The invention relates to a bone morphogenetic protein 9 (BMP9) variant lacking osteogenic activity, and its use in the treatment of a vascular disease or a respiratory disease.
FIGURE 1
Signal peptide

prodomain BMP9

Pre-pro-BMP9

Ionomerisation

prodomain BMP9

Unprocessed Pro-BMP9

Furin cleavage

Pro-BMP9

Endothelial signalling

Fold change in ID1

BMP9 concentrations (ng/ml)

WT

D366A

D408A

Endothelial signalling

Fold change in ID1

BMP9 concentrations (ng/ml)

WT

D366A

D408A

Osteogenic signalling

AlP activity (pM/100 minutes)

BMP9 concentrations (ng/ml)

WT

D366A

D408A

FIGURE 2
A. ID1 gene expression

B. ID2 gene expression

C. BMPR-II gene expression

D. TNF/CHX-induced apoptosis assay

E. Osteogenic signalling

FIGURE 3
Apatent application publication

**Figure 4**

**A.**

Signal peptide

Prodomain BMP10

Pre-pro-BMP10

Pro-BMP10

Unprocessed Pro-BMP10

Furin cleavage

**B.**

MW (kDa)

100, 75, 50, 37, 25, 20

Pro-BMP10

**C.**

MW (kDa)

116, 59, 66, 55

Pro-BMP10

**D.**

R&D BMP9

R&D BMP10

Pro-BMP10

pSmad1/5/8

α-tubulin

**E.**

ID1 expression, 8 hours

Fold change

0, 0.05, 0.1, 1, 5

BMP concentrations (ng/ml)

ID2 expression, 8 hours

Fold change

0, 0.05, 0.1, 1, 5

BMP concentrations (ng/ml)

ID3 expression, 8 hours

Fold change

0, 0.05, 0.1, 1, 5

BMP concentrations (ng/ml)
**BMP9 alanine-scanning mutagenesis screen**

- ID1 induction in HMEC-1 (endothelial signalling)
- ALP activity in C2C12 (osteogenic signalling)

N=3

**FIGURE 5**
PAEC BMPR2 Expression

FIGURE 6

% early apoptosis

FIGURE 7
FIGURE 8
FIGURE 10

(A) 

(B)
THERAPEUTIC USE OF BONE MORPHOGENETIC PROTEINS

FIELD OF THE INVENTION

[0001] The invention relates to a polypeptide selected from bone morphogenetic protein 10 (BMP10), or a bone morphogenetic protein 9 (BMP9) variant lacking osteogenic activity, for use in the treatment of a vascular disease or a respiratory disease. The invention also relates to novel BMP9 variants and to pharmaceutical compositions comprising said polypeptides.

BACKGROUND OF THE INVENTION

[0002] Vascular disease is a pathological state of large and medium sized muscular arteries and is triggered by endothelial cell dysfunction. Because of factors like pathogens, oxidized LDL particles and other inflammatory stimuli, endothelial cells become activated. This leads to changes in their characteristics: endothelial cells start to secrete cytokines and chemokines and express adhesion molecules on their surface. This in turn results in recruitment of white blood cells (monocytes and lymphocytes), which can infiltrate the blood vessel wall. Stimulation of the smooth muscle cell layer with cytokines produced by endothelial cells and recruited white blood cells causes smooth muscle cells to proliferate and migrate towards the blood vessel lumen. This process causes thickening of the vessel wall, forming a plaque consisting of proliferating smooth muscle cells, macrophages and various types of lymphocytes. This plaque results in obstructed blood flow leading to diminished amounts of oxygen and nutrients, that reach the target organ. In the final stages, the plaque may also rupture causing the formation of clots, and as a result, strokes.

[0003] Respiratory disease is a medical term that encompasses pathological conditions affecting the organs and tissues that make gas exchange possible in higher organisms, and includes conditions of the upper respiratory tract, trachea, bronchi, bronchioles, alveoli, pleura and pleural cavity, and the nerves and muscles of breathing. Respiratory diseases range from mild and self-limiting, such as the common cold, to life-threatening entities like bacterial pneumonia, pulmonary embolism, and lung cancer.

[0004] Pulmonary arterial hypertension (PAH) is a rare vascular disease for which there is currently no cure. Heritable and idiopathic pulmonary arterial hypertension (PAH) are characterized by narrowing and obliteration of precapillary pulmonary arteries, secondary to proliferation and apoptosis resistance of smooth muscle cells, fibroblasts and endothelial cells (Morrell et al (2009) J Am Coll Cardiol 54, S20-31). The resulting increase in pulmonary vascular resistance causes severe elevation of pulmonary artery pressure, leading to right ventricular hypertrophy and ultimately death from right heart failure (Gaine and Rubin (1998) Lancet 352, 719-725).


[0007] While in vitro studies using pulmonary artery smooth muscle cells (PASMCs) have demonstrated that increasing concentrations of BMP ligand can overcome the loss of function associated with mutations in the BMP signaling pathway (Yang et al (2008) Circ Res 102, 1212-1221), to date, no study has therapeutically delivered BMP ligand in vivo to provide proof-of-concept for such an approach in the treatment of PAH. The complexity of the BMP signaling family, which is comprised of four type-II receptors, five type-I receptors and over twenty BMP ligands (Miyazono et al (2005) Cytokine Growth Factor Rev 16, 251-263), may account for the absence of such studies. Identifying an appropriate ligand to selectively target the pulmonary endothelium presents a significant challenge. Recently, BMPR-II was found to form a signaling complex with ALK-1 and signal specifically in response to BMP9 and 10 in microvascular endothelial cells (David et al (2007) Blood 109, 1953-1961).

[0008] WO 2005/113590 describes the use of BMP10 antagonists for the treatment of heart disorders. WO 2013/152213 describes the use of BMP9 and/or BMP 10 polypeptides for increasing red blood cell and/or hemoglobin levels in vertebrates. WO 2006/130022 describes an agonist or antagonist of BMPKII which is useful in the modulation...
of folliculogenesis and ovulation rate in female mammals. WO 2010/114833 describes pharmaceutical compositions for treating heart disease that include a bone morphogenetic protein. WO 94/26893 describes BMP-10 proteins, processes for producing them and their use in the treatment of bone and cartilage defects and in wound healing and related tissue repair. WO 95/24474 and WO 96/39431 describe the human BMP-10 polypeptide and DNA (RNA) encoding such polypeptide which are claimed to be useful in inducing de novo bone formation. WO 95/00432 and WO 95/33830 describe BMP-9 proteins, processes for producing them and their use in the treatment of bone and cartilage defects, wound healing and related tissue repair and in hepatic growth and function. WO 2010/115874 describes methods for treating pulmonary arterial hypertension by administering apelin/ARI targeting drugs. WO 2009/114180 and WO 2014/160203 describe small molecule inhibitors of BMP signaling which are claimed to be useful in the modulation of cell growth, differentiation, proliferation, and apoptosis, and thus may be useful for treating diseases or conditions associated with BMP signaling, including inflammation, cardiovascular disease, hematological disease, cancer, and bone disorders, as well as for modulating cellular differentiation and/or proliferation. The small molecule inhibitors are also claimed to be useful in reducing circulating levels of ApoB-100 or LDL and treating or preventing acquired or congenital hypercholesterolemia or hyperlipoproteinemia; diseases, disorders, or syndromes associated with defects in lipid absorption or metabolism; or diseases, disorders, or syndromes caused by hyperlipidemia.

[0009] There is therefore a need to provide an effective treatment for vascular and respiratory diseases, in particular pulmonary arterial hypertension (PAH).

SUMMARY OF THE INVENTION

[0010] According to a first aspect of the invention, there is provided a polypeptide selected from bone morphogenetic protein 10 (BMP10), or a bone morphogenetic protein 9 (BMP9) variant lacking osteogenic activity, for use in the treatment of a vascular disease or a respiratory disease.

[0011] According to a further aspect of the invention, there is provided a method of treating a vascular disease or a respiratory disease which comprises administering to a subject in need thereof a therapeutically effective amount of a polypeptide selected from bone morphogenetic protein 10 (BMP10), or a bone morphogenetic protein 9 (BMP9) variant lacking osteogenic activity, for example an isolated polypeptide which is a BMP9 variant lacking osteogenic activity and comprising the amino acid sequence of SEQ ID NO: 7 and/or a variant thereof wherein one or more of the following amino acids of the polypeptide of claim 1 have been substituted with Alanine: H326, D342, S343, W344, K346, K349, F362, K372, I375, L379, H381, L382, K383, K390, S402, L404, K406, V411, 1413, L1414, Y414 and Y418.

