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(54) Title: BMP-7 VARIANTS WITH IMPROVED PROPERTIES

(57) Abstract: The invention relates to variants of BMP-7 with improved properties and methods for their use.

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BMP-7 VARIANTS WITH IMPROVED PROPERTIES

[001] This application claims benefit under 35 U.S.C. §119(e) to USSN US 60/558,189 filed on March 31, 2004, entitled *"Cysteine Knot Cytokine Variants with Improved Properties"*; US 60/570,520 filed on May 11, 2004 entitled *" Cysteine Knot Cytokine Variants with Improved Properties"*; US 60/578,432 filed on June 9, 2004 entitled: *"Cysteine Knot Cytokine Variants with Improved Properties"; and* US 60/587,464, filed on July 13, 2004 entitled: *Cysteine Knot Cytokine Variants with Improved Properties"* all *of* which are expressly incorporated by reference in their entirety.

FIELD OF THE INVENTION

[002] The invention relates to variants of bone morphogenetic proteins and other cysteine knot growth factors with improved properties, and to methods of making and using compositions utilizing these variants.

BACKGROUND OF THE INVENTION

[003] Bone morphogenetic proteins (BMPs) are a well-known family of growth factors that contribute to developmental processes such as pattern formation and tissue specification as well as promoting wound healing and repair processes in adult tissues. BMPs were initially isolated by their ability to induce bone and cartilage formation and are now known to regulate cell proliferation, migration, differentiation, and apoptosis in a number of tissues and organs.

[004] BMPs include a number of related human proteins, such as BMP-2, BMP-3 (osteogenin), BMP-3b (GDF-10), BMP-4 (BMP-2b), BMP-5, BMP-6, BMP-7 (osteogenic protein-1 or OP-1), BMP-8 (OP-2), BMP-8B (OP-3), BMP-9 (GDF-2), BMP-10, BMP-11 (GDF-11), BMP-12 (GDF-7), BMP-13 (GDF-6, CDMP-2), BMP-15 (GDF-9), BMP-16, GDF-1, GDF-3, GDF-5 (CDMP-1), and GDF-8 (myostatin). BMPs may be grouped into subfamilies. For example, BMP-2 and BMP-4 are closely related, as are BMP-5, BMP-6, BMP-7, BMP-8, and BMP-8B. BMP-13, BMP-14, and BMP-12 also constitute a subfamily. BMPs are also present in other animal species. Furthermore, there is some allelic variation in BMP sequences among different members of the human population.

[005] BMPs are a subset of the transforming growth factor-β (TGF-β) family, which also includes TGFs (TGF-β1, TGF-β2, and TGF-β3), activins (activin A) and inhibins, macrophage inhibitory cytokine-1 (MIC-1), Mullerian inhibiting substance, anti-Mullerian hormone, and glial cell line derived neurotrophic factor (GDNF). The TGF-β family is in turn a subset of the cysteine knot cytokine superfamily. Additional members of the cysteine knot cytokine superfamily include, but are not limited to, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), noggin, neurotrophins (BDNF, NT3, NT4, and βNGF), gonadotropin, follitropin, lutropin, interleukin-17, and coagulogen.

[006] BMPs have demonstrated utility in the treatment of a variety of conditions and diseases. BMP-2 and BMP-7 have been used to promote bone formation, bone fracture healing, and spinal fusion. BMP-4 (Rundle et. al. (2003) Bone 32: 591-601), BMP-5 (Arosarena and Collins (2003) Arch. Otolaryngol. Head Neck Surg. 129: 1125-1130), BMP-6 (Helm (2003) Gene

Ther. 10: 1735-1743), and BMP-9 (Li et. al. (2003) J. Gene Med. 5: 748-756), have also been demonstrated to promote bone healing in animal models. Animal studies indicate that BMP-7 may be used to treat renal fibrosis and renal failure (Wang et. al. (2001) J. Am. Soc. Nephrol. 12: 2392-2399; Wang and Hirshberg (2003) Am. J. Physiol. Renal Physiol. 284: 1006-1013; Zeisberg et. al. (2003) Nat. Med. 9: 964-968; and Zeisberg et. al. (2003) Am. J. Physiol. Renal Physiol. 285: F1060-F1067), ischemic stroke (Chang et. al. (2003) Stroke 34: 558-564 and Harvey et. al. Pharmacol. Ther. (2005) 105: 113-125) and inflammatory bowel diseases (Maric et. al. (2003) J. Cell Physiol. 196: 258-264).

[007] Structurally, BMPs are dimeric cysteine knot proteins. Each BMP monomer comprises multiple intramolecular disulfide bonds. An additional intermolecular disulfide bond mediates dimerization in most BMPs. BMPs may form homodimers; furthermore some BMPs may form heterodimers. BMPs are expressed as pro-proteins comprising a long pro-domain, one or more cleavage sites, and a mature domain. The pro-domain is believed to aid in the correct folding and processing of BMPs. Furthermore, in some but not all BMPs, the pro-domain may noncovalently bind the mature domain and may act as an inhibitor (eg. Thies et. al. (2001) Growth Factors 18: 251-259).

[008] BMP signal transduction is initiated when a BMP dimer binds two type I and two type II serine/threonine kinase receptors. Type I receptors include but are not limited to ALK-1, ALK-2 (also called ActRIa or ActRI), ALK-3 (also called BMPRIa), and ALK-6 (also called BMPRIb) and type II receptors include but are not limited to ActRIIa (also called ActRII), ActRIIb, and BMPRII. Following BMP binding, the type II receptors phosphorylate the type I receptors, the type I receptors phosphorylate members of the Smad family of transcription factors, and the Smads translocate to the nucleus and activate the expression of a number of genes.

[009] BMPs also interact with inhibitors, soluble receptors, and decoy receptors, including BAMBI (BMP and activin membrane bound inhibitor), BMPER (BMP-binding endothelial cell precursor-derived regulator), Cerberus, cordin, cordin-like, Dan, Dante, follistatin, follistatin-related protein (FSRP), ectodin, gremlin, noggin, protein related to Dan and cerberus (PRDC), sclerostin, sclerostin-like, and uterine sensitization-associated gene-1 (USAG-1). Furthermore, BMPs may interact with co-receptors, for example BMP-2 and BMP-4 bind the co-receptor DRAGON (Samad et. al. (2005) J. Biol. Chem.), and extracellular matrix components such as heparin sulfate and heparin (Irie et. al. (2003) Biochem. Biophys. Res. Commun. 308: 858-865)

[010] For further background on the BMP family, see Balemans and Hul (2002) Dev. Biol. 250: 231-250; Bubnoff and Cho (2001) Dev. Biol. 239: 1-14; Celeste et. al. (1990) Proc. Nat. Acad. Sci. USA 87: 9843-9847; and Cheng et. al. (2003) J. Bone Joint Surgery 85A: 1544-1552

[011] A number of unfavorable properties of naturally occurring BMPs limit the development and use of BMP therapeutics. BMP expression yields are typically poor and suitable expression hosts are limited, hindering development and production. BMPs often possess multiple biological effects, including unwanted side effects. Many BMPs are poorly soluble, reducing storage stability and bioavailability. Finally, BMPs may induce unwanted immune responses.

[012] Earlier studies have identified BMP variants with a number of interesting properties. BMP variants with improved yield in the context of E. coli expression and subsequent refolding from inclusion bodies have been disclosed (US 5,399,677; US 5,804,416; and US 6,677,432). Consensus BMP variants with BMP-like activity have also been described (US 5,011,691; US 6,395,883; US 6,531,445). A BMP-2 point mutant, L51P, has been described that does not bind type I receptors but binds type II receptors normally (Keller et. al. (2004) Nat. Struct. Mol. Biol. 11: 481-488). Deletion mutants of BMP-4 that act as competitive inhibitors of BMP signaling have been disclosed (Weber et. al. (2003) J. Bone Miner. Res. 18: 2142–2151). Mutagenesis experiments have also been performed on BMP-2 to identify residues important for receptor binding; some of these variants were found to act as antagonists (Kirsch et. al. (2000) EMBO J. 19: 3314-3324 and Nickel et. al. (2001) J. Bone Joint Surg. Am. 83-A: S7-S14). In addition, methods for identifying analogs of morphogenetic proteins have been claimed (US 6,273,598). Furthermore, several point mutants of ActA with reduced ALK-4 binding have been identified: S60P, I63P, M91E, I105E, and M108A (Harrison et. al. (2004) J. Biol. Chem. 279: 28036-28044).

SUMMARY OF THE INVENTION

[013] The present invention is related to variants of human bone morphogenetic proteins and other cysteine knot cytokine proteins with improved properties, including increased expression yield, expression in the absence of a pro-domain, increased solubility, increased specific activity, altered receptor, co-receptor, and inhibitor specificity, and decreased immunogenicity.

