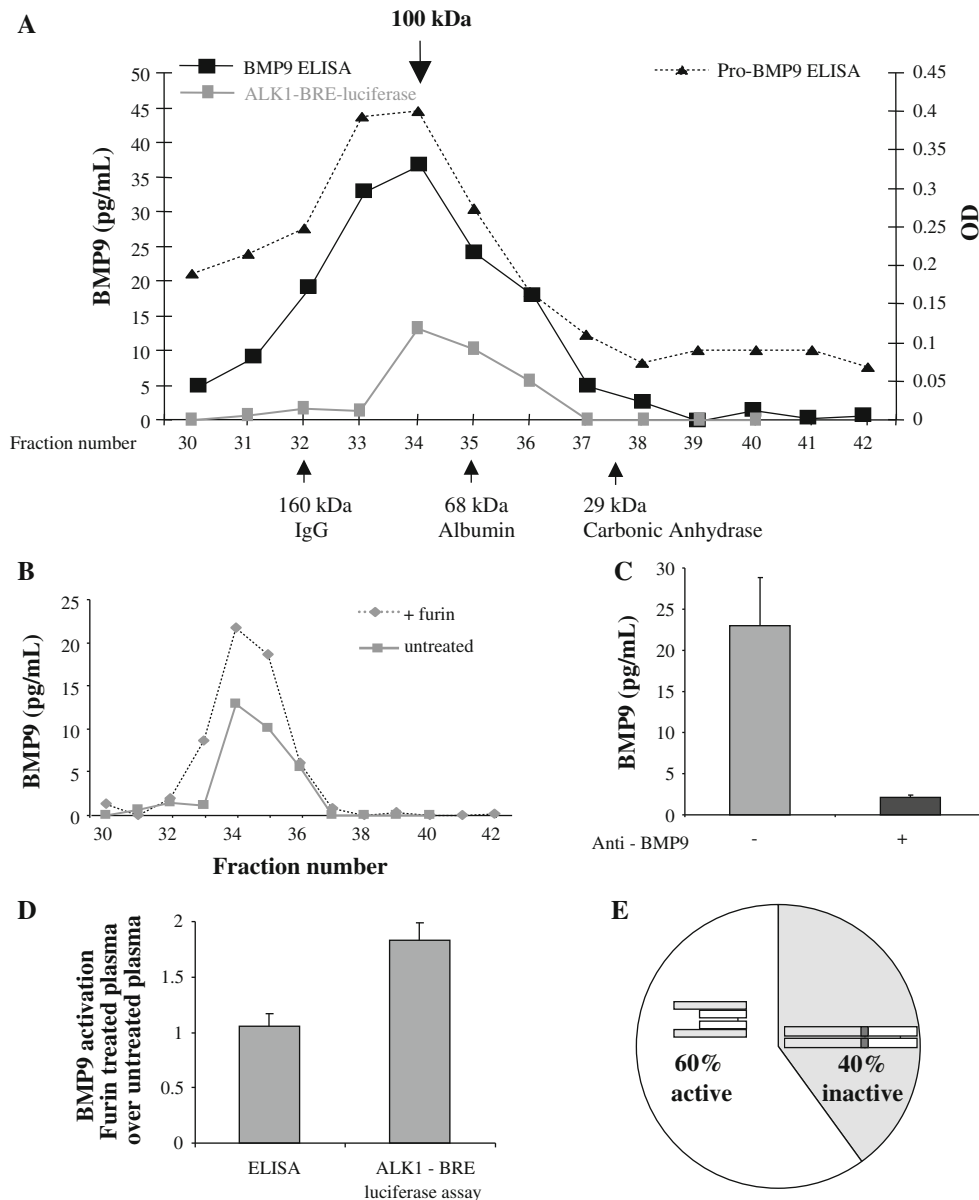


**Fig. 2** BMP9 circulates in plasma under a high molecular mass form. **a** BMP9 biosynthesis. BMP9 is synthesized as a precursor protein (*Pre-pro-BMP9*) composed of 429 amino acids (aa) that include a 22 aa signal peptide, a 297 aa prodomain (33 kDa) and a 110 aa mature protein (12.5 kDa). The pre-pro-BMP9 then homodimerizes (pro-BMP9) and is subsequently cleaved by serine endoproteases. This generates two active forms: the short mature form (25 kDa) and the complexed form (100 kDa) in which the prodomain remains associated with the mature form. **b** Proteins from human plasma were separated by gel filtration chromatography. In a parallel experiment, recombinant human BMP9 was passed onto the same column. BMP9