[0012] According to a further aspect of the invention, there is provided a pharmaceutical composition comprising BMP10, or a BMP9 variant lacking osteogenic activity, for use in the treatment of a vascular disease or a respiratory disease.

[0013] According to a further aspect of the invention, there is provided a BMP9 variant having the amino acid sequence of SEQ ID NO: 5.

[0014] According to a further aspect of the invention, there is provided a BMP9 variant having the amino acid sequence of SEQ ID NO: 6.

[0015] According to a further aspect of the invention, there is provided a BMP9 variant having the amino acid sequence of SEQ ID NO: 7.

[0016] According to a further aspect of the invention, there is provided a variant of the polypeptide of SEQ ID NO: 7 wherein the variant has one or more of the following amino acids of SEQ ID NO: 7 substituted with Alanine: H326, D342, S343, W344, K346, K349, F362, K372, I375, L379, H381, L382, K383, K390, S402, L404, K406, V411, 1413, L1414, Y414 and Y418.

[0017] According to a further aspect of the invention, there is provided a pharmaceutical composition comprising the BMP9 variants as defined herein.

BRIEF DESCRIPTION OF THE FIGURES

[0018] FIG. 1. Expression vector and system for pro. BMP9 and pro.BMP10.

[0019] FIG. 2. Generation of non-osteogenic BMP9 variants. A. Schematic drawing of BMP9 synthesis and post-translational processing. B-D. Two BMP9 variants (D366A and D408A) have comparable signaling activity as the wild type in endothelial cells in inducing ID1 and ID2 expression (B&C), but lack osteogenic signaling activity in the C2C12 cells (D).

[0020] FIG. 3. Comparison of endothelial cell signalling activity and C2C12 cell osteogenic activity between BMP9 and BMP10. A-C: BMP9 and BMP10 have similar signalling activity HMEC-1. After serum-starvation, HMEC-1 cells were treated with BMP9 or BMP10 at indicated concentrations. 8 hours after treatment, mRNA was extracted and the expression levels of ID1, ID2 or BMPR-11 were measured by quantitative PCR. B2-microglobulin was used as control and fold changes relative to non-treated samples were plotted. Mean±SEM is shown, N=2. D. Similar to BMP9, BMP10 can also protect hPAEC against TNFα-CHX induced apoptosis. Methods are as FIG. 3A, N=1; E. BMP9 and BMP10 inhibit endothelial cell proliferation to similar extents. hPAECs were treated with BMP9 or BMP10 (both R&D Systems) in EBM2/2% FBS for 24 hours. Cells were incubated with 0.5 µCi/well 3H-Thymidine for the final 6 hours. Cells were then lysed and 3H-Thymidine uptake measured by liquid scintillation counting. N=1 experiment, mean±SEM of 4 wells. F. Unlike BMP9, BMP10 has no detectable osteogenic activity measured as ALP activity in C2C12 cells. C2C12 cells were treated with BMP9 or BMP10 at indicated concentrations for 64 hours. Cells were lysed in 1% Triton X-100/PBS and ALP activity in the cell lysate was measured using the chromogenic phosphate substrate 4-nitrophenyl phosphate disodium salt (Sigma, S0942) and the soluble product was measured at 405 nm on a plate reader. In all assays, both BMP9 and BMP10 were purchased from R&D Systems. Pro.BMP9 was produced in-house and its concentration (mature ligand) was determined by ELISA, using BMP9 from R&D system as standard.

[0021] FIG. 4. In-house generated pro.BMP10 is fully active. A. Schematic drawing of BMP10 synthesis and post-translational processing. B. BMP10 expressing conditioned medium, blotted by anti-BMP10 antibody (R&D Systems). C. Non-reducing SDS-PAGE showing the purification of pro.BMP10 from an S200 gel filtration column.
The identities of prodomain and BMP10 have been verified by western blot and mass spec peptide mapping. Comparing pro-BMP10 signalling capacity with BMP9 and BMP10 (from R&D Systems) in HMEC-1 by monitoring Smad1/5/8 phosphorylation and ID1/2/3 gene expression. Methods are as in FIG. 3A to C. BMP concentrations in D are 0.05, 0.1, 1, 5 ng/ml and treatment time is 1 hour. Mean±SEM is shown. N=2.

FIG. 5. Summary of BMP9 alanine scanning mutagenesis. Twenty-four BMP9 variants were generated and tested in both HMEC-1 cells for ID1 gene induction and C2C12 cells for alkaline phosphatase activity. All results were normalized to wild type (WT) BMP9 and average of three experiments are shown. * denotes untreated cells.

FIG. 6. BMP variants can induce BMPR2 gene expression in hPACs.

FIG. 7. BMP D408A variant can rescue TNFα/CHX induced early apoptosis in hPACs.

FIG. 8. BMP9 and BMP10 inhibit blood outgrowth endothelial cell (BOEC) tube formation in a collagen:fibronectin matrix. (A) Representative images of BOEC tubes in collagen gels stained with DAPI and FITC-ULEX. Networks form when in media alone (2% BDM2−EBM2 containing 2% FBS). Addition of increasing concentrations of BMP9 inhibits BOEC network formation. (B) Quantification of BOEC network parameters determined for 3 separate experiments demonstrating that BMP9 inhibits tube length and number, branching and loop formation in a concentration-dependent manner. (C) BMP9 and BMP10 that both ligands inhibit BOEC tube formation.

FIG. 9. BMP9 D366A/D408A double mutant variant has potent endothelial cell signalling activity, comparable with D366A and D408A single mutants and the wild type. hPACs were treated with pBMP9 wild type or pBMP9 variants for 8 hours and the induction of ID1 (A) and BMPR2 (B) gene expression was measured using reverse-transcription coupled with qPCR. In both graphs, X-axes represent ligand concentration (in ng/ml) and Y-axes represent fold changes in gene expression relative to PBS-treated cells. N=3, mean±SEM are shown.

FIG. 10. BMP9 D366A/D408A double mutant variant does not have osteogenic signalling activity. A C2C12 cells were treated with increasing dose of BMP ligands (3.7, 18.7, 37.4, 74.8, 149.6 & 374 ng/ml) and ALP activities were measured as described in the methods. ALP activity readings were normalized to the wild type pBMP9 top concentration (374 ng/ml) treatment. The fractional changes from four experiments were averaged and shown on Y-axis. X-axis represents ligand concentration (in ng/ml). N=3, mean±SEM are shown. B. Human periodontal ligament fibroblast cells (hPLFs) were serum-starved overnight before treated with BMP ligands. After six days of treatment, cells were harvested and changes in OSTERIX gene expression were measured using qPCR. X-axis represents ligand concentration (in ng/ml) and Y-axis represent changes in gene expression relative to PBS-treated samples. N=1.

DETAILED DESCRIPTION OF THE INVENTION

According to a first aspect of the invention, there is provided a polypeptide selected from bone morphogenetic protein 10 (BMP10), or a bone morphogenetic protein 9 (BMP9) variant lacking osteogenic activity, for use in the treatment of a vascular disease or a respiratory disease.

The BMP9 variant lacking osteogenic activity may comprise the amino acid sequence of SEQ ID NO: 7. Alternatively, the BMP9 variant may comprise the amino acid sequence of SEQ ID NO: 7 in which one or more of the following amino acids have been substituted with Alanine: H326, D342, S343, W344, I346, K349, F362, K372, I375, L379, H381, L382, K383, K390, S402, L404, K406, V411, T413, L414, Y416 and Y418.

The present invention is directed to the therapeutic use of bone morphogenetic proteins which maintain endothelial cell signalling activity (for example, as which may be evidenced by the induction of ID1, ID2 and/or BMPR-II gene expression) but which lack osteogenic activity (for example as which may be measured by alkaline phosphatase (ALP) activity in the mouse myoblast cell line C2C12). For example, BMP10 and the BMP9 variants disclosed herein not maintain endothelial cell signalling activity but are synergistically devoid of osteogenic activity. Thus, native BMP10 and the BMP9 variants disclosed herein represent a more desirable agonist than native BMP9 for treating a vascular disease or a respiratory disease, in particular PAH via virtue of lacking the ability to promote bone formation.