[014] In one aspect, the invention provides variant BMP-7 protein comprising the sequence:

Fx(1-20)-Vb(21)-Fx(22-38)-Vb(39)-Fx(40-64)-Vb(65)-Fx(66-71)-Vb(72)-Fx(73-77)-Vb(78)-Fx(79-92)-Vb(93)-Fx(94-119)-Vb(120)-Fx(121-134)-Vb(135)

wherein

Fx1(1-20) corresponds to amino acid residues 1-20 of human BMP-7 (SEQ ID NO:5);

Vb(21) is selected from the group consisting of L and G;

Fx(22-38) corresponds to amino acid residues 22-38 of human BMP-7 (SEQ ID NO:5);

Vb(39) is selected from the group consisting of K, A and S;

Fx(40-64) corresponds to amino acid residues 40-64 of human BMP-7 (SEQ ID NO:5);

Vb(65) is selected from the group consisting of Y and N;

Fx(66-71) corresponds to amino acid residues 66-71 of human BMP-7 (SEQ ID NO:5);

Vb(72) is selected from the group consisting of A and D;

Fx(73-77) corresponds to amino acid residues 73-77 of human BMP-7 (SEQ ID NO:5);

Vb(78) is selected from the group consisting of Y and H;

Fx(79-92) corresponds to amino acid residues 79-92 of human BMP-7 (SEQ ID NO:5);

Vb(93) is selected from the group consisting of F, H, S and T;

Fx(94-119) corresponds to amino acid residues 94-119 of human BMP-7 (SEQ ID NO:5);

Vb(120) is selected from the group consisting of S and D;

Fx(121-134) corresponds to amino acid residues 121-134 of human BMP-7 (SEQ ID NO:5);

Vb(135) is selected from the group consisting of A and E;

wherein the variant comprises an amino acid substitution as compared to human BMP-7 (SEQ ID NO:5).

[015] In a further aspect, the invention provides variant BMP-7 proteins comprising a substitution as compared to human BMP-7 (SEQ ID NO:5) selected from the group consisting of: L21G, K39A, K39S, Y65N, A72D, Y78H, F93H, F93S, F93T, S120D and A135E. In both cases, particular variants with single substitutions include, L21G, K39A, K39S, Y65N, A72D, Y78H, F93H, F93S, F93T, S120D and A135E. Particular sets of substitutions include, K39S-F93S; K39S-S120D; K39S-S120D-Y65N; K39S-S120D-A72D; K39S-S120D-Y78H; K39S-S120D-F93H; K39S-S120D-F93S; Y65N-L21G; Y65N-L21R; Y65N-K39S; Y65N-Y78H; Y65N-S120D; Y78H-A72D; Y78H-F93H-Y65N; Y78H-F93H-A72D; Y78H-F93H-S120D; Y78H-S120D and F93H-K39S.

[016] In an additional aspect, the invention provides variant BMP-7 proteins having altered receptor binding affinity compared to wild-type BMP-7 (SEQ ID NO:5), the variant BMP-7 protein comprising one or more substitutions selected from the group consisting of: M23N, Q53G, Q53H and I86D.

BRIEF DESCRIPTION OF THE DRAWINGS

[017] Figure 1 shows sequence alignments of the mature domains of human BMP and GDF proteins. The consensus BMP sequence and the MIC-1 sequence are shown for reference. The N-terminal most residues of the mature domain, which are significantly less well aligned, are not shown.

[018] Figure 2 shows the hexameric structure comprising BMP-7 dimer bound to two type I receptors and two type II receptors. The structure was generated by superimposing BMP-2 (white) bound to ALK-3 (gray, upper right and lower left) and BMP-7 (black) bound to ActRIIa (gray, upper left and lower right).

[019] Figure 3 shows alignments of human type I BMP receptors and human type II BMP receptors used for homology modeling.

[020] Figure 4 shows 12-point binding curves for wild-type BMP-7 (Image clone) binding to ActRIIa, BMPRII, BMPRIa, and BMPRIb.

[021] Figure 5 shows 12-point binding curves for (A) BMPRIa (ALK-3), (B) BMPRIb (ALK-6), (C) ActRIIa, and (D) BMPRII. The thick black lines are wild type BMP-7 (Image clone). The thin gray lines correspond to variants.

[022] Figure 6 shows dose-response C2C12 biassay data for selected Library 1 variants. Highlighted variants in (A) include: F93Q (black hollow circles), F93S (gray filled diamonds), N110D (gray filled squares), S120D (black hollow squares), A135E (black hollow triangles), A135S (black filled circles) and wild type (thick black line, no markers). Additional variants are shown in (A) and (B)

[023] Figure 7 shows 12-point binding curves for (A) BMPRIa (ALK-3), (B) BMPRIb (ALK-6), (C) ActRIIa, and (D) BMPRII. Two replicates of wild type (Image clone) are shown (thick black line, filled black circles). Variants are shown in thin gray lines, no markers.

[024] Figure 8 shows a summary of 12-point receptor binding curves for wild type human BMP-7 (Image clone) and variants.

[025] Figure 9 shows dose-response C2C12 biassay data for selected Library 2 variants.

[026] Figure 10 shows dose-response C2C12 biassay data for selected Library 3 variants, Library 1 variants (thick gray lines, no markers), and wild type (thick black line, no markers).

[027] Figure 11 shows (A) ELISA quantitation of the expression yield of the best single, double, and triple variants in 293T cells (black bars) and CHO cells (white bars) and (B) that enhanced expression yield of the best-expressing single, double, and triple mutant variants results in increased C2C12 bioactivity from serially diluted conditioned media.

[028] Figure 12 shows appropriate correction factors when using a commercial ELISA (R&D Systems) to determine the concentration of selected BMP-7 variants.

[029] Figure 13 shows purification of selected BMP-7 variants.

[030] Figure 14 shows fluorescence images of SDS-PAGE gels showing (A) Alexa 568 labeling of BMP-7 variant Y65N/S120D as a function of dye concentration; and (B) scale-up of Alexa-568 labeled Y65N/S120D BMP-7.

[031] Figure 15 shows fluorescence anisotropy as a function of receptor concentration.

[032] Figure 16 shows that binding of Alexa568-labeled Y65N/S120D BMP-7 to the receptor ActRIIa can be competed with unlabeled BMP-7.

[033] Figure 17 shows competitive binding of (A) recombinant human BMP-7 (R&D Systems), (B) BMP-7 variant 565 (Y65N/F93T/R129D), (C) BMP-7 variant 526 (K39S/S120D/R134E), and (D) BMP-7 variant 504 (Y65N/S120D) to the BMP receptors and inhibitors BMPRIb (open circles), ActRIIa (closed diamonds), BMPRII (closed triangles), and Noggin (stars) determined using AlphaScreen.

[034] Figure 18 shows a bar graph indicating the EC50 of binding for four BMP-7 variants to three receptors and one inhibitor.

DETAILED DESCRIPTION OF THE INVENTION

[035] By "**BMP responsive disorders**" and grammatical equivalents herein is meant diseases, disorders, and conditions that may benefit from treatment with one or more BMPs. Examples of BMP responsive disorders include, but are not limited to, cartilage, bone, and tooth disorders or conditions including but not limited to bone fractures, bone degeneration, osteoporosis, spinal fusion, spinal degenerative disc disease, osteotomy, orthopedic and reconstructive surgery, and periodontal disease; repair of tendons and ligaments; renal disease including but not limited to chronic or acute renal failure, renal injury due to reperfusion, drug-induced renal toxicity, renal fibrosis, renal osteodystrophy, and vascular complications resulting from kidney disease; liver disease including but not limited to cirrhosis and hepatic fibrosis; lung disease including but not limited to asthma, emphysema, and pulmonary fibrosis; wound healing; cancers including but not limited to prostate cancer; inflammatory bowel disease; conditions that would benefit from a neuroprotective agent including but not limited to stroke, Parkinson's disease, traumatic brain injury, and amyotrophic lateral sclerosis; and skin and hair disorders. By "**exposed residues**" and grammatical equivalents herein are meant those