activity was then measured in the different fractions using the ALK1-BRE-luciferase assay as described in “Materials and methods”. The data from one representative experiment (out of 4) are represented. **c** and **d** Plasma was passed through an anti-human serum albumin column. The plasma (1), the eluate (2) and the flow-through (3) were then analyzed by 10% SDS-PAGE and stained by Coomassie blue (**c**) and their BMP9 activity using the ALK1-BRE-luciferase assay was also quantified as described in “Materials and methods” (**d**). Data are expressed as the mean luciferase value  $\pm$  SD obtained from two independent experiments

form complexed to its prodomain) (Fig. 3a). We also developed an ELISA using a monoclonal anti-human mature BMP9 antibody as a capture antibody and the

previously used biotinylated polyclonal anti-human mature BMP9 protein as a detection antibody (BMP9 ELISA). Using this ELISA, we also detected circulating BMP9 in



**Fig. 3** BMP9 circulates in plasma under both active and inactive high molecular mass forms. **a** Proteins from a pool of five human plasmas were separated through gel filtration chromatography. BMP9 levels were then measured in the different fractions by three different means: the ALK1-BRE-luciferase assay, the BMP9 ELISA and the pro-BMP9 ELISA as described in “Materials and methods”. The ALK1-BRE-luciferase assay data and the data obtained from the BMP9 ELISA are presented as pg/mL of BMP9. The data obtained with the pro-BMP9 ELISA are presented as optical densities (OD). The data obtained in one representative experiment out of 3 are presented. **b, c** Human fractions (30–42) were treated with or without furin and BMP9 levels were then measured in the different fractions with the ALK1-BRE-luciferase assay (fraction 34, corresponding to

the peak of BMP9 activity, was also measured **c** in the absence (gray square) or presence of anti-BMP9 neutralizing antibodies (black square). The ALK1-BRE-luciferase assay data are presented as pg/mL of BMP9. The data obtained in one representative experiment out of 2 are presented. **d** Nine human plasma (0.3%) were treated with or without furin and BMP9 activation was then measured using the ALK1-BRE-luciferase assay and the BMP9 ELISA as described in “Materials and methods”. Data are expressed as furin-treated plasma over untreated plasma mean ± SEM from duplicate determinations. **e** Pie chart representing the percentage of each BMP9 complex circulating in human plasma, as calculated from (d) measured with the ALK1-BRE luciferase assay

the fractions 33–35 (Fig. 3a) but not in the fractions 38–41 corresponding to the short mature form (Fig. 2b), confirming that there is no free mature BMP9 circulating in plasma.