References herein to “BMP10” and “bone morphogenetic protein 10” refer to a human polypeptide belonging to the TGF-β superfamily of proteins which is encoded by the BMP10 gene (having the sequence shown in SEQ ID NO: 1) and which has the 424 amino acid sequence shown in SEQ ID NO: 2 wherein amino acid residues 1 to 21 comprise the signal peptide, amino acid residues 22 to 315 comprise the propeptide and amino acid residues 317 to 424 comprise mature BMP10.

References herein to “a BMP9 variant” and “bone morphogenetic protein 9 variant” refer to a human polypeptide belonging to the TGF-β superfamily of proteins which is encoded by the BMP9 gene (having the sequence shown in SEQ ID NO: 3) and which has a variant of the 429 amino acid sequence shown in SEQ ID NO: 4 wherein amino acid residues 1 to 22 comprise the signal peptide, amino acid residues 23 to 319 comprise the propeptide and amino acid residues 320 to 429 comprise mature BMP9. For the avoidance of doubt, it should be stressed that such BMP9 variant must maintain endothelial cell signalling activity but lack osteogenic activity.

References to “variant” include a genetic variation in the native, non-mutant or wild type sequence of BMP9. Examples of such genetic variations include mutations selected from: substitutions, deletions, insertions and the like.

References to “lacking osteogenic activity” or “lack osteogenic activity” as used herein refer to a BMP9 variant comprising one or more, mutations of the sequence of SEQ ID NO: 4 which results in elimination, minimization and/or suppression of osteogenic activity (for example, which may be measured by alkaline phosphatase (ALP) activity in the mouse myoblast cell line C2C12). Advantageous BMP9 variants will be those which maintain endothelial specific signaling (i.e. those which have at least 0.75 fold ID1 induction compared to wild type BMP9, as measured by ID1 gene expression in HMEC-1 cells) and which have a lower value of osteogenic activity (i.e. less than 0.5 fold compared to wild type BMP9, as measured by ALP activity in the mouse myoblast cell line C2C12).
More desirable BMP9 variants will be those which maintain endothelial specific signaling (i.e. those which have at least 0.75 fold ID1 induction compared to wild type BMP9, as measured by ID1 gene expression in HMEC-1 cells) and negligible osteogenic activity (i.e. less than 0.1 fold compared to wild type BMP9, as measured by ALP activity in the mouse myoblast cell line C2C12).

Most desirable BMP9 variants will be those which have increased endothelial specific signaling (i.e. those which have higher levels of ID1 induction compared to wild type BMP9, as measured by ID1 gene expression in HMEC-1 cells) and negligible osteogenic activity (i.e. less than 0.1 fold compared to wild type BMP9, as measured by ALP activity in the mouse myoblast cell line C2C12).

In one embodiment, the vascular disease is selected from: pulmonary hypertension; pulmonary arterial hypertension; hereditary haemorrhagic telangiectasia; atherosclerosis; and hepatopulmonary syndrome.

In a further embodiment, the vascular disease is selected from: pulmonary hypertension; pulmonary arterial hypertension; hereditary haemorrhagic telangiectasia; and hepatopulmonary syndrome.

In a further embodiment, the vascular disease is selected from pulmonary arterial hypertension.

In one embodiment, the respiratory disease is selected from: obstructive lung diseases such as chronic obstructive pulmonary disease (COPD), chronic bronchitis and emphysema; pulmonary vascular diseases such as pulmonary edema and pulmonary hemorrhage; respiratory failure and respiratory distress syndrome, such as acute lung injury and acute respiratory distress syndrome; and interstitial lung diseases, such as idiopathic pulmonary fibrosis.

Thus, according to a further aspect of the invention there is provided BMP10 for use in the treatment of a vascular disease or a respiratory disease. Data is provided herein which shows that BMP10 is as potent as BMP9 in inducing ID1, ID2 and BMPR-II gene expression (see FIGS. 3A to 3C). Furthermore, BMP10 has been shown herein to exhibit the same anti-angiogenic activity as BMP9 in protecting hiPAECs against TNFα-CHX induced apoptosis (see FIG. 3D).

In a further embodiment, the polypeptide is BMP10 comprising the amino acid sequence of SEQ ID NO: 2.

In a further embodiment, the polypeptide is BMP10 encoded by the nucleotide sequence of SEQ ID NO: 1.

In a further embodiment, the polypeptide is the prodomain bound form of BMP10 (pro.BMP10). Data is provided herein which demonstrates that the pro.BMP10 complex is very stable (see FIGS. 4B and 4C) and is likely to be the preferred form for the treatment of vascular and respiratory diseases, such as PAH.

In a further embodiment, the pro.BMP10 comprises a propeptide sequence having the amino acid sequence of residues 22-316 of SEQ ID NO: 2: non-covalently bound to a mature BMP10 sequence having the amino acid sequence of residues 317-424 of SEQ ID NO: 2.

In a further embodiment, the pro.BMP10 comprises a tetramer containing two of said propeptide sequences and two of said mature BMP10 sequences.

In one embodiment, the polypeptide is a BMP9 variant lacking osteogenic activity. Thus, according to a further aspect of the invention there is provided a BMP9 variant lacking osteogenic activity for use in the treatment of a vascular disease or a respiratory disease.

In a further embodiment, the polypeptide is a variant of the prodomain bound form of BMP9 (pro.BMP9).

In a further embodiment, the variant of pro.BMP9 comprises a variant of: the propeptide sequence having the amino acid sequence of residues 23-319 of SEQ ID NO: 4; non-covalently bound to a mature BMP9 sequence having the amino acid sequence of residues 320-429 of SEQ ID NO: 4.

In a further embodiment, the variant of pro.BMP9 comprises a tetramer containing two of said propeptide sequences and two of said mature BMP9 sequences.

In one embodiment, the BMP9 variant lacking osteogenic activity comprises a substitution, deletion or insertion mutant of the amino acid sequence of SEQ ID NO: 4.

In a further embodiment, the BMP9 variant lacking osteogenic activity comprises a substitution mutant of the amino acid sequence of SEQ ID NO:

In one embodiment, the substitution mutant of the amino acid sequence of SEQ ID NO:


In a further embodiment, the substitution mutant of the amino acid sequence of SEQ ID NO: 4 comprises one or more (i.e. single, double, triple mutants etc.) of the following substitutions: H326A, S343A, K349A, F362A, D366A, I375A, L379A, L382A, K390A, S402A, D408A, Y416A and Y418A.

In a further embodiment, the substitution mutant of the amino acid sequence of SEQ ID NO: 4 comprises one or more (i.e. single, double, triple mutants etc.) of the following substitutions: H326A, S343A, K349A, F362A, D366A, I375A, L379A, L382A, K390A, S402A, D408A, Y416A and Y418A.

In a further embodiment, the substitution mutant of the amino acid sequence of SEQ ID NO: 4 comprises one or more (i.e. single, double, triple mutants etc.) of the following substitutions: H326A, S343A, K349A, F362A, D366A, I375A, L379A, L382A, K390A, S402A, D408A, Y416A and Y418A.

In a further embodiment, the substitution mutant of the amino acid sequence of SEQ ID NO: 4 comprises one or more (i.e. single, double, triple mutants etc.) of the following substitutions: H326A, S343A, K349A, F362A, D366A, I375A, L379A, L382A, K390A, S402A, D408A, Y416A and Y418A.

In a further embodiment, the substitution mutant of the amino acid sequence of SEQ ID NO: 4 comprises one or more (i.e. single, double, triple mutants etc.) of the following substitutions: H326A, S343A, K349A, F362A, D366A, I375A, L379A, L382A, K390A, S402A, D408A, Y416A and Y418A.
BMP9 variants of SEQ ID NO: 4: F362A, D366A, I375A, L379A, S402A, D408A, Y416A and Y418A. Data is provided herein which demonstrates that these mutant sequences maintain the beneficial effect of endothelial specific signaling but lack osteogenic signaling (as evidenced by at least 0.75 fold ID1 induction and negligible (i.e. less than 0.1 fold) ALP activity when compared to wild type BMP9 in Fig. 5).

In a further embodiment, the substitution mutant of the amino acid sequence of SEQ ID NO: 4 comprises one or both (i.e. a single or double mutant) of the following substitutions: D366A or D408A.