residues whose side chains have at least 50 $Å^2$ (square Angstroms) of solvent accessible surface area in the context of a specified protein structure, preferably an x-ray crystal structure. As will be appreciated by those skilled in the art, other values such as 75 $Å^2$ (square Angstroms) or fractional values such as 50% could be used instead. Furthermore, alternative methods such as contact models, among others, may be used to identify exposed residues. By "**expression yield**" and grammatical equivalents herein is meant the amount of protein, preferably in mg/L or PCD (picograms per cell per day) that is produced or secreted under a given expression protocol (that is, a specific expression host, transfection method, media, time, etc.). By "**improved expression yield**" and grammatical equivalents herein is meant an increase in expression yield, relative to a wild type or parent protein, under a given set of expression conditions. In a preferred embodiment, at least a 50% improvement is achieved, with improvements of at least 100%, 5-fold, 10-fold, or more being especially preferred. In another preferred embodiment, the expression yield is improved to yields of at least 1 µg/ml, with at least 10 µg/ml or 100 µg/ml being especially preferred. By "**hydrophobic residues**" and grammatical equivalents are meant valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, and tryptophan. By "**interface residues**" and grammatical equivalents herein are meant those residues located within 8 Å (Angstroms) of a protein-protein contact. Distances of less than 5 Å (Angstroms) are especially preferred. Distances may be measured in the context of any structure, with high-resolution crystal structures being especially preferred. By "**library**" as used herein is meant a collection of protein sequences that are likely to take on a particular fold or have particular protein properties. The library preferably comprises a set of sequences resulting from computation, which may include energy calculations or statistical or knowledge based approaches. Libraries that range in size from about 5 to about 10^{13} sequences are preferred. Libraries are generally generated experimentally and analyzed for the presence of members possessing desired protein properties. By "**mature domain**" herein is meant, in the context of BMP-7, a domain substantially comprising residues 1-137 of BMP-7. In wild type BMP-7, the mature domain is cleaved from the pro-domain by furin or a furin-like proprotein convertase. By "**modification**" and grammatical equivalents is meant one or more insertions, deletions, or substitutions to a protein or nucleic acid sequence. By "**naturally occurring**" or "**wild type**" or "**wt**" and grammatical equivalents thereof herein is meant an amino acid sequence or a nucleotide sequence that is found in nature, including allelic variations. In a preferred embodiment, the wild type sequence is the most prevalent human sequence. However, the wild type BMP nucleic acids and proteins may be a less prevalent human allele or BMP nucleic acids and proteins from any number of organisms, including but not limited to rodents (rats, mice, hamsters, guinea pigs, etc.), primates, and farm animals (including sheep, goats, pigs, cows, horses, etc). By "**nucleic acid**" and grammatical equivalents herein is meant DNA, RNA, or molecules which contain both deoxy- and ribonucleotides. Nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. Nucleic acids may also contain modifications, such as modifications in the ribose-phosphate backbone that confer increased stability and half-life. By "**polar residues**" and grammatical equivalents herein are

meant aspartic acid, asparagine, glutamic acid, glutamine, lysine, arginine, histidine, serine, and threonine. By "**pro-domain**" herein is meant, in the context of a BMP or other TGF-β family member, the N-terminal domain that is removed following cleavage by furin or a furin-like proprotein convertase. The presence of the pro-domain may promote proper folding and processing. By "**protein**" herein is meant a molecule comprising at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures such as peptoids (see Simon et al. (1992) Proc. Natl. Acad. Sci. USA 89: 9367-9371). For example, homo-phenylalanine, citrulline, and noreleucine are considered amino acids for the purposes of the invention, and both D- and L- amino acids may be utilized. By "**protein properties**" herein are meant physical, chemical, and biological properties including but not limited to physical properties (including molecular weight, hydrodynamic properties such as radius of gyration, net charge, isoelectric point, and spectral properties such as extinction coefficient), structural properties (including secondary, tertiary, and quaternary structural elements) stability (including thermal stability, stability as a function of pH or solution conditions, storage stability, and resistance or susceptibility to ubiquitination, proteolytic degradation, or chemical modifications such as methionine oxidation, asparagine and glutamine deamidation, sidechain racemerization or epimerization, and hydrolysis of peptide bonds), solubility (including susceptibility to aggregation under various conditions, oligomerization state, and crystallizability), kinetic and dynamic properties (including flexibility, rigidity, folding rate, folding mechanism, allostery, and the ability to undergo conformational changes and correlated motions), binding affinity and specificity (to one or more molecules including proteins, nucleic acids, polysaccharides, lipids, and small molecules, and including affinities and association and dissociation rates), enzymatic activity (including substrate specificity; association, reaction, and dissociation rates; reaction mechanism; and pH profile), ammenability to synthetic modification (including PEGylation and attachment to other molecules or surfaces), expression properties (such as yield in one or more expression hosts, soluble versus inclusion body expression, subcellular localization, ability to be secreted, and ability to be displayed on the surface of a cell), processing and posttranslational modifications (including proteolytic processing, N- or C-linked glycosylation, lipidation, sulfation, and phosphorylation), pharmacokinetic and pharmacodynamic properties (including bioavailability following subcutaneous, intramuscular, oral, or pulmonary delivery; serum half-life, distribution, and mechanism and rate of elimination) and ability to induce altered phenotype or changed physiology (including immunogenicity, toxicity, ability to signal or inhibit signaling, ability to stimulate or inhibit cell proliferation, differentiation, or migration, ability to induce apoptosis, and ability to treat disease). By "**solubility**" and grammatical equivalents herein is meant the maximum possible concentration of protein, in the desired or physiologically appropriate oligomerization state, in a solution of specified condition (i.e. pH, temperature, concentration of any buffer components, salts, detergents, osmolytes, etc.). Unless otherwise noted, dimeric BMPs are the desired species. By "**soluble expression**" and grammatical equivalents herein is meant that the protein is able to be

produced at least partially in soluble form rather than in inclusion bodies when expressed in a prokaryotic host. It is preferred that at least 1 μg soluble protein is produced per 100 mL culture, with at least 10 μg or 100 μg being especially preferred. By "**improved solubility**" and grammatical equivalents herein is meant an increase in the maximum possible concentration of protein, in the desired or physiologically appropriate oligomerization state, in solution. For example, if the naturally occurring protein can be concentrated to 1 mM and the variant can be concentrated to 5 mM under the same solution conditions, the variant can be said to have improved solubility. In a preferred embodiment, solubility is increased by at least a factor of 2, with increases of at least 5-fold or 10-fold being especially preferred. As will be appreciated by those skilled in the art, solubility is a function of solution conditions. For the purposes of this invention, solubility should be assessed under solution conditions that are pharmaceutically acceptable. Specifically, pH should be between 6.0 and 8.0, salt concentration should be between 50 and 250 mM. Additional buffer components such as excipients may also be included; although it is preferred that albumin is not required. By "**variant BMP nucleic acids**" and grammatical equivalents herein is meant nucleic acids that encode variant BMPs. Due to the degeneracy of the genetic code, an extremely large number of nucleic acids may be made, all of which encode the variant BMPs of the present invention, by simply modifying the sequence of one or more codons in a way that does not change the amino acid sequence of the variant BMP. By "**variant BMPs**" or "**non-naturally occurring BMPs**" and grammatical equivalents thereof herein is meant non-naturally occurring BMPs which differ from a wild type or parent BMP by at least one (1) amino acid insertion, deletion, or substitution. It should be noted that unless otherwise stated, all positional numbering of variant BMPs and variant BMP nucleic acids is based on these sequences. BMP variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the BMP sequence. BMP variants must retain at least 50 % of wild type BMP activity in one or more cell types, as determined using an appropriate assay described below. Variants that retain at least 75 % or 90 % of wild type activity are more preferred, and variants that are more active than wild type are especially preferred. Alternatively, in some embodiments BMP variants may be engineered to have different activities than a wild type BMP. For example, competitive inhibitors may be designed. A variant BMP may contain insertions, deletions, and/or substitutions at the N-terminus, C-terminus, or internally. In a preferred embodiment, variant BMPs have at least 1 residue that differs from the most similar human BMP sequence, with at least 2, 3, 4, or 5 different residues being more preferred. Variant BMPs may contain further modifications, for instance mutations that alter additional protein properties such as stability or immunogenicity or which enable or prevent posttranslational modifications such as PEGylation or glycosylation. Variant BMPs may be subjected to co- or post-translational modifications, including but not limited to synthetic derivatization of one or more side chains or termini, glycosylation, PEGylation, circular permutation, cyclization, fusion to proteins or protein domains, and addition of peptide tags or labels.

[036] Naturally occurring BMPs regulate cell proliferation, migration, differentiation, and apoptosis in a number of tissues and organs; as a result BMPs may serve many therapeutic uses. However, naturally occurring BMPs are difficult to produce in large amounts, are sparingly soluble, exhibit pleiotropic activities, and may induce unwanted immune responses.

[037] Here, are disclosed novel variants of human BMPs. These BMP variants comprise one or more modifications that were selected to improve biophysical properties and clinical performance.

[038] Strategies for improving expression yield

[039] Reported expression yields for BMPs are typically very low. Reported yields range from 2-6 ng/mL for transiently transfected COS-1 cells in roller bottle culture to 100-200 ng/mL in DHFR-amplified stably transfected CHO cells, *see* U.S. Patent No. 6,048,964 to John C. Lee, *et al.* To facilitate the development and therapeutic use of BMPs, it would be desirable to increase the expression yield to at least 10 µg/ml, with at least 100 µg/ml being more preferred and at least 1000 µg/ml being especially preferred.

[040] A number of nucleic acid properties and protein properties may influence expression yields; furthermore the expression host and expression protocol contribute to yields. Any of these parameters may be optimized to improve expression yields. Also, expression yield may be improved by the incorporation of one or mutations that confer improved stability and/or solubility, as discussed further below. Furthermore, interactions between the pro-domain and the mature domain may influence folding efficiency, and so the pro-domain may also be targeted for modification.