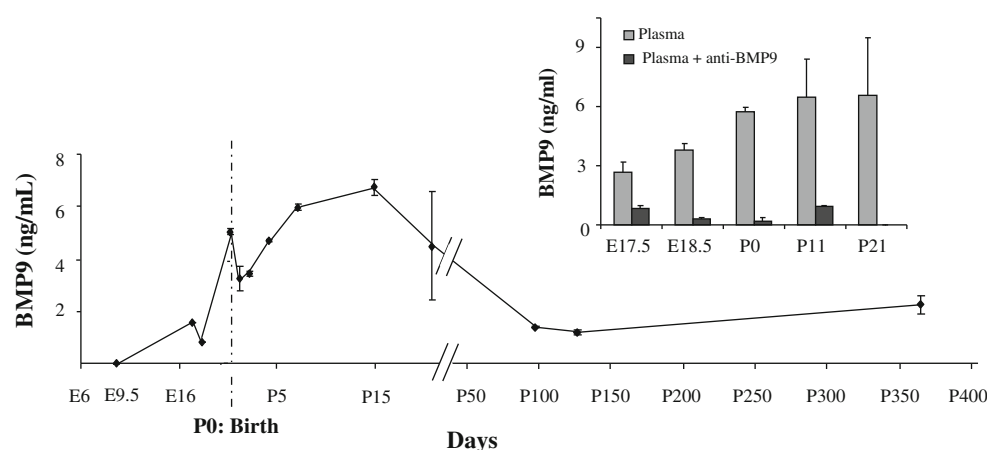
Interestingly, we always measured lower levels of circulating BMP9 using the cellular ALK1-BRE-luciferase assay, which measures only active BMP9, than with the BMP9 ELISA (12.9 vs. 36.9 pg/mL, respectively, calculated from fraction 34 in Fig. 3a). As some members of the BMP family such as myostatin have been shown to be expressed as immature unprocessed proteins [41], we wondered whether BMP9 might be circulating under both an activated form and an inactive unprocessed form. To test this, we used recombinant furin, a member of the proprotein serine endoprotease family that is known to proteolytically process the precursor forms of the TGF $\beta$ /BMP superfamily members [10]. Addition of recombinant furin to the different gel filtration fractions increased the BMP9 activity measured by the ALK1-BRE-luciferase assay (Fig. 3b). This suggested that some BMP9 is circulating as an inactive unprocessed form. To make sure that the increase in the BRE activity measured in presence of furin is due to BMP9, we added neutralizing anti-BMP9 antibody and showed that it completely inhibited this furin-revealed activity, which can therefore be totally attributed to BMP9 (Fig. 3c). Addition of furin to unfractionated plasma also significantly increased the level of active BMP9 measured with the ALK1-BRE-luciferase assay, while it did not change the level of BMP9 measured with the ELISA (Fig. 3d). This allowed us to determine that, in human adult plasma, 60% of BMP9 is circulating under an active complexed form and 40% under an inactive

unprocessed form (Fig. 3e). We were not able to confirm the presence of these two circulating forms by western blot analysis in human plasma due to insufficient sensitivity. However, western blot analysis of the supernatant of 293 cells expressing recombinant BMP9 showed the presence of mature BMP9, BMP9 prodomain, and uncleaved BMP9 (Suppl Fig. 1). These data support that BMP9 can be secreted in an uncleaved form as we show in plasma.

We also analyzed the circulating form of BMP9 in murine plasma. We could show that it also circulates as a large complex, peaking in fractions 33–35, as human plasma (Suppl Fig. 2) and that this large complex could be activated by furin (data not shown). These data suggest that BMP9 circulating forms in human and mouse are identical.

#### Ontogeny of circulating blood BMP9 levels during murine development

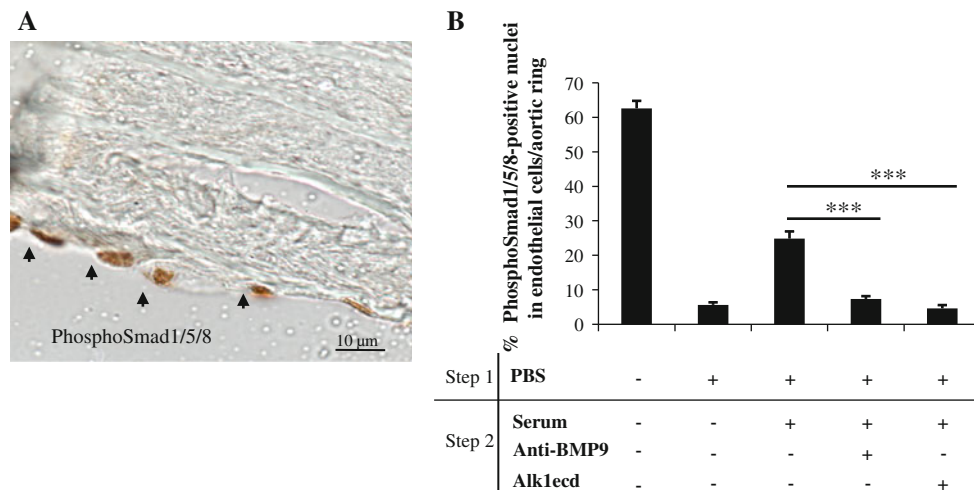
We next measured circulating BMP9 levels in mouse plasmas throughout development from embryonic day 9.5 up to adulthood (1 year) using the ALK1-BRE-luciferase assay. For the assay, plasmas were diluted to 0.5% in DMEM. We found that BMP9 was absent from plasma in E9.5 embryos, was present in E17.5 embryos, peaked in newborn mice (6 ng/mL), and remained high for the first three postnatal weeks before decreasing in adult mice (2 ng/mL) (Fig. 4). The use of BMP9 neutralizing antibodies proved that the measured activity is only attributable to BMP9 (inset in Fig. 4). We could further demonstrate that there was no difference in BMP9 circulating levels between males and females, nor any variation during the circadian cycle (data not shown).