In a further embodiment, the BMP9 variant lacking osteogenic activity is selected from one of the following BMP9 variants of SEQ ID NO: 4: D366A or D408A. Data is provided herein which demonstrates that these mutant sequences maintain the beneficial effect of BMP9 but are not able to initiate the osteogenic signaling and hence remove the potential risk of bone formation by administration of BMP9 in vivo (see the results shown in Fig. 2). Data is also provided herein which demonstrates that these mutant sequences have increased endothelial specific signaling but lack osteogenic signaling (as evidenced by a greater than 1 fold ID1 induction and negligible (i.e. less than 0.1 fold) ALP activity when compared to wild type BMP9 in Fig. 5).

In one embodiment, the BMP9 variant lacking osteogenic activity is a BMP9 variant of SEQ ID NO: 4 comprising D366A and D408A substitutions. Data is provided herein which demonstrates that the BMP9 variant maintains the beneficial effect of endothelial specific signaling but lacks osteogenic signaling (as seen in Figs. 9 and 10).

In a further embodiment, the BMP9 variant lacking osteogenic activity is selected from a D408A BMP9 variant of SEQ ID NO: 4. Data is provided herein which demonstrates that this mutant sequence has been shown to be able to rescue PAEC early apoptosis induced by tumor necrosis factor α (TNFα) and cycloheximide (CHX) (see the results shown in Fig. 7).

In a further embodiment, the BMP9 variant lacking osteogenic activity is selected from a D366A BMP9 variant comprising the amino acid sequence of SEQ ID NO: 5 or a D408A BMP9 variant comprising the amino acid sequence of SEQ ID NO: 6.

In another embodiment, the BMP9 variant lacking osteogenic activity is selected from a D366A/D408A BMP9 variant comprising the amino acid sequence of SEQ ID NO: 7.

In a further embodiment, the BMP9 variant lacking osteogenic activity is a polypeptide comprising the amino acid sequence of SEQ ID NO: 7 in which one or more of the following amino acids have been substituted with Alanine: H326, D342, S343, W344, I346, K349, F362, K372, I375, L379, I381, L382, K383, K390, 0402, L404, K406, V411, T413, L414, Y416 and Y418.

It will be appreciated that the BMP9 variants disclosed herein constitute previously unknown polypeptides which therefore form novel aspects of the invention. Thus, according to a further aspect of the invention there is provided a BMP9 variant comprising the amino acid sequence of SEQ ID NO: 5. According to a further aspect of the invention there is provided a BMP9 variant comprising the amino acid sequence of SEQ ID NO: 6. According to another aspect of the invention there is provided a BMP9 variant comprising the amino acid sequence of SEQ ID NO: 7.

In another aspect there is provided a BMP9 variant lacking osteogenic activity which is a polypeptide comprising the amino acid sequence of SEQ ID NO: 7 wherein one or more of the following amino acids have been substituted with Alanine: H326, D342, S343, W344, I346, K349, F362, K372, I375, L379, I381, L382, K383, K390, S402, L404, K406, V411, T413, L414, Y416 and Y418.

While it is possible for the active polypeptide to be administered alone, it is preferable to present it as a pharmaceutical composition (e.g. formulation). In one embodiment this is a sterile pharmaceutical composition.

The invention further provides pharmaceutical compositions, as defined above, and methods of making a pharmaceutical composition comprising (e.g. admixing) at least one polypeptide of the invention together with one or more pharmaceutically acceptable excipients and optionally other therapeutic or prophylactic agents.

Thus, according to a further aspect of the invention there is provided a pharmaceutical composition comprising BMP10, or a BMP9 variant lacking osteogenic activity, for use in the treatment of a vascular disease or a respiratory disease.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising the BMP9 variants as defined herein, such as the D366A BMP9 variant comprising the amino acid sequence of SEQ ID NO: 5 or the D408A BMP9 variant comprising the amino acid sequence of SEQ ID NO: 6 or the D366A/D408A BMP9 variant comprising the amino acid sequence of SEQ ID NO: 7 or its variant.

The pharmaceutically acceptable excipient(s) can be selected from, for example, carriers (e.g. a solid, liquid or semi-solid carrier), adjuvants, diluents, fillers or bulking agents, granulating agents, coating agents, release-controlling agents, binding agents, disintegrants, lubricating agents, preservatives, antioxidants, buffering agents, suspending agents, thickening agents, flavoring agents, sweeteners, taste masking agents, stabilizers or any other excipients conventionally used in pharmaceutical compositions. Examples of excipients for various types of pharmaceutical compositions are set out in more detail below.

The term “pharmaceutically acceptable” as used herein pertains to compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of a subject (e.g. human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, excipient, etc. must also be “acceptable” in the sense of being compatible with the other ingredients of the formulation.

Pharmaceutical compositions containing the polypeptides of the invention can be formulated in accordance with known techniques, see for example, Remington’s Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA.

The pharmaceutical compositions can be in any form suitable for oral, parenteral, topical, intranasal, intrabronchial, sublingual, ophthalmic, otic, rectal, intra-vaginal, or transdermal administration. Where the compositions are intended for parenteral administration, they can be formu-
lated for intravenous, intramuscular, intraperitoneal, subcutaneous administration or for direct delivery into a target organ or tissue by injection, infusion or other means of delivery. The delivery can be by bolus injection, short term infusion or longer term infusion and can be via passive delivery or through the utilization of a suitable infusion pump or syringe driver.

Pharmaceutical formulations adapted for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats, co-solvents, surface active agents, organic solvent mixtures, cyclodextrin complexation agents, emulsifying agents (for forming and stabilizing emulsion formulations), liposome components for forming liposomes, gelable polymers for forming polymeric gels, lyophilization protectants and combinations of agents for, inter alia, stabilizing the active ingredient in a soluble form and rendering the formulation isotonic with the blood of the intended recipient. Pharmaceutical formulations for parenteral administration may also take the form of aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents (R. G. Strickly, Solubilizing Excipients in oral and injectable formulations, Pharmaceutical Research, Vol 21(2) 2004, p 201-230).

It will be appreciated that gene therapy comprising the BMP10 or BMP9 variant of the invention is within the scope of the invention. For example, a vector encoding the BMP10 or BMP9 variant nucleotide sequence is administered to the host human subject resulting in endogenous expression (such as endogenous expression in the liver) of the BMP10 or BMP9 variant polypeptide for release into the circulation. Thus, according to a further aspect of the invention there is provided a vector comprising a nucleotide sequence encoding BMP10 or a BMP9 variant for use in the treatment of a vascular disease or a respiratory disease. In a further embodiment, the vector comprises the nucleotide sequence of SEQ ID NO: 1.

In one embodiment, the vector is a viral vector. In a further embodiment, the viral vector is selected from a: retrovirus, adenovirus, lentivirus, herpes simplex, vaccinia and adenovirus-associated virus. In a further embodiment, the vector is a viral vector is an adenovirus-associated virus.

In an alternative embodiment, the vector is a non-viral vector. The use of non-viral vectors has a number of advantages over the use of viral vectors, such as ease of large scale production and low immunogenicity in the host. Examples of non-viral gene therapy methods include: injection of naked DNA, electroporation, gene gun, sonoporation, magnetofection and the use of oligonucleotides, lipoplexes, dendrimers, and inorganic nanoparticles. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules, vials and prefilled syringes, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

The pharmaceutical formulation can be prepared by lyophilizing a polypeptide of the invention. Lyophilization refers to the procedure of freeze-drying a composition. Freeze-drying and lyophilization are therefore used herein as synonyms.

Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.
Capsule formulations may be of the hard gelatin or soft gelatin variety and can contain the active component in solid, semi-solid, or liquid form. Gelatin capsules can be formed from animal gelatin or synthetic or plant derived equivalents thereof.

The solid dosage forms (e.g.; tablets, capsules etc.) can be coated or un-coated. Coatings may act either as a protective film (e.g. a polymer, wax or varnish) or as a mechanism for controlling drug release or for aesthetic or identification purposes. The coating (e.g. a Eudragit® type polymer) can be designed to release the active component at a desired location within the gastrointestinal tract. Thus, the coating can be selected so as to degrade under certain pH conditions within the gastrointestinal tract, thereby selectively release the polypeptide in the stomach or in the ileum, duodenum, jejunum or colon.