[041] In a preferred embodiment, nucleic acid properties are optimized to improve expression yields using one or more of the following strategies: 1) replace imperfect Kozak sequence, 2) reduce 5' GC content and secondary structure of the RNA, 3) optimize codon usage, 4) use an alternate leader sequence, 5) include a chimeric intron, or 6) add an optimized poly-A tail to the C-terminus of the message. In another preferred embodiment, protein properties are optimized to improve expression yields using one or more of the following strategies: 1) optimize the signal sequence, 2) optimize the proteolytic processing site, 3) replace one or more cysteine residues in order to minimize formation of improper disulfide bonds, 4) improve the rate or efficiency of protein folding, or 5) increase protein stability, especially proteolytic stability. In an alternate preferred embodiment, alternate pro-domain sequences are used. For example, the pro-domain from BMP-2 may be used to aid in the expression of BMP-4 (Wozney et. al. (1988) Science 242: 1528-1534). Pro-domains that may be used include but are not limited to the pro-domains from any BMP sequence and the MIC-1 pro-domain. The pro-domain may be expressed in cis or in trans.

[042] In an additional preferred embodiment, transfection or expression conditions are optimized to increase expression yields. For example, since furin and other pro-protein convertase enzymes require calcium, the addition of calcium to the media during expression may increase the yield of properly processed protein. As another example, proteosome inhibitors may be added to minimize proteosomal degradation. Fetal calf serum or heparin may

also be used. In a further preferred embodiment, the expression host is selected to optimize expression yields. Folding and processing of BMPs is relatively complex and may be assisted by appropriate chaperones. These chaperones may not be expressed equally in all mammalian cell lines. BMP-7 is naturally produced in the kidney and several well-established expression lines are derived from the kidney; in a preferred embodiment BMP-7 is expressed in a kidney cell line including but not limited to 293T, 239-EBNA, COS, and BHK. In another preferred embodiment, the cleavage site in BMP-7 is optimized to promote more efficient proteolytic processing by furin and related subtilisin-like proprotein convertase enzymes. Substrate preferences for furin have been well-characterized (Henrich et. al. (2003) Nat. Struct. Biol. 10: 520-526; Holyoak et. al. (2004) Biochem. 43: 2412-2421; and Duckert et. al. (2004) PEDS 17: 107-112), and cleavage sites in BMP and TGF-β proteins have been analyzed (Constam and Robertson (1999) JBC 144: 139-149). In an alternate preferred embodiment, BMP-7 is expressed in a cell line that is co-transfected with one or more chaperone or processing proteins, including but not limited to furin.

STRATEGIES FOR ENABLING THE USE OF ALTERNATE EXPRESSION HOSTS

[043] BMPs are typically expressed in mammalian cells. In order to enable the use of alternate expression systems, including but not limited to yeast expression systems, it would be desirable to 1) eliminate the N-linked glycosylation site, 2) eliminate potential O-linked glycosylation sites, 3) enable expression in the absence of the pro-domain, and 4) enable processing by an alternate protease present in the desired expression host. In a preferred embodiment, one or more N- or O-linked glycosylation sites is removed. Removal of glycosylation sites from variant BMP polypeptides may be accomplished, for example, by the elimination of one or more serine or threonine residues to the native sequence or variant BMP polypeptide (for O-linked glycosylation sites) or by the modification of a canonical N-linked glycosylation site, N-X-Y-X, where X is any amino acid except for proline and Y is threonine, serine or cysteine. In another preferred embodiment, pro-domain dependence is reduced or eliminated by 1) introducing mutations that stabilize the folded state of the BMP; 2) reducing the exposed hydrophobic surface area of BMP; 3) stabilizing one or more intermediates along the folding pathway of BMP; or 4) replacing one or more pairs of cysteine residues forming a disulfide bond. In an additional preferred embodiment, the furin cleavage site is modified to allow recognition by an alternate protease that is present in the desired expression host. For example, the furin cleavage site may be changed to a kexin cleavage site to facilitate yeast expression. Kexin cleavage sites have been well characterized; see for example Holyoak et. al. (2004) Biochem. 43: 2412-2421.

[044] In an alternate preferred embodiment, the BMPs are expressed using in vitro translation. A number of factors may be added to the reaction to improve the yield of total protein and of correctly folded protein, including but not limited to 1) pro-domains from any TGF-β family member, including but not limited to BMP-2, BMP-4, BMP-7, and MIC-1; 2) accessory factors and chaperones including but not limited to cysteine isomerases, proline isomerases, BiP, heat shock proteins, furin, and other proprotein convertases; 3) redox agents including but

not limited to glutathione; 4) monovalent and divalent cations including but not limited to sodium, potassium, calcium, zinc, and magnesium; and 5) microsomes.

STRATEGIES FOR IMPROVING SOLUBILITY

[045] A variety of strategies may be utilized to design BMP variants with improved solubility and expression yield. In a preferred embodiment, one or more of the following strategies are used: 1) reduce hydrophobicity by substituting one or more solvent-exposed hydrophobic residues with suitable polar residues, 2) increase polar character by substituting one or more neutral polar residues with charged polar residues, 3) increase protein stability, for example by one or more modifications that improve packing in the hydrophobic core, increase beta sheet forming propensity, improve helix capping and dipole interactions, or remove unfavorable electrostatic interactions (increasing the stability of a protein may improve solubility by decreasing the population of partially folded or misfolded states that are prone to aggregation), and 4) modify one or more residues that can affect the isoelectric point of the protein (that is, aspartic acid, glutamic acid, histidine, lysine, arginine, tyrosine, and cysteine residues). Protein solubility is typically at a minimum when the isoelectric point of the protein is equal to the pH of the surrounding solution. Modifications that perturb the isoelectric point of the protein away from the pH of a relevant environment, such as serum, may therefore serve to improve solubility. Furthermore, modifications that decrease the isoelectric point of a protein may improve injection site absorption (Holash et. al. (2002) Proc. Nat. Acad. Sci. USA 99: 11393-11398).

STRATEGIES FOR ALTERING RECEPTOR BINDING AFFINITY OR SPECIFICITY

[046] Several strategies may be used to design BMP variants with improved receptor binding affinity or specificity. In a preferred embodiment, diversity is incorporated at one or more receptor interface positions. However, as is known in the art, modifications at positions distal to the receptor binding interface may also alter binding affinity or specificity. In an especially preferred embodiment, modifications are made to positions in BMP that contact one or more non-conserved receptor positions. For example, Arg 48, Gln 53, and Glu 60 in BMP-7 contacts position 76 in the type II receptor; this position is Glu in ActRIIb, Lys in ActRII, and Thr in BMPRII. Some BMPs, such as BMP-2, bind more tightly to type I receptors while other BMPs, such as BMP-7, have higher affinity for type II receptors. In an additional preferred embodiment, a BMP is modified such that its lower affinity receptor binding site is made more similar to a BMP for which that site is the higher affinity site. For example, the type I receptor interface of BMP-7 may be modified to be more similar to BMP-2, or the type II receptor interface of BMP-2 may be altered to be more BMP-7-like. In an alternate embodiment, modifications are made to stabilize the bound conformation of BMP versus the free conformation. For example, BMP-7 binds first to the type II receptor and then to the type I receptor, and binding to the type II receptor may increase binding affinity for the type I receptor. Binding to the type II receptor causes a conformational change in BMP, producing what may be a higher affinity state. Accordingly, mutations may be introduced to stabilize the bound state.

STRATEGIES FOR EVADING BMP INHIBITORS

[047] A number of soluble and membrane bound proteins function as endogenous inhibitors of BMP action. In a preferred embodiment, BMPs are engineered to reduce or eliminate binding affinity for one or more BMP inhibitors while retaining affinity for one or more of the BMP receptors. Rational alteration of inhibitor specificity may be used to control the site of BMP action, as many of the inhibitors are expressed in specific tissues or organs. Similar approaches may be used to alter specificity for co-receptors such as DRAGON, extracellular matrix components such as heparin and heparin sulfate, and serum components such as alpha2-macroglobulin.

PROTEIN DESIGN AND ENGINEERING METHODS

[048] A number of methods can be used to identify modifications (that is, insertion, deletion, or substitution mutations) that will yield BMP variants with improved properties. These methods include, but are not limited to, sequence profiling (Bowie and Eisenberg (1991) Science 253: 164-170), rotamer library selections (Dahiyat and Mayo (1996) Protein Sci 5: 895-903; Dahiyat and Mayo, Science (1997) 278: 82-87; Desjarlais and Handel (1995) Prot. Sci. (1995) 4: 2006-2018; Harbury et. al. (1995) Proc. Nat. Acad. Sci. USA 92: 8408-8412; Kono et al., Proteins (1994) 19: 244-255; Hellinga and Richards (1994) Proc. Nat. Acad. Sci. USA 91: 5803-5807); and residue pair potentials (Jones (1994) Prot. Sci. 3: 567-574).