**Fig. 4** Ontogeny of BMP9 circulating levels in mice. BMP9 levels were measured from pooled diluted plasma (0.5%) taken from mice at the indicated developmental stages using the ALK1-BRE-luciferase assay as described in “Materials and methods”. In order to check that the activity measured by the ALK1-BRE-luciferase assay was

attributable to BMP9, the assay was performed (*inset*) in the absence (*gray squares*) or the presence of anti-BMP9 neutralizing antibodies (*black squares*). The results are presented as means  $\pm$  SD from triplicate determinations (*E* embryonic day, *P* post-natal day)





**Fig. 5** Rat aortic endothelial cells are physiologically Smad1/5/8 phosphorylated in response to circulating BMP9. **a** Immunostaining for phosphoSmad1/5/8 of rat aorta cross-sections. The aortas were fixed immediately after surgical removal from the killed animal and processed for phospho-Smad1/5/8 immunostaining. Note the nuclear staining of endothelial cells. **b** Quantification of the number of phosphoSmad1/5/8-positive nuclei. Rat aortic slices were either fixed immediately after sacrificing the animal or incubated ex vivo for 1 h

with PBS (step 1) and then for another hour with rat serum in the absence or presence of either anti-BMP9 neutralizing antibodies or recombinant ALK1ecd (step 2). The rat aorta slices were then fixed and immunostained for phosphoSmad1/5/8. Results are presented as the percentage of phosphoSmad1/5/8 nuclei in endothelial cells per aortic ring. Data are expressed as the mean  $\pm$  SD of values obtained in 3 independent experiments (\*\* $p < 0.001$ )

Endothelial cells from rat aorta cross sections are Smad1/5/8 phosphorylated by circulating BMP9

We have previously shown that BMP9 is present in human adult plasma at levels (2–12 ng/mL) above its  $EC_{50}$  (50 pg/mL) for ALK1 [13]. This implies that circulating BMP9 should constantly activate the Smad1/5/8 pathway in endothelial cells. To demonstrate this, we performed immunostaining for phospho-Smad1/5/8 on rat aortic rings fixed immediately after sacrifice. We observed that most nuclei of endothelial cells were positively marked by the anti-phosphoSmad1/5/8 antibody in the rat aortic cross sections (60–80%; Fig. 5a). Interestingly, we could show that ex vivo incubation of this rat aorta section for 1 h in PBS resulted in a strong decrease of phosphoSmad1/5/8 immunoreactivity, whereas subsequent incubation in the presence of serum for 1 h could partially restore it (Fig. 5b). When we added serum together with either a BMP9 neutralizing antibody or recombinant ALK1ecd, this immunoreactivity was not restored (Fig. 5b). Taken together, these data clearly demonstrate that circulating BMP9 induces a permanent physiological activation of the Smad1/5/8 pathway in endothelial cells.