Instead of, or in addition to, a coating, the drug can be presented in a solid matrix comprising a release controlling agent, for example a release delaying agent which may be adapted to release the polypeptide in a controlled manner in the gastrointestinal tract. Alternatively the drug can be presented in a polymer coating e.g. a polymethacrylate polymer coating, which may be adapted to selectively release the polypeptide under conditions of varying acidity or alkalinity in the gastrointestinal tract. Alternatively, the matrix material or release retarding coating can take the form of an erodible polymer (e.g. a maleic anhydride polymer) which is substantially continuously eroded as the dosage form passes through the gastrointestinal tract. In another alternative, the coating can be designed to disintegrate under microbial action in the gut. As a further alternative, the active polypeptide can be formulated in a delivery system that provides osmotic control of the release of the polypeptide. Osmotic release and other delayed release or sustained release formulations (for example formulations based on ion exchange resins) may be prepared in accordance with methods well known to those skilled in the art.


The pharmaceutical compositions typically comprise from approximately 1% (w/w) to approximately 95% (w/w) active ingredient and from 99% (w/w) to 5% (w/w) of a pharmaceutically acceptable excipient or combination of excipients. Particularly, the compositions comprise from approximately 20% (w/w) to approximately 90% (w/w) active ingredient and from 80% (w/w) to 10% of a pharmaceutically acceptable excipient or combination of excipients. The pharmaceutical compositions comprise from approximately 1% to approximately 95%, particularly from approximately 20% to approximately 90%, active ingredient. Pharmaceutical compositions according to the invention may be, for example, in unit dose form, such as in the form of ampoules, vials, suppositories, pre-filled syringes, drages, tablets or capsules.
The pharmaceutical formulations may be presented to a patient in “patient packs” containing an entire course of treatment in a single package, usually a blister pack. Patient packs have an advantage over traditional prescriptions, where a pharmacist divides a patient’s supply of a pharmaceutical from a bulk supply, in that the patient always has access to the package insert contained in the patient pack, normally missing in patient prescriptions. The inclusion of a package insert has been shown to improve patient compliance with the physician’s instructions.

Compositions for topical use and nasal delivery include ointments, creams, sprays, patches, gels, liquid drops and inserts (for example intraocular inserts). Such compositions can be formulated in accordance with known methods.

Examples of formulations for rectal or intra-vaginal administration include pessaries and suppositories which may be, for example, formed from a shaped moldable or waxy material containing the active polypeptide. Solutions of the active polypeptide may also be used for rectal administration.

Compositions for administration by inhalation may take the form of inhalable powder compositions or liquid or powder sprays, and can be administered in standard form using powder inhaler devices or aerosol dispensing devices. Such devices are well known. For administration by inhalation, the powdered formulations typically comprise the active polypeptide together with an inert solid powdered diluent such as lactose.

The polypeptides of the invention will generally be presented in unit dosage form and, as such, will typically contain sufficient polypeptide to provide a desired level of biological activity. For example, a formulation may contain from 1 nanogram to 2 grams of active ingredient, e.g. from 1 nanogram to 2 milligrams of active ingredient. Within these ranges, particular sub-ranges of polypeptide are 0.1 milligrams to 2 grams of active ingredient (more usually from 10 milligrams to 1 gram, e.g. 50 milligrams to 500 milligrams), or 1 microgram to 20 milligrams (for example 1 microgram to 10 milligrams, e.g. 0.1 micrograms to 2 milligrams of active ingredient).

For oral compositions, a unit dosage form may contain from 1 milligram to 2 grams, more typically 10 milligrams to 1 gram, for example 50 milligrams to 1 gram, e.g. 100 milligrams to 1 gram, of active polypeptide.

The active polypeptide will be administered to a patient in need thereof (for example a human or animal patient) in an amount sufficient to achieve the desired therapeutic effect.

According to a further aspect of the invention, there is provided a method of treating a vascular disease or a respiratory disease which comprises administering to a subject in need thereof a therapeutically effective amount of a polypeptide selected from bone morphogenetic protein 10 (BMP10), and/or a bone morphogenetic protein 9 (BMP9) variant lacking osteogenic activity, and/or.

The following studies illustrate the invention:

**ABBREVIATIONS**

ActR-IIA(B); activin receptor type-IIA(B);
ALK1, 2, 3, 6; activin receptor-like kinase 1, 2, 3, 6;
ALP: alkaline phosphatase;
BMP: bone morphogenetic protein;
BMPR-II: bone morphogenetic protein receptor type II;
ECM: extracellular domain;
FBS: fetal bovine serum;
HMEC-1: human microvascular endothelial cells;
hPAEC: human pulmonary artery endothelial cells;
MSC: mesenchymal stem cells;
PAH: pulmonary arterial hypertension;
pro.BMP9: prodomain bound BMP9;
pro.BMP10: prodomain bound BMP10; and
qPCR: quantitative PCR.

**EXAMPLE 1**

Materials and Methods

Generating Recombinant Human pro.BMP9 and pro.BMP10

The full-length cDNA containing open reading frame of human pro-pre-BMP9 was cloned into the expression vector pCEP4 between HindIII and Xhol sites (see FIG. 1). Similarly, the full-length cDNA of human pre-pre-BMP10 was cloned into the expression vector pCEP4 between Xhol and BamHI sites (see FIG. 1). The inserts were verified by DNA sequencing. Pro.BMP9 variants were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and all mutations were verified by DNA sequencing.

Plasmids containing pro-pre-BMP9 (or pro-pre-BMP10) were transfected into HEK-EBNA cells using polyethyleneimine in DMEM medium containing 5% fetal bovine serum (FBS). Plasmids expressing human furin were co-transfected to facilitate the processing of pro-BMP9 and pro-BMP10. Cells were changed into CDCHO medium without serum the following day and conditioned media were harvested after 3-4 days. The identities of pro.BMP9 and pro.BMP10 in the conditioned media were confirmed by Western blotting using anti-BMP9 (MAB3209, R&D Systems), anti-BMP9 prodomain (AF3879, R&D Systems) or anti-BMP10 (MAB2926, R&D Systems) antibodies, respectively.

To purify pro.BMP10, 1-5 liters of conditioned media were loaded onto a Q-Sepharose column pre-equilibrated in 50 mM Tris.HCl, pH 7.4, 50 mM NaCl. Bound proteins were eluted with a NaCl gradient (50-2000 mM). Fractions were analysed by a non-reducing SDS-PAGE and those containing pro.BMP10 were pooled and concentrated before loading onto an S200 gel filtration column. Pro. BMP10 from the S200 column was over 90% pure and the identities of BMP10 prodomain and mature BMP10 were further confirmed by in-gel digestion and mass spectrometric identifications.

Signalling Assays by Quantitative PCR (qPDCR) and Smad1/5/8 Phosphorylation in Endothelial Cells

For the signalling assays, the concentration of pro.BMP9 was determined by ELISA using R&D BMP9 as standards; and the concentration of pro.BMP10 was quantified by western blot and ImageJ using R&D BMP10 as standards.

After serum-starvation, HMEC-1 cells were treated with BMP ligands at indicated concentrations. 8 hours after treatment, mRNA was extracted and the expression levels of ID1, ID2 or BMPR-II were measured by quantitative PCR. β2-microglobulin was used as control and fold changes relative to non-treated samples were plotted. Mean±SEM is shown, N=2. For Smad1/5/8 phosphorylation assay, serum-
starved HMEC-1 cells were treated with BMP ligands at indicated concentrations for 1 hour and the signalling was stopped by placing the dishes on dry ice. Lysis buffer (125 mM Tris.HCl, pH 6.8, 296 SDS and 10% glycerol) was added and the protein concentration in the cell lysate was determined using DCTM protein assay (Bio-Rad). 25-35 μg of total cell protein was used for immunoblotting and the phosphorylation of Smad1/5/8 was monitored by anti-ps-madr1/5/8 antibody (Cell Signaling, cat No: 9516), α-tubulin was used as an internal control.

[0135] Alkaline Phosphatase (ALP) Activity in Mouse Myoblast Cell Line C2C12

[0136] C2C12 cells were seeded at 20,000 cells/well in 24-well plate in DMEM with 10% FBS. After 48 hours, cells were quiesced with DMEM containing 0.25% FBS for 16 hours and treated with BMP ligands at indicated concentrations for 64 hours. Cells were lysed in 1% Triton X-100/PBS and the total protein concentration in the cell lysate was determined using DCTM protein assay (Bio-Rad). ALP activity in the cell lysate was measured using the chromogenic phosphatase substrate 4-nitrophenyl phosphate disodium salt (Sigma: S0942) and the soluble product was measured at 405 nm on a plate reader. In all assays, control BMP9 and BMP10 were purchased from R&D Systems.