[049] In a preferred embodiment, one or more sequence alignments of BMPs and related proteins is analyzed to identify residues that are likely to be compatible with each position. In a preferred embodiment, the PFAM or BLAST alignment algorithm is used to generate alignments of the BMP subfamily, the TGF-β family, or the cysteine knot cytokine superfamily. For each variable position, suitable substitutions may be defined as those residues that are observed at the same position in homologous sequences. Especially preferred substitutions are those substitutions that are frequently observed in homologous sequences. In an additional preferred embodiment, an Analogous Contact Environment (ACE) algorithm, U.S. Patent application 11/00/,647, filed December 8. 2004, is used in conjunction with the sequence alignment information to identify alternate suitable residues that are located in structurally similar environments in other BMPs or homologs. In an especially preferred embodiment, rational design of improved BMP variants is achieved by using Protein Design Automation® (PDA®) technology; see U.S. Patent Nos. 6,188,965; 6,269,312; 6,403,312; 6,708,120; WO98/47089 and USSNs 09/058,459, 09/127,926, 60/104,612, 60/158,700, 09/419,351, 60/181,630, 60/186,904, 09/419,351, 09/782,004 and 09/927,790, 60/347,772, and 10/218,102; and PCT/US01/218,102 and U.S.S.N. 10/218,102, U.S.S.N. 60/345,805; U.S.S.N. 60/373,453 and U.S.S.N. 60/374,035, or using the sequence prediction algorithm (SPA) (Raha et al. (2000) Protein Sci., 9: 1106-1119; USSN 09/877,695, filed June 8, 2001 and 10/071,859, filed February 6, 2002).

STRUCTURAL ANALYSIS OF BMPS

OBTAINING STRUCTURES OF BMPS

[050] PDA® technology calculations require a template protein structure. In one embodiment, the structure of a human BMP is obtained by x-ray crystallography or NMR.

Structures of BMPs include BMP-2 (PDB code 3BMP, Scheufler et. al. (1999) J. Mol. Biol. 287: 103), BMP-2 mutant L51P (PDB code 1REU, Keller et. al. (2004) Nat. Struct. Mol. Biol. 11: 481) and wild type human BMP-7 (PDB code 1LXI Griffith et. al. (1996) Proc. Nat. Acad. Sci. USA 93: 878-883). It is also possible to use the crystal structure of another cysteine knot cytokine protein, such as TGF-β2 (PDB code 1TFG, Schlunegger and Grutter (1992) Nature 358: 430), or NMR structures of cysteine knot cytokine proteins such as TFG-β1 (PDB codes 1KLA, 1KLC, and 1KLD; Hinck et. al. (1996) Biochem. 35: 5817) In an especially preferred embodiment, the crystal structure is a co-crystal structure comprising a BMP and a BMP receptor. High-resolution structures are available for BMP-7 in complex with the receptor ActRIIa (PDB code 1LX5, Greenwald et. al. (2003) Mol. Cell 11: 605-617), activin A bound to ActRIIb (PDB codes 1NYS and 1NYU, Thompson et. al. (2003) EMBO J. 22: 1555-1566), and BMP-2 bound to ALK-3 (PDB code 1ES7, Kirsch et. al. (2000) Nat. Struct. Biol. 7: 492; and PDB code 1REW, Keller et. al. (2004) Nat. Struct. Mol. Biol. 11: 481). In another preferred embodiment, the crystal structure is a co-crystal structure comprising BMP and a BMP inhibitor. A high resolution structure is available for BMP-7 bound to the soluble inhibitor noggin (PDB code 1M4U, Groppe et. al. (2002) Nature 420: 636). Structures of additional BMPs alone and bound to one or more receptors or inhibitors may be built using NMR or x-ray crystal structures including but not limited to those described above in conjunction with homology modeling, structural alignment, and protein-protein docking methods known in the art.

IDENTIFYING FURIN CLEAVAGE SITES

[051] The furin cleavage sites of BMPs may be determined by scanning for the consensus furin cleavage site, R-X-X-K/R, in the residues located N-terminally relative to the aligned regions of the BMP mature domains. The sequence of BMP-7 in the region of the cleavage site is (from P8 to P1 before the cleavage site and from P1' to P4' after the cleavage site, with "|" indicating the cleaved bond) is EVHLRSIR | STGG, wherein the residues "STGG" comprise residues 1 through 4 of the BMP-7 mature domain. The most favored furin cleavage site comprises R at P4, R at P1, and K or R at P2. It is also favorable to have at least one basic residue in residues P5-P8. Some BMPs have multiple cleavage sites. For example, BMP-4 has two cleavage sites, and sequential cleavage is thought to provide a mechanism for regulation of activation and signaling range (Degnin et. al. (2004) Mol. Biol. Cell 15: 5012-5020).

IDENTIFYING GLYCOSYLATION SITES

[052] BMP-7 has an N-linked glycosylation site at Asn 80. When expressed in mammalian cells, the mature domain of BMP-7 does not include any O-linked glycosylation sites. However, it is possible that one or more serine or threonine residues would be susceptible to glycosylation in alternate expression hosts, including but not limited to yeast and Baculovirus expression systems. The presence and location of such O-linked glycosylation sites may be determined experimentally, for example using mass spectrometry.

IDENTIFYING SOLVENT-EXPOSED HYDROPHOBIC RESIDUES

[053] As used herein, exposed hydrophobic residues in BMP-7 include but are not limited to Tyr 44, Trp 52, Trp 55, Ile 57, Phe 73, Tyr 78, Ile 86, Leu 90, Phe 93, Ile 94, Leu 115, Tyr

116, Tyr 117, Val 123, Leu 125, and Tyr 128. BMP-7 also contains three hydrophobic residues in the disordered N-terminal region (that is, residues 1-35). While these residues are not observed in the crystal structures of BMP-7, it is highly likely that they are significantly exposed to solvent. In a preferred embodiment, these additional hydrophobic residues, Leu 21, Met 23, and Val 26, are also considered solvent exposed hydrophobic residues.

IDENTIFYING RESIDUES AT THE RECEPTOR BINDING SITES

[054] In a preferred embodiment, residues that mediate intermolecular interactions between BMPs and their receptors are replaced with structurally and functionally compatible residues that confer improved receptor binding affinity or specificity. Preferred residues at the BMP / type I receptor interface include, but are not limited to, residues Lys 39, Phe 47, Asp 49, Leu 50, Gly 51, Pro 74, Leu 75, Asn 76, Ser 77, Tyr 78, Asn 80, Asn 83, Ile 86, Leu 90, Phe 93, Ile 94, Pro 96, Tyr 116, Lys 126, Tyr 128, Arg 129, Asn 130, and Met 131. Preferred residues at the BMP / type II receptor interface include, but are not limited to, residues Tyr 44, Arg 48, Gln 53, Ile 57, Ala 58, Pro 59, Glu 60, Gly 61, Tyr 62, Ala 63, Gln 108, Asn 110, Ala 111, Ile 112, Ser 113, Val 114, Leu 115, Phe 117, Asn 122, Val 123, Leu 125, Lys 127, and Arg 134.

IDENTIFYING RESIDUES AT INHIBITOR BINDING SITES

[055] In a preferred embodiment, residues that mediate intermolecular interactions between BMPs and their inhibitors are replaced with structurally and functionally compatible residues that confer reduced inhibitor binding affinity or increased inhibitor binding specificity. Preferred residues at the noggin / BMP-7 interface include, but are not limited to, Phe 73, Pro 74, Leu 75, Asn 76, Ser 77, Asn 83, Ile 86, Val 87, and Leu 90.

IDENTIFYING RESIDUES IN REGIONS OF HIGH ELECTROSTATIC POTENTIAL

[056] Proteins may be destabilized by the presence of unfavorable electrostatic interactions or stabilized by the presence of favorable electrostatic interactions. Accordingly, a protein may be stabilized by removing unfavorable electrostatic interactions or by incorporating favorable electrostatic interactions. Modifying regions of high electrostatic potential may also modulate interactions with serum and extracellular matrix components, which may affect pharmacokinetics properties. In a preferred embodiment, the electrostatic potential that is present at each residue position is determined, for example by using Debye-Huckel calculations. Residues in BMP-7 that are located in regions of electrostatic potential greater than 0.25 or less than –0.25 include, but are not limited to, Lys 40, Ser 46, Arg 48, Tyr 62, Ala 64, Tyr 65, Tyr 66, Cys 67, Glu 68, Gly 69, Glu 70, Cys 71, Ala 72, Tyr 78, Asn 80, Ala 81, Thr 82, Asn 83, His 84, Ala 85, Val 87, Gln 88, Thr 89, Ile 94, Pro 100, Cys 104, Ala 105, Pro 106, Thr 107, Gln 108, Leu 109, Asn 110, Ala 111, Ile 112, Ser 113, Asn 122, Ile 124, Asn 130, Met 131, Val 132, Val 133, Arg 134, Ala 135, Cys 136, Gly 137, and His 139.

DESIGN OF OPTIMIZED BMP VARIANTS

IDENTIFYING SUITABLE MODIFICATIONS OF THE FURIN CLEAVAGE SITE

[057] The furin cleavage sequences of several BMPs differ somewhat from the consensus furin cleavage sequence. In BMP-7, preferred modifications to improve proteolytic processing include, but are not limited to, (P8) E→Q or K; (P6) H→K or R; (P5) L→K; and (P2) I→K or R.