## Discussion

The aim of the present work was to better characterize BMP9 expression in humans and mice. In agreement with a

previous work, reporting that BMP9 expression occurs predominantly in the rat's liver [24], we show here that BMP9 mRNA is predominantly expressed in human and mouse liver tissues. However, in contrast to this previous work [24], we did not detect BMP9 in hepatic endothelial cells nor in hepatic stellate cells but rather in hepatocytes and biliary epithelial cells. As hepatocytes are much more abundant than biliary epithelial cells, our data suggest that hepatocytes are the major cellular source of circulating BMP9. We have previously shown that BMP9 binds with a high affinity to ALK1 [14], which is predominantly expressed on endothelial cells; thus our data would suggest that BMP9 acts mostly in a paracrine manner. ALK1 mutations are linked to a genetic vascular disease named hereditary hemorrhagic telangiectasia (HHT), which affects vessels in specific tissues [15]. It is interesting to note that we detected BMP9 mRNA levels in the liver, but also, although to a much lower level, in the lungs and the brain, which are the three tissues that are mostly affected in HHT. This could suggest a local role for BMP9 in the development of the disease. It is also interesting to note that BMP9 mRNA level was 100 times and 10 times less abundant in isolated cells (hepatocytes and biliary epithelial cells, respectively) than in liver tissue (Tables 1 and 2). Similar data were obtained using two different reporter genes for normalization (HPRT and RPL13a, data not shown). These differences might be due to mRNA degradation occurring during cell isolation procedures and could suggest that BMP9 mRNA might have a very rapid

turnover. Further work will be necessary to understand the regulation of BMP9 expression.

The analysis of the BMP9 circulating forms in human and murine plasma by ELISA and luciferase assay after gel filtration demonstrated that BMP9 is circulating as a high molecular mass complex of around 100 kDa. Using an ELISA with an antibody directed against BMP9 prodomain and another antibody directed against BMP9 mature form, we could demonstrate that this 100 kDa complex is composed of BMP9 mature short form and BMP9 prodomain. Further, using the serine endoprotease furin which cleaves the prodomain, we showed that we could increase the level of active BMP9 detected by the luciferase assay without modifying the level of BMP9 detected with the BMP9 ELISA. Taken together, these data suggested that BMP9 circulates as two large complexes. The first complex consists of the short mature BMP9 dimer non-covalently associated to two BMP9 prodomains. The second complex consists of the dimeric uncleaved form of BMP9. Because of the estimated size of the complex, we can rule out that other proteins such as fibrillin or Latent TGF $\beta$  binding protein 1 are associated with mature BMP9, as has been shown for BMP7 [17]. We could also experimentally exclude albumin as a potential binding protein (Fig. 2d). We also found that the addition of recombinant mature BMP9 to plasma did not induce a shift in its apparent molecular mass, suggesting that it does not bind to other circulating plasma proteins (data not shown). This first large complex is biologically active. This result is in accordance with a previous work that described that overexpressed recombinant BMP9 is secreted as a large complex comprising mature BMP9 and its prodomain and that this complex has a similar biological activity to that of mature BMP9 [5]. However, our work demonstrates for the first time that this complex does exist under physiological conditions. The significance of prodomain association lies in the observation that, following secretion, the complex is directly targeted to elements of the extracellular matrix. This has not yet been shown for the prodomain of BMP9 and it will be interesting to know in future if it binds to extracellular matrix proteins. Our data would suggest that it does not, as we find large amounts of circulating soluble BMP9. The second BMP9 circulating form is an inactive unprocessed form. Unfortunately, we were unable to perform western blots directed against BMP9 from plasma. However, we could show that the supernatant of 293 cells expressing recombinant BMP9 contains cleaved and uncleaved forms detected by using antibodies directed at the BMP9 prodomain and BMP9 mature form (Suppl Fig. 1). These two forms had already been shown by Coomassie staining from a supernatant of Chinese hamster ovary cell line expressing recombinant BMP9 [5]. This inactive form can be experimentally cleaved and activated by