[0137] Results


[0139] Engineering of BMP9 Variants that Retain Endothelial Protective Properties but that are Devoid of Bone Forming Activity


[0141] The inventors have hypothesized that by mutating the type I and type II receptor binding sites on BMP9, BMP9 variants can be generated that retain ALK1 binding but lose ALK2 binding. Such BMP9 variants are likely to retain endothelial protective function but lack osteogenic activity. The inventors have already identified two such BMP9 variants that maintain endothelial cell signalling activity, as evidenced by the induction of ID1 and ID2 gene expression, but lack osteogenic signalling activity, assessed by the alkaline phosphatase assay in the C2C12 cells (FIG. 2). These BMP9 variants (D366A and D408A) are likely to maintain the beneficial effect in vivo, since they have normal signalling activity in endothelial cells, but they will not be able to initiate the osteogenic signalling and hence remove the potential risk of bone formation by administration of BMP9 in vivo.

[0143] BMP9 Alkaline Scanning Mutagenesis

[0144] Twenty four BMP9 alanine variants (H326A, D342A, 3343A, W344A, I346A, K349A, F362A, D366A, K372A, I375A, L379A, H381A, L382A, K385A, K390A, S402A, L404A, K406A, D408A, V411A, T413A, L414A, Y416A and Y418A) were generated and tested in both HMEC-1 cells for ID1 gene induction and C2C12 cells for alkaline phosphatase activity. The results of this study are summarized in FIG. 5 where it can be seen that thirteen BMP9 variants (H326A, S343A, K349A, F362A, D366A, I375A, L379A, L382A, K390A, S402A, D408A, Y416A and Y418A) were identified as maintaining the beneficial effect of endothelial specific signaling and having greatly reduced osteogenic signaling (as evidenced by at least 0.75 fold ID1 induction and less than 0.5 fold ALP activity when compared to wild type BMP9). In addition, the results shown in FIG. 5 demonstrate that eight BMP9 variants (F362A, D366A, I375A, L379A, S402A, D408A, Y416A and Y418A) were identified as maintaining endothelial specific signalling but lacking osteogenic signaling (as evidenced by at least 0.75 fold ID1 induction and negligible (i.e. less than 0.1 fold) ALP activity when compared to wild type BMP9). Furthermore, the results shown in FIG. 5 demonstrate that two BMP9 variants (D366A or D408A) increased endothelial specific signaling but lack osteogenic signaling (as evidenced by a greater than 1 fold ID1 induction and negligible (i.e. less than 0.1 fold) ALP activity when compared to wild type BMP9).

[0145] Validation of BMP9 Variants in Primary Endothelial Cells

[0146] The eight BMP9 variants (F362A, D366A, I375A, L379A, S402A, D408A, Y416A and Y418A) which were identified above as maintaining endothelial specific signalling but lacking osteogenic signaling were further validated in primary endothelial cells. These mutants were all found to induce BMPRII gene expression in human pulmonary arterial endothelial cells (hPAECs, FIG. 6). At least one variant, D408A, has been shown to be able to rescue PAEC early apoptosis induced by tumor necrosis factor c. (TNFα) and cycloheximide (CTX) (FIG. 7).

[0147] BMP10 Signalling in Endothelium

BMP10 regulates cardiac ventricular wall development through the transcription factor Tbx20 (Zhang et al. 2011) J. Biol. Chem. 286(42):36820-36829) and overexpression of BMP10 in myocardium disrupts cardiac postnatal hypertrophic growth (Chen et al. 2006) J. Biol. Chem. 281(37):27481-27491). In the adult, BMP10 is only expressed in the right atrium (Chen et al. 2004 supra). It has been shown that circulating BMP10 mediates flow-dependent arterial quiescence (Lau et al. 2013) Development 140(16):3403-3412.

0149 The circulating level of BMP10 is controversial. While BMP10 protein has been detected in human sera using proteomic approaches (Souza et al. 2008) Mol. Endocrinol., 22(12):2689-2702) and can be measured by ELISA (Ricard et al. 2012) Blood 119(25):6162-6171), other studies using activity assays could not detect circulating BMP10 (Bidart et al. 2012) and Herrera and Inman (2009), supra). However, a recent report has demonstrated BMP10 activity in the circulation (Chen et al. 2013) Proc. Natl. Acad. Sci. U. S. A. 110(29):11887-11892). Such controversy could be due to circulating BMP10 being in active/inactive states, incomplete processing, inhibition by a serum factor, or the different activity assays used in published reports. The prodomain of BMP10 could play a role in this. For example, it has been reported that the prodomain of BMP10, unlike other BMPs, can inhibit BMP10-induced gene expression in C2C12 cells (Sengle et al. 2011) J. Biol. Chem. 286(45):5087-5099). In addition, Biscare measurements have shown that BMP10 has higher affinity for ALK1/BMPR-11 than BMP9 (Townson et al. 2012) J. Biol. Chem. 287(33):27313-27325) and the loss of BMPR-11 protein during the onset of PAH will clearly have an impact on BMP10 signalling. Importantly, in vitro and in vivo studies, BMP10 is devoid of osteogenic activity. Thus, native BMP10 represents a more desirable agonist than native BMP9 for treating PAH.

0150 Comparison of BMP9 and BMP10 Activity

0151 A concentration-response signalling assay in human microvascular endothelial cells (HMEC-1) showed that BMP10 is as potent as BMP9 in inducing ID1, ID2 and BMPR-II gene expression (FIG. 3A to 3C). Importantly, BMP10 exhibits the same anti-apoptotic activity as BMP9 in protecting hPACs against TNFα-CHX induced apoptosis (FIG. 3D). BMP10 is reported to maintain the stability of the vasculature by suppressing endothelial cell proliferation (David et al. 2008) supra). Both BMP9 and BMP10 repress DNA synthesis to similar extents, measured as 3H-thymidine uptake, in hPACs (FIG. 3E). Alkaline phosphatase (ALP) is a key enzyme in the osteogenic pathway and BMP9-induced ALP activity can be detected in C2C12 cells at 5 ng/ml BMP9. However, under identical conditions, BMP10 did not induce any ALP activity at the highest concentration tested (20 ng/ml, FIG. 3F), consistent with the previous study using the adenovirus-expressed BMPs in C2C12 cells (Kang et al. 2004 supra).

0152 Potential of Administration of BMP10 and Prodomain Bound BMP10 for Treating PAH and Other Cardiovascular Diseases

0153 BMPs are synthesized as pre-pro-proteins and the prodomain is cleaved upon secretion (FIG. 4A). A previous report showed that the prodomain of BMP10 can inhibit BMP10 activity and BMP10 is likely to circulate in an inactive form. The inventors have generated a large quantity of prodomain bound BMP10 (pro.BMP10). In contrast to the previous report, the prodomain was found to remain bound to BMP10 when BMP10 is produced from mammalian cells and that the pro.BMP complex is very stable (FIG. 4B and 4C). This indicates that pro.BMP10 is likely to be the circulating form. Furthermore, the inventors have demonstrated in the HMEC-1 cells (FIG. 4D) and hPACs that pro.BMP10 have comparable activities to BMP9 and BMP10 purchased from a commercial source (R&D Systems). Since the prodomain protects the hydrophobic surface of BMP10 and hence stabilizes the circulating form of BMP10, pro.BMP10 is likely to be a preferred form for the in vivo administration for treating PAH and other cardiovascular diseases.

0154 BMP9 and BMP10 Inhibit Blood Outgrowth Endothelial Cell (BOEC) Tube Formation in Collagen Gels

0155 Blood outgrowth endothelial cells can be isolated from the peripheral blood of most individuals and represent a highly proliferative cell type that are highly representative of human endothelial cells. It has been shown that, like endothelial cells, BOECs form vacuolated capillary-like structures in a 3-dimensional collagen:fibronectin matrix. The results of this analysis are shown in FIG. 8 which not only demonstrates the anti-angiogenic role of BMP9 and BMP10 but also shows that BMP9 as well as being anti-proliferative for endothelial cells, protects endothelial cells from apoptosis, and protects endothelial cells from increased permeability. The inhibition by BMP9 is evident even at low concentrations.