IDENTIFYING SUITABLE REPLACEMENTS FOR GLYCOSYLATION SITES

[058] In a preferred embodiment, residues comprising a N- or O-linked glycosylation site are replaced with structurally and functionally compatible residues that do not comprise a glycosylation site. As is known in the art, N-linked glycosylation sites are specified by the sequence N-X-(S/T)-X, where X may be any residue other than proline. Accordingly, an N-linked glycosylation site may be eliminated by 1) replacing the N with any other residue, 2) replacing either X with proline, or 3) replacing the S or T with any residue other than T, S, or C. Preferred modifications that remove the N-linked glycosylation site include, but are not limited to, replacing Asn 80 with Asp, Gln, Ser, or Thr; replacing Thr 82 with Val, and replacing Asn 83 with Pro.

IDENTIFYING SUITABLE POLAR RESIDUES FOR EACH EXPOSED HYDROPHOBIC POSITION

[059] In a preferred embodiment, solvent exposed hydrophobic residues are replaced with structurally and functionally compatible polar residues. Alanine and glycine may also serve as suitable replacements, constituting a reduction in hydrophobicity. Furthermore, mutations that increase polar character, such as Phe to Tyr, and mutations that reduce hydrophobicity, such as Ile to Val, may be appropriate. In a preferred embodiment, preferred suitable polar residues are defined as those polar residues: 1) whose energy in the optimal rotameric configuration, as determined using PDA® technology, is more favorable than the energy of the exposed hydrophobic residue at that position and 2) whose energy in the optimal rotameric configuration is among the most favorable of the set of energies of all polar residues at that position. In a preferred embodiment, the polar residues that are included in the library at each variable position are deemed suitable by both PDA® technology calculations and by sequence alignment data. Alternatively, one or more of the polar residues that are included in the library are deemed suitable by either PDA® technology calculations or sequence alignment data.

[060] Especially preferred modifications to BMP-7 include, but are not limited to, the following substitutions: L21D, L21G, L21K, L21N, L21R, L21S, M23D, M23G, M23K, M23N, M23R, M23S, V26D, V26E, V26G, V26K, V26N, V26S, Y44A, Y44D, Y44E, Y44G, Y44H, Y44K, Y44N, Y44P, Y44Q, Y44R, Y44S, W52A, W52E, W52K, W52Q, W55A, W55E, W55H, W55K, W55N, W55Q, I57A, I57D, I57E, I57H, I57K, I57L, I57T, I57V, E60R, F73A, F73D, F73E, F73H, F73Q, F73R, F73S, Y78D, Y78G, Y78H, Y78N, Y78R, Y78S, Y78T, I86A, I86D, I86E, I86K, I86Q, I86T, L90E, L90K, L90N, L90Q, L90R, L90S, L90T, F93A, F93D, F93E, F93G, F93H, F93Q, F93R, F93S, F93T, I94A, I94E, I94H, I94K, I94Q, I94R, I94T, L115E, L1115K, L115T, Y116A, Y116D, Y116E, Y116H, Y116K, Y116S, Y116T, F117A, F117D, F117E, F117H, F117K, F117Q, F117R, F117Y, V123A, V123D, V123N, V123R, V123T, L125A, L125E, L125K, L125Q, Y128D, Y128E, Y128H, Y128K, and Y128Q. Most especially preferred modifications are those modifications that confer improved properties, such as improved expression yield or activity. Most especially preferred modifications to exposed hydrophobic residues in BMP-7 include, but are not limited to, L21G, L21R, M23G, M23N, M23R, M23S, V26G, V26N, Y65N, Y78H, Y78R, I86A, F93D, F93E, F93G, F93H, F93S, F93T, I94R, Y116H, F117H, F117Y, and Y128D.

IDENTIFYING SUITABLE RESIDUES FOR EACH INTERFACE POSITION

[061] Suitable residues for interface residues as used herein are meant all amino acid residues that are compatible with the structure of a BMP and that retain appreciable binding affinity for at least one of the BMP receptors. Alternatively, competitive inhibitor variants may be generated by identifying alternate residues that are compatible with the structure of a BMP but that substantially eliminate binding affinity for at least one of the BMP receptors. Suitable residues may confer binding specificity by maintaining or increasing affinity for one or two receptors or inhibitors while substantially reducing binding affinity for the other receptors, inhibitors, or additional binding partners. In other cases, modifications are selected to confer other desired properties, for example improved expression yield, while maintaining binding affinities that are substantially similar to the wild type protein. Typically, the interface positions will be substantially exposed to solvent. In such cases, preferred substitutions include the polar residues, alanine, and glycine. However, for interface positions that are substantially buried in the dimer structure, hydrophobic replacements are preferred. Suitable polar residues may also include the subset of polar residues that are observed in analogous positions in homologous proteins, especially other BMPs. In an especially preferred embodiment, suitable polar residues include the subset of polar residues with low or favorable energies as determined using PDA® technology calculations or SPA calculations (described above).

[062] Especially preferred modifications to polar BMP-7 interface residues include, but are not limited to, K39D, K39E, K39G, K39N, K39R, K39S, K39T, R48D, R48E, R48H, R48K, R48N, R48Q, Q53A, Q53D, Q53E, Q53G, Q53H, Q53K, Q53R, Q53S, Q53T, E60H, E60K, E60N, E60P, E60Q, E60R, E60S, E60T, N76A, N76D, N76S, N76T, S77A, S77D, S77E, S77H, S77K, S77N, S77P, S77Q, S77T, K126D, K126E, K126G, K126Q, K126R, K127A, K127D, K127E, K127H, K127N, K127P, K127Q, K127S, K127T, R129D, R129E, R129K, R129N, R129S, R134D, R134E, R134K, R134Q, and R134S. Most especially preferred modifications are those modifications that confer improved properties, such as improved expression yield, improved activity, or enhanced receptor binding specificity. Most especially preferred modifications to residues in regions of high electrostatic potential in BMP-7 include, but are not limited to, K39A, K39S, R48H, R48N, R48Q, Q53A, Q53K, Q53D, Q53G, Q53S, Q53T, E60R, K126R, K127E, R129D, R129N, R134E, and R134S. Additional especially preferred modifications are those modifications that reduce binding to either type I or type II receptors, thereby potentially acting as a competitive inhibitor of BMP. Additional especially preferred modifications to receptor interface residues in BMP-7 include, but are not limited to, Y44T, W52E, and I57Q.

[063] Further preferred modifications are those modifications that alter binding affinity or specificity to a BMP inhibitor. Preferred modifications that reduce binding to noggin include, but are not limited to, W55I, W55L, W55K, W55R, I57M, I57Y, I57E, I57H, I57K, I57Q, I57R, A58I, A58L, A58M, A58Y, A58V, A58E, A58H, A58K, A58Q, A58R, P59Y, N76E, N76Q, N76R, S77E, S77Q, N83F, N83W, N83Y, N83H, N83K, N83R, I86L, I86M, I86F, I86Y, I86R, V87H, S113I, S113L, S113M, S113F, S113Y, S113E, S113H, S113K, S113Q, S113R, L115M, L115K, L115R, V123M, V123Y, V123H, K127I, K127V, K127H, Y128I, and Y128R. Preferred modifications that

increase binding to noggin include, but are not limited to, R48M, P59M, E60I, E60L, E60M, E60V, P74M, N76I, N76V, N76A, S77T, D119I, D119L, K126W, and K127M.

IDENTIFYING SUITABLE RESIDUES FOR REGIONS OF HIGH ELECTROSTATIC POTENTIAL

[064] Regions of high electrostatic potential may be modified in order to increase protein stability or to alter receptor binding affinity and specificity. In a preferred embodiment, residues that are located in a region of high electrostatic potential are replaced by structurally and functionally compatible residues that are predicted to interact favorably with the local electrostatic field. In a preferred embodiment, suitable polar residues include the subset of electrostatically favorable residues that are observed in analogous positions in homologous proteins, especially other BMPs. In an especially preferred embodiment, suitable polar residues include the subset of polar residues with low or favorable energies as determined using PDA® technology calculations or SPA calculations (described above).

[065] Especially preferred modifications to BMP-7 residues located in regions of high electrostatic potential include, but are not limited to, Q88E, N110D, N110E, N110H, A111D, A111S, N130D, A135D, A135E, and A135S. Most especially preferred modifications are those modifications that confer improved properties, such as improved expression yield, improved activity, or enhanced receptor binding specificity. Most especially preferred modifications to receptor interface residues in BMP-7 include, but are not limited to, N110D, A135E, and A135S.

[066] Identifying suitable residues for additional surface positions

[067] Additional residues on the surface of a BMP may be modified in order to improve stability, solubility, or expression yield. In a preferred embodiment, suitable polar residues include the subset of polar residues that are observed in analogous positions in homologous proteins, especially other BMPs. In an especially preferred embodiment, suitable polar residues include the subset of polar residues with low or favorable energies as determined using PDA® technology calculations or SPA calculations (described above).