recombinant furin, suggesting that circulating inactive BMP9 can be cleaved and activated extracellularly. Although extracellular maturation of TGF $\beta$  ligands is not believed to be the rule, several cases have been reported. Secretion of pro-TGF $\beta$ 1 has been observed in bovine adrenocortical cells [35]. GDF15 was also shown to be secreted as both processed and unprocessed forms [2]. Pro-myostatin has been reported to be secreted by skeletal muscle cells and cleaved extracellularly by furin proteases [1]. Pro-nodal is secreted in a different region of the embryo than the proprotein convertases and therefore first encounter each other in the extracellular space, and this was shown to be a critical step for localized nodal activation and early embryo patterning [3]. Seven members of the proconvertase family have been identified [9]. They have been shown to cycle between the trans-Golgi network and cell surface. Some are secreted and can be found in the extracellular matrix and as such could play a role in the spatiotemporal regulation of BMPs biological activity [37]. The role of this uncleaved inactive circulating BMP9 form is not yet clear. It might render the protein more stable, and/or it might allow its activation to occur locally where secreted proconvertases will be expressed. Our data demonstrate that we have here identified another potential control mechanism that might be important for regulating BMP9 bioavailability.

Analysis of BMP9 blood level during mouse development using the ALK1-BRE-luciferase assay shows that neither circulating BMP9 nor any other BMP activating the Smad1/5/8 pathway is detectable at E9.5 (Fig. 4). This is intriguing as ALK1 deletion is embryonic lethal at E10.5 [29, 38]. This suggests that the ALK1 ligand necessary at this embryonic stage might be stored and act locally. Interestingly, we detected circulating BMP9 at E17.5 and this level increases until birth (6 ng/mL) and remains high during the first 3 weeks after birth. This suggests that high circulating BMP9 might be necessary for post-natal development. In line with this hypothesis, it was recently shown that the blockade of ALK1 signaling resulted in defective vascular and lymphatic development in early postnatal mice [28]. BMP9 circulating levels decrease a few weeks after birth but remain around 2.5 ng/mL, i.e., higher than its EC<sub>50</sub> for ALK1 activation (50 pg/mL). This suggests that endothelial cells are constantly activated by BMP9. This is indeed what we observed when we looked at the Smad1/5/8 phosphorylation status of rat aortic cross-sections. This result is in accordance with a previous work that showed the presence of phosphorylated Smad1/5/8 in endothelial cells from various types of blood vessels [39]. These data further emphasize the role of circulating BMP9 on endothelial cells, which contributes to vascular quiescence in adults. Taken together, our results show that BMP9 is mainly produced by hepatocytes, and that it is circulating as inactive and active complexes controlling endothelial cell activation.

In the present work, we have characterized the circulating forms of BMP9 and developed two assays that allow the measurement of either total BMP9 (BMP9 ELISA) or active BMP9 (ALK1-BRE-luciferase assay) in blood. Other BMPs have been described to be present in human or bovine serum (BMP4 and BMP6) [18, 19]. However, it should be emphasized that, although the ALK1-BRE-luciferase assay allows the detection of all BMPs, as they all activate Smad1/5/8 phosphorylation, performing this assay in ALK1-overexpressing cells with very low quantities of serum or plasma (0.5%) allows the specific detection of BMP9. This is due to the much higher affinity of BMP9 for its receptor ALK1 than that of the other BMPs for their receptors ALK2, ALK3 and ALK6 ( $EC_{50} = 50$  pg/mL for ALK1 and 50 ng/mL for the other ALKs). In the future, it will therefore be very interesting to measure either active or total BMP9 circulating levels and to test whether they can represent biomarkers of the angiogenic response, especially since two recent works support the view that ALK1-Fc is a powerful antiangiogenic agent capable of blocking vascularization [11, 25]. BMP9 measurements could therefore be particularly useful in the follow-up of cancer patients or other vascular diseases (HHT, Pulmonary Arterial Hypertension) treated with antiangiogenic drugs, as well as in liver diseases, or in other diseases where deregulation of BMP signaling has been shown to lead to pathological consequences [16].

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