EXAMPLE 2

0156 Materials and Methods

0157 Generation of pBMP9 D366A/D408A Variant

0158 BMP9 alanine-scanning results (see FIG. 5) illustrated that D366A and D408A have maintained endothelial signalling but reduced osteogenic signalling activity. Double mutant containing both D366A and D408A (pBMPD366AD408A) were generated using site-directed mutagenesis, and sequence was verified by DNA sequencing. Large-scale transfection of HEK-EBNA cells was carried out as described previously. Mutant protein was purified as described for BMP10 (see Jiang et al. 2016) J. Biol Chem 291, 2954-2966, which is hereby incorporated by reference in its entirety).

0159 Endothelial Cell Signaling Assays

0160 Human pulmonary artery endothelial cells (hPACs) were grown in EGM-2 with 10% FBS. hPACs were serum-starved overnight in EGM-2 with 0.1% FBS and treated with BMP ligands at indicated concentrations. After 8 hours of treatment, cells were harvested and mRNA was extracted. The expression levels of ID1 and BMPR2 were measured by quantitative PCR (qPCR) with 132-microglobulin as control. Fold changes relative to PBS-treated samples were plotted. N=3, mean±SEM is shown.

0161 Osteogenic Signaling in C2C12 Cells

0162 C2C12 cells were seeded in DMEM with 10% FBS and antibiotic : antimycotic (AA) at 4×10^4 cells/well in 24-well plate. Cells were washed twice with DMEM 0.25% FBS +AA before ink of treatment (DMEM 0.25%+AA containing different amount of ligands as indicated) was added. Cells were incubated for 65 hours before harvesting. Alkaline phosphatase (ALP) activities were measured as previously described in the PCT application.
[0163] Osteogenic Signalling in Human Periodontal Ligament Fibroblast Cells (hPLF)

[0164] hPLFs were seeded in Eagles minimal medium (eMM)/Fglutamax/AA/10%FBS at 2.5x10^5 cells/well in 6-well dishes and grown to ~90% confluent. Cells were serum-starved in eMEM with 0.25% FBS overnight before ligands were added. Media were refreshed after three days and cells harvested 6 days later. RNA extraction and qPCR analysis are the same as in PAEC signalling assay.

[0165] Results

[0166] The BMP9 double mutant variant D366A/D408A has potent endothelial cell signalling activity, comparable with D366A and D408A single mutants and the wild type. As shown in

Fig. 9, in a dose-dependent signalling assay in hPAECs, BMP9 D366A/D408A can induce ID1 (Fig. 9A) and BMP2R (Fig. 9B) gene expression with similar EC50 as the wild type BMP9.

[0168] However, the BMP9 double mutant variant D366ND408A does not show any osteogenic signalling activity in vitro. As shown in FIG. 10A, while the wild type BMP9 started to induce ALP activity at 18 ng/ml, BMP9 D366A/D408A does not induce any ALP activity even at 374 ng/ml. Similarly, in hPLF cells, in contrast to the wild type, BMP9 double mutant variant D366ND408A did not induce OSTERIX gene expression even at 187 ng/ml (FIG. 10B).

[0169] Although the present invention has been described with reference to preferred or exemplary embodiments, those skilled in the art will recognize that various modifications and variations to the same can be accomplished without departing from the spirit and scope of the present invention and that such modifications are clearly contemplated herein. No limitation with respect to the specific embodiments disclosed herein and set forth in the appended claims is intended nor should any be inferred.

[0170] All documents cited herein are incorporated by reference in their entirety.
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1290
-continued

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20 25  30
Ala Gly Gly Asn Ala His Ser Pro Leu Gly Val Pro Gly Gly Gly Leu
35 40  45
Pro Glu His Thr Phe Asn Leu Lys Met Phe Leu Gln Asn Val Lys Val
50 55  60
Asp Phe Leu Arg Ser Leu Asn Leu Ser Gly Val Pro Ser Gln Asp Lys
65 70  75  80
Thr Arg Val Glu Pro Pro Glu Thr Met Ile Asp Leu Tyr Asn Arg Tyr
85 90  95
Thr Ser Asp Lys Ser Thr Pro Ala Ser Asn Ile Val Arg Ser Phe
100 105 110
Ser Met Glu Asp Ala Ile Ser Ile Thr Ala Thr Glu Asp Phe Pro Phe
115 120 125
Gln Lys His Ile Leu Leu Phe Asn Ile Ser Ile Pro Arg His Glu Gln
130 135 140
Ile Thr Arg Ala Glu Leu Arg Leu Tyr Val Ser Cys Glu Asn His Val
145 150 155 160
Asp Pro Ser His Asp Leu Lys Ser Val Ile Tyr Asp Val Leu
165 170 175
Asp Gly Thr Asp Ala Trp Asp Ser Ala Thr Glu Thr Lys Thr Phe Leu
180 185 190
Val Ser Gln Asp Ile Gln Asp Glu Gly Trp Glu Val Leu Glu Val Ser
195 200 205
Ser Ala Val Lys Arg Trp Val Arg Ser Asp Ser Thr Lys Ser Lys Asn
210 215 220
Lys Leu Glu Val Thr Val Glu Ser His Arg Lys Gly Cys Asp Thr Leu
225 230 235 240
Asp Ile Ser Val Pro Pro Gly Ser Arg Asn Leu Pro Phe Phe Val Val
245 250 255
Phe Ser Asn Asp His Ser Ser Gly Thr Lys Glu Thr Arg Leu Glu Leu
260 265 270
Arg Glu Met Ile Ser His Glu Gln Glu Ser Val Leu Lys Leu Ser
275 280 285
Lys Asp Gly Ser Thr Glu Ala Gly Glu Ser His Glu Glu Asp Thr
290 295 300
Asp Gly His Val Ala Ala Gly Ser Thr Leu Ala Arg Arg Lys Arg Ser
305 310 315 320
Ala Gly Ala Gly Ser His Cys Glu Thr Ser Leu Arg Val Asn Phe
325 330 335
Glu Asp Ile Gly Trp Asp Ser Trp Ile Ile Ala Pro Lys Glu Tyr Glu
340 345 350
Ala Tyr Glu Cys Lys Gly Gly Cys Phe Phe Pro Leu Ala Asp Asp Val
355 360 365
Thr Pro Thr Lys His Ile Val Glu Thr Leu Val His Leu Lys Phe
370 375 380
Pro Thr Lys Val Gly Ala Cys Val Pro Thr Lys Leu Ser Pro
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<210> SEQ ID NO 5
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<212> TYPE: PRT
<213> ORGANISM: Artificial
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Pro Glu His Thr Phe Asn Leu Lys Met Phe Leu Glu Asn Val Lys Val 50 55 60
Asp Phe Leu Arg Ser Leu Asn Leu Ser Gly Val Pro Ser Gln Asp Lys 65 70 75 80
Thr Arg Val Glu Pro Pro Gln Tyr Met Ile Asp Leu Tyr Asn Arg Tyr 85 90 95
Thr Ser Asp Lys Ser Thr Pro Ala Ser Asn Ile Val Arg Ser Phe 100 105 110
Ser Met Glu Asp Ala Ile Ser Ile Thr Ala Thr Glu Asp Phe Pro Phe 115 120 125
Gln Lys His Ile Leu Leu Phe Asn Ile Ser Ile Pro Arg His Glu Gln 130 135 140
Ile Thr Arg Ala Glu Leu Arg Leu Tyr Val Ser Cys Gln Asn His Val 145 150 155 160
Asp Pro Ser His Asp Leu Lys Gly Ser Val Val Ile Tyr Asp Val Leu 165 170 175
Asp Gly Thr Asp Ala Trp Asp Ser Ala Thr Glu Thr Lys Thr Phe Leu 180 185 190
Val Ser Glu Asp Ile Gln Asp Gly Glu Trp Glu Thr Leu Glu Val Ser 195 200 205
Ser Ala Val Lys Arg Trp Val Arg Ser Asp Thr Lys Ser Lys Asn 210 215 220
Lys Leu Glu Val Thr Val Glu Ser His Arg Lys Gly Cys Thr Leu 225 230 235 240
Asp Ile Ser Val Pro Pro Gly Ser Arg Asn Leu Pro Phe Phe Val Val 245 250 255
Phe Ser Asn Asp His Ser Ser Gly Thr Lys Glu Thr Arg Leu Glu Leu 260 265 270
Arg Glu Met Ile Ser His Glu Glu Ser Val Leu Lys Lys Ser Ser 275 280 285
Lys Asp Gly Ser Thr Glu Ala Gly Glu Ser His Glu Glu Asp Thr 290 295 300
Asp Gly His Val Ala Ala Gly Ser Thr Leu Ala Arg Arg Lys Arg Ser 305 310 315 320
Ala Gly Ala Gly Ser His Cys Gln Lys Thr Ser Leu Arg Val Asn Phe
Glu Asp Ile Gly Trp Asp Ser Trp Ile Ile Ala Pro Lys Glu Tyr Glu
325 330 335
Ala Tyr Glu Cys Lys Gly Gly Cys Phe Phe Pro Leu Ala Ala Asp Val
340 345
Thr Pro Thr Lys His Ala Ile Val Gln Thr Leu Val His Leu Lys Phe
355 360 365
Pro Thr Lys Val Gly Lys Ala Cys Val Pro Thr Lys Leu Ser Pro
370 375 380
Ile Ser Val Leu Tyr Lys Asp Met Gly Val Pro Thr Leu Lys Tyr
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<210> SEQ ID NO 6
<211> LENGTH: 429
<212> TYPE: PRT
<213> ORGANISM: Artificial
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Ala Gly Gly Ala Ala His Ser Pro Leu Gly Val Pro Gly Gly Gly Leu
35 40 45
Pro Glu His Thr Phe Asn Leu Lys Met Phe Leu Glu Asn Val Lys Val
50 55 60
Asp Phe Leu Arg Ser Leu Asn Leu Ser Gly Val Pro Ser Gln Asp Lys
65 70 75 80
Thr Arg Val Glu Pro Gln Tyr Met Ile Asp Leu Tyr Asn Arg Tyr
95 100 105 110
Thr Ser Asp Lys Ser Thr Thr Pro Ala Ser Asn Ile Val Arg Ser Phe
115 120 125
Ser Met Glu Asp Ala Ile Ser Ile Thr Ala Thr Glu Asp Phe Pro Phe
130 135 140
Gln Lys His Ile Leu Leu Phe Asn Ile Ser Ile Pro Arg His Gln
145 150 155 160
Ile Thr Arg Ala Glu Leu Arg Tyr Val Ser Cys Glu Asn His Val
165 170 175
Asp Pro Ser His Asp Leu Lys Gly Ser Val Ile Tyr Asp Val Leu
180 185 190
Asp Gly Thr Asp Ala Trp Asp Ser Ala Thr Glu Thr Lys Thr Phe Leu
195 200 205
Val Ser Gln Asp Ile Gln Asp Gly Trp Glu Thr Leu Glu Val Ser
210 215 220
Ser Ala Val Lys Arg Trp Val Arg Ser Asp Ser Thr Lys Ser Lys Asn
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Lys Leu Glu Val Trp Val Glu Ser His Arg Lys Gly Cys Asp Thr Leu
Asp Ile Ser Val Pro Pro Gly Ser Arg Asn Leu Pro Phe Phe Val Val
<210> SEQ ID NO: 7
<211> LENGTH: 429
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 7