[068] Additional especially preferred modifications to BMP-7 surface residues include but are not limited to Q36E, Q36N, Q36R, E42D, E42Q, E42R, E42T, D49E, D49S, D54K, D54N, D54R, D54S, E70A, E70Q, N95D, N95K, N95Q, N95R, E97D, E97K, E97R, T98A, T98E, T98K, T98R, Q108D, Q108K, Q108S, D119E, D119N, D119S, D119T, S120D, S120E, S120N, S120R, S121D, S121E, S121K, S121N, S121T, N122E, N122Q, and N122R. Furthermore, residue T98 may be deleted. Most especially preferred modifications are those modifications that confer improved properties, such as improved expression yield, improved activity, or enhanced receptor binding specificity. Most especially preferred modifications to additional residues in BMP-7 include, but are not limited to, S120D.

IDENTIFYING SUITABLE COMBINATIONS OF MUTATIONS

[069] In a preferred embodiment, variants comprising two or more mutations, including but not limited to those disclosed above, are made. Such variants may exhibit greater improvements in expression yield, solubility, or receptor specificity than point mutants. Such variants may also exhibit improvements in more than one protein property.

[070] Especially preferred variants comprising two mutations include but are not limited to L21G/F93H, L21R/F93H, M23N/Y65N, M23R/Y65N, K39A/Y65N, K39A/F93H, K39S/Y78H, K39S/F93H, K39S/N110D, K39S/S120D, K39S/N130D, K39S/R134E, K39S/A135E, R48N/F93H, Q53D/Y65N, Q53G/Y65N, Q53G/Y78H, Q53S/Y65N, Q53T/Y65N, I57L/Y65N, Y65N/Y78H, Y65N/Y78R, Y65N/S120D, Y65N/A135E, Y65N/A135S, A72D/F93H, Y78H/F93H, Y78H/A105V, Y78H/Q108D, Y78H/Y116H, Y78H/F117Y, Y78H/S120D, Y78H/N130D, Y78H/R134E, Y78H/R134S, Y78H/A135E, Y78H/A135S, Y78H/H139R, Y78R/F93H, F93H/F117Y, F93H/S120D, F93H/R134S, and F93H/H139R. Especially preferred variants comprising three mutations include but are not limited to L21G/K39S/S120D, M23R/K39S/S120D, K39S/Y65N/S120D, K39S/A72D/S120D, K39S/Y78H/S120D, K39S/Q108D/S120D, Y65N/Y78H/F93H, Y65N/Y78H/R134E, A72D/Y78H/F93H, Y78H/F93H/Q108D, Y78H/F93H/F117H, Y78H/F93H/S120D, and Y78H/F93H/R134E. Especially preferred variants comprising four modifications include but are not limited to K39S/F93S/Q108D/S120D, K39S/F93S/S120D/R129D, K39S/Y65N/F93S/S120D, K39S/Y78H/F93S/S120D, K39S/F93S/S120D/R134E, K39S/A72D/F92S/S120D, Y65N/Y78H/F93S/R134E, A72D/Y78H/F93S/R134E, M23R/Y65N/F93S/R129D, Y65N/F93S/Q108D/R129D, K39S/F93T/Q108D/S120D, K39S/F93T/S120D/R129D, K39S/Y65N/F93T/S120D, K39S/Y78H/F93T/S120D, K39S/F93T/S120D/R134E, K39S/A72D/F93T/S120D, Y65N/Y78H/F93T/R134E, A72D/Y78H/F93T/R134E, M23R/Y65N/F93T/R129D, and Y65N/F93T/Q108D/R129D.

ADDITIONAL MODIFICATIONS

[071] Additional modifications that might favorably impact expression yield and/or activity can be deduced by observing significant trends in the data obtained. Once such trend is the observation that introduction of a negatively charged amino (E or D) within the Finger 2 region of BMP7 (positions 105-139) leads in most cases to enhanced expression or activity, exemplified by the expression and activity of variants such as Q108D, N110D, N110E, S120D, K127E, Y128D, R129D, N130D, and A135E. Analysis of additional positions within this region indicates that BMP7 substitutions T107D, T107E, S113D, S113E will most likely also possess superior expression and/or activity. A second trend is that the substitution of exposed hydrophobic amino acids with more polar or less hydrophobic alternatives generally leads to enhanced expression yield or activity, exemplified by variants such as Y78H, I86A, Y128D, and multiple substitutions of F93. Application of this trend to I124, another exposed hydrophobic residue, suggests the additional expression- or activity-enhancing variants I124A, I124D, I124E, I124K, I124N, I124Q, I124R, I124S, I124T, and I124V.

[072] Additional insertions, deletions, and substitutions may be incorporated into the variant BMPs of the invention in order to confer other desired properties. In a preferred embodiment, the BMP variant comprises insertions, deletions, or substitutions that reduce immunogenicity, as described in "Antibodies And Fc Fusion Proteins With Altered Immunogenicity," U.S.S.N. 60/643,313, filed January 12, 2005. In an alternate preferred embodiment, the BMP variant is further modified to increase stability. As discussed above, modifications that improve stability

can also improve solubility, for example by decreasing the concentration of partially unfolded, aggregation-prone species. For example, modifications can be introduced to the protein core that improve packing or remove polar or charged groups that are not forming favorable hydrogen bond or electrostatic interactions. It is also possible to introduce modifications that introduce stabilizing electrostatic interactions or remove destabilizing interactions. Additional stabilizing modifications also may be used. In another preferred embodiment, one or more cysteine, lysine, histidine, or other reactive amino acids are added to or eliminated from variant BMPs in order to incorporate or remove sites that are susceptible to covalent modification. For example, see "Rational Chemical Modification," U.S. Patent Application No. 10/956,352, filed September 30, 2004. As is known in the art, variant BMPs may be modified by adding an epitope tag (e.g. a poly-histidine (poly-His), c-myc, or FLAG-tag) or a fusion partner (e.g. an immunoglobulin, the Fc region of an immunoglobulin, albumin, other BMPs, other cytokine proteins, the extracellular domain of a BMP receptor protein, etc). For further details see the descriptions of tags and fusion partners in "Optimized Fc Variants," U.S. Patent Application No. 60/627,774, filed November 12, 2004.

BMP FORMS

[073] BMPs are naturally expressed as pro-proteins comprising a long pro-domain, one or more cleavage sites, and a mature domain. This pro-protein is then processed by the cellular machinery to yield a dimeric mature BMP molecule. In a preferred embodiment, the variants of the invention are produced in a similar manner. The pro-domain is believed to aid in the correct folding and processing of BMPs. Furthermore, in some but not all BMPs, the pro-domain may noncovalently bind the mature domain and may act as a chaperone, as well as an inhibitor (eg. Thies et. al. (2001) Growth Factors 18: 251-259). In additional preferred embodiments, the variants of the invention are produced and/or administered therapeutically in this form. In alternative embodiments, BMPs may be produced in other forms, including, but not limited to, mature domain produced directly or refolded from inclusion bodies, or full-length intact pro-protein. The variants of the invention are expected to find use in these and other forms.

GENERATING THE VARIANTS

[074] Variant BMP nucleic acids and proteins of the invention may be produced using a number of methods known in the art, as elaborated upon below.

PREPARING NUCLEIC ACIDS ENCODING THE BMP VARIANTS

[075] In a preferred embodiment, nucleic acids encoding BMP variants are prepared by total gene synthesis, or by site-directed mutagenesis of a nucleic acid encoding wild type or variant BMP. Methods including template-directed ligation, recursive PCR, cassette mutagenesis, site-directed mutagenesis or other techniques that are well known in the art may be utilized (see for example Strizhov et. al. PNAS 93:15012-15017 (1996), Prodromou and Perl, Prot. Eng. 5: 827-829 (1992), Jayaraman and Puccini, Biotechniques 12: 392-398 (1992), and Chalmers et. at. Biotechniques 30: 249-252 (2001)).

EXPRESSION VECTORS

[076] In a preferred embodiment, an expression vector that comprises the components described below and a gene encoding a variant BMP is prepared. Numerous types of appropriate expression vectors and suitable regulatory sequences for a variety of host cells are known in the art. The expression vectors may contain transcriptional and translational regulatory sequences including but not limited to promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, transcription terminator signals, polyadenylation signals, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences. In addition, the expression vector may comprise additional elements. For example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences, which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art. In addition, in a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome.

[077] The expression vector may include a secretory leader sequence or signal peptide sequence that provides for secretion of the variant BMP from the host cell. Suitable secretory leader sequences that lead to the secretion of a protein are known in the art. The signal sequence typically encodes a signal peptide comprised of hydrophobic amino acids, which direct the secretion of the protein from the cell. The protein is either secreted into the growth media or, for prokaryotes, into the periplasmic space, located between the inner and outer membrane of the cell. For expression in bacteria, bacterial secretory leader sequences, operably linked to a variant BMP encoding nucleic acid, are usually preferred.