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Ala Gly Asn Ala His Ser Pro Leu Gly Val Pro Gly Gly Gly Gly Leu 35 40 45

Pro Glu His Thr Phe Asn Leu Lys Met Phe Leu Glu Asn Val Val Lys Val 50 55 60

Asp Phe Leu Arg Ser Leu Asn Leu Ser Gly Val Pro Ser Gln Asp Lys 65 70 75 80

Thr Arg Val Glu Pro Pro Gln Tyr Met Ile Asp Leu Tyr Asn Arg Tyr 95 100 90 96

Thr Ser Asp Lys Ser Thr Thr Pro Ala Ser Asn Ile Val Arg Ser Phe 105 110

Ser Met Glu Asp Ala Ile Ser Ile Thr Ala Thr Glu Asp Phe Pro Phe 115 120 125

Gln Lys His Ile Leu Leu Phe Asn Ile Ser Ile Pro Arg His Glu Gln 130 135 140

Ile Thr Arg Ala Glu Leu Arg Leu Tyr Val Ser Cys Glu Asn His Val 145 150 155 160

Asp Pro Ser His Asp Leu Lys Gly Ser Val Val Ile Tyr Asp Val Leu
1. An isolated polypeptide which is a BMP9 variant lacking osteogenic activity and comprising the amino acid sequence of SEQ ID NO: 7.

2. A variant of the polypeptide according to claim 1, wherein one or more of the following amino acids of the polypeptide of claim 1 have been substituted with Alanine: H326, 1)342, S343, W344, I346, K349, F362, K372, I375, L379, H381, L382, K383, K390, S402, L404, K406, V411, T413, L414, Y416 and Y418.

3. A pharmaceutical composition comprising the polypeptide according to claim 1.

4. An isolated nucleotide sequence encoding the polypeptide according to claim 1.

5. A vector comprising a nucleotide sequence encoding the polypeptide according to claim 1.

6. The vector according to claim 5, wherein the vector is a viral vector selected from the group consisting of: retrovirus, adenovirus, lentivirus, herpes simplex, vaccinia and adeno-associated virus.

7. A method of treating a vascular disease or a respiratory disease, comprising administering to a subject in need thereof a therapeutically effective amount of a polypeptide as defined in claim 1.

8. The method according to claim 7, wherein the vascular disease is selected from: pulmonary hypertension, pulmonary arterial hypertension, hereditary haemorrhagic telangiectasia, atherosclerosis and hepatopulmonary syndrome.

9. The method according to claim 7, wherein the respiratory disease is selected from: obstructive lung diseases such as chronic obstructive pulmonary disease (COPD), chronic bronchitis and emphysema; pulmonary vascular diseases such as pulmonary edema and pulmonary hemorrhage; respiratory failure and respiratory distress syndrome, such as acute lung injury and acute respiratory distress syndrome; and interstitial lung diseases, such as idiopathic pulmonary fibrosis.

10. A method of treating a vascular disease or a respiratory disease, comprising administering to a subject in need thereof a therapeutically effective amount of a vector as defined in claim 5.
11. The method according to claim 10, wherein the vascular disease is selected from: pulmonary hypertension, pulmonary arterial hypertension, hereditary haemorrhagic telangiectasia, atherosclerosis and hepatopulmonary syndrome.

12. The method according to claim 10, wherein the respiratory disease is selected from: obstructive lung diseases such as chronic obstructive pulmonary disease (COPD), chronic bronchitis and emphysema; pulmonary vascular diseases such as pulmonary edema and pulmonary hemorrhage; respiratory failure and respiratory distress syndrome, such as acute lung injury and acute respiratory distress syndrome; and interstitial lung diseases, such as idiopathic pulmonary fibrosis.

13. A pharmaceutical composition comprising the variant according to claim 2.

14. An isolated nucleotide sequence encoding the variant according to claim 2.

15. A vector comprising a nucleotide sequence encoding the variant according to claim 2.

16. The vector according to claim 15, wherein the vector is a viral vector selected from the group consisting of: retrovirus, adenovirus, lentivirus, herpes simplex, vaccinia and adeno-associated virus.

17. A method of treating a vascular disease or a respiratory disease, comprising administering to a subject in need thereof a therapeutically effective amount of a variant as defined in claim 2.

18. The method according to claim 17, wherein the vascular disease is selected from: pulmonary hypertension, pulmonary arterial hypertension, hereditary haemorrhagic telangiectasia, atherosclerosis and hepatopulmonary syndrome.

19. The method according to claim 17, wherein the respiratory disease is selected from: obstructive lung diseases such as chronic obstructive pulmonary disease (COPD), chronic bronchitis and emphysema; pulmonary vascular diseases such as pulmonary edema and pulmonary hemorrhage; respiratory failure and respiratory distress syndrome, such as acute lung injury and acute respiratory distress syndrome; and interstitial lung diseases, such as idiopathic pulmonary fibrosis.

20. A method of treating a vascular disease or a respiratory disease, comprising administering to a subject in need thereof a therapeutically effective amount of a vector as defined in claim 15.

21. The method according to claim 20, wherein the vascular disease is selected from: pulmonary hypertension, pulmonary arterial hypertension, hereditary haemorrhagic telangiectasia, atherosclerosis and hepatopulmonary syndrome.

22. The method according to claim 20, wherein the respiratory disease is selected from: obstructive lung diseases such as chronic obstructive pulmonary disease (COPD), chronic bronchitis and emphysema; pulmonary vascular diseases such as pulmonary edema and pulmonary hemorrhage; respiratory failure and respiratory distress syndrome, such as acute lung injury and acute respiratory distress syndrome; and interstitial lung diseases, such as idiopathic pulmonary fibrosis.