TRANSFECTION/TRANSFORMATION

[078] The variant BMP nucleic acids are introduced into the cells either alone or in combination with an expression vector in a manner suitable for subsequent expression of the nucleic acid. The method of introduction is largely dictated by the targeted cell type. Exemplary methods include $CaPO_4$ precipitation, liposome fusion (eg. using the reagent Lipofectin® or FuGene), electroporation, viral infection (eg. as outlined in PCT/US97/0101 9,), dextran-mediated transfection, polybrene mediated transfection, protoplast fusion, direct microinjection, etc. The variant BMP nucleic acids may stably integrate into the genome of the host cell or may exist either transiently or stably in the cytoplasm.

HOSTS FOR THE EXPRESSION OF BMP VARIANTS

[079] Appropriate host cells for the expression of BMP variants include yeast, bacteria, archaebacteria, fungi, and insect and animal cells, including mammalian cells. Of particular

interest are fungi such as *Saccharomyces cerevisiae* and *Pichia pastoris* and mammalian cell lines including 293 (eg. 293-T and 293-EBNA), BRK, CHO (eg. CHOK1 and DG44), COS, Jurkat, NIH3T3, etc. (see the ATCC cell line catalog). BMP variants can also be produced in more complex organisms, including but not limited to plants (such as corn, tobacco, and algae) and animals (such as chickens, goats, cows); see for example Dove, Nature Biotechnol. 20: 777-779 (2002). In one embodiment, the cells may be additionally genetically engineered, that is, contain exogenous nucleic acid other than the expression vector comprising the variant BMP nucleic acid.

EXPRESSION METHODS

[080] The variant BMPs of the present invention are produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a variant BMP, under the appropriate conditions to induce or cause expression of the variant BMP. Either transient or stable transfection methods may be used. The conditions appropriate for variant BMP expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation.

PURIFICATION

[081] In a preferred embodiment, the BMP variants are purified or isolated after expression. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, a BMP variant may be purified using a standard anti-recombinant protein antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see Scopes, R., Protein Purification, Springer-Verlag, NY, 3d ed. (1994). The degree of purification necessary will vary depending on the desired use, and in some instances no purification will be necessary.

POSTTRANSLATIONAL MODIFICATION AND DERIVATIZATION

[082] Once made, the variant BMP may be covalently modified. Covalent and non-covalent modifications of the protein are thus included within the scope of the present invention. Such modifications may be introduced into a variant BMP polypeptide by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Optimal sites for modification can be chosen using a variety of criteria, including but not limited to, visual inspection, structural analysis, sequence analysis and molecular simulation. Sites for modification may be located in the pro-domain or the mature domain.

[083] In one embodiment, the variant BMP of the invention are labeled with at least one element, isotope or chemical compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position. Labels include but are not limited to biotin, tag (e.g. FLAG, Myc) and fluorescent labels (e.g. fluorescein). Derivatization with bifunctional agents is useful, for

instance, for cross linking a variant BMP to a water-insoluble support matrix or surface for use in the method for purifying anti-variant BMP antibodies or screening assays, as is more fully described below. Commonly used cross linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio] propioimidate. Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. Such derivatization may improve the solubility, absorption, transport across the blood brain barrier, serum half-life, and the like. Modifications of variant BMP polypeptides may alternatively eliminate or attenuate any possible undesirable side effect of the protein. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Pa. (1980).

[084] Another type of covalent modification of variant BMP comprises linking the variant BMP polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol ("PEG"), polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. A variety of coupling chemistries may be used to achieve PEG attachment, as is well known in the art. Examples, include but are not limited to, the technologies of Shearwater and Enzon, which allow modification at cysteine residues and primary amines, including but not limited to histidine groups, lysine groups and the N-terminus (see, Kinstler et al, Advanced Drug Deliveries Reviews, 54, 477-485 (2002) and MJ Roberts et al, Advanced Drug Delivery Reviews, 54, 459-476 (2002)). Both labile and non-labile PEG linkages may be used. An additional form of covalent modification includes coupling of the variant BMP polypeptide with one or more molecules of a polymer comprised of a lipophililic and a hydrophilic moiety. Such composition may enhance resistance to hydrolytic or enzymatic degradation of the BMP. Polymers utilized may incorporate, for example, fatty acids for the lipophilic moiety and linear polyalkylene glycols for the hydrophilic moiety. The polymers may additionally incorporate acceptable sugar moieties as well as spacers used for BMP attachment. Polymer compositions and methods for covalent conjugation are described, for example, in U.S. Patent Nos. 5,681,811; 5,359,030.

[085] Another type of modification is chemical or enzymatic coupling of glycosides to the variant BMP. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981). Alternatively, removal of carbohydrate moieties present on the variant BMP polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52

(1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

ASSAYING THE EXPRESSION YIELD OF THE VARIANTS

[086] A primary object of the current invention is the identification of variant BMPs with increased expression yield. Accordingly, the yield, using one or more set of expression conditions, of the variant and wild type BMPs is determined. In one embodiment, expression yields are determined using ELISA. One limitation of this technique is that some variants may confer increased or decreased antibody binding affinity. Accordingly, in a preferred embodiment ELISAs are performed using at least two monoclonal antibodies that recognize distinct epitopes. It is also possible to derive ELISA correction factors for selected variants by purifying said variants and determining their concentration through orthogonal methods, such as UV absorption or BCA assay. Alternatively, the BMPs may be engineered to contain a tag, such as a FLAG tag or His tag, and anti-tag antibodies may be used in the ELISA. In another embodiment, expression yields are determined using Western blotting. As with ELISA, a limitation is that some mutations may confer increased or decreased antibody binding affinity.

ASSAYING THE SOLUBILITY OF THE VARIANTS

[087] In a preferred embodiment, the variant BMPs are assayed for solubility using methods including but not limited to those described below. In all preferred embodiments, the variant and wild type proteins are compared directly in the same assay system and under the same conditions in order to evaluate the solubility of each variant. The solubility of the BMP variants may be determined under a number of solution conditions. A variety of excipients, including solubilizing and stabilizing agents, may be tested for their ability to promote the highest soluble BMP concentration. In addition, different salt concentrations and varying pH may be tested. In a preferred embodiment, solubility is assayed under pharmaceutically acceptable conditions.

[088] Differential light scattering (DLS) may be used to determine oligomerization state. DLS determines diffusion coefficients based on signal correlation from fluctuation of laser light scattered from Brownian motion of particles in solution (Heimenz, Chapter 10 in Polymer Chemistry, Marcel Dekker, Inc., NY, 1984, pp. 659-701). Commercially available instruments provide graphical or table readouts of particle population(s) by size(s) after transforming the diffusion coefficient(s) measured by deconvolution/autocorrelation of laser light scattering data using the Stokes-Einstein equation. The size is therefore the hydrodynamic radius. The distribution of particle sizes within a population(s) is the dispersity, and this factor provides data on the uniformity of the particle population(s). Both dispersity and the appearance of aggregates over time may be monitored to test for solubility. Aggregated protein may be easily resolved by DLS, and readily detected at low levels due to the physical property of aggregates: they scatter more laser light per unit due to the greater target surface area. The sample may be directly introduced into the cuvette (i.e. it is not necessary to perform a chromatographic step first). A relative ratio of monodisperse to aggregate particle population may be determined. Optionally,

this ratio may be weighted by mass or by light scattering intensity. Thus, DLS is a preferred technique to monitor formation of aggregates, and holds the advantage in that it is a non-intrusive technique.

[089] In another preferred embodiment analytical ultracentrifugation (AUC) is used to determine the oligomerization state of the variant BMPs. AUC can be performed in two different 'modes', either velocity or equilibrium. Equilibrium AUC is the most preferred method for determining protein molecular weight and oligomeric state measurement.

[090] A further preferred embodiment is to use size-exclusion chromatography (SEC) to determine the oligomerization state of the BMP variants. Utilizing high performance liquid chromatography, sample may be introduced to an isocratic mobile phase and separated on a gel permeation matrix designed to exclude protein on the basis of size. Thus, the samples will be "sieved" such that the aggregated protein will elute first with the shortest retention time, and will be easily separated from the remainder. This can identify aggregates and allow a relative quantification by peak integration using the peak analysis software provided with the instrument.

[091] In an alternate embodiment, protein concentration is monitored as a function of time. In the case of poor solubility, aggregates will form over time in the protein solution, and eventually precipitate entirely. This may be performed following centrifugation and sampling of the solution phase, in which case insolubility can be measured as a drop in solution protein concentration over time will be observed following centrifugation.

[092] In an alternate embodiment, the oligomerization state is determined by monitoring relative mobility on native gel electrophoresis.

[093] In another embodiment, the amount of protein that is expressed solubly is determined. While factors other than the solubility of the native protein can impact levels of soluble expression, improvements in soluble expression may correlate with improvements in solubility. Any of a number of methods may be used; for example, following expression, SDS-polyacrylamide gel electrophoresis and/or western blots can be done on the soluble fraction of crude cell lysates or the expression media.

[094] Furthermore, any of a number of high throughput screens for soluble expression may be used. In one embodiment, the protein of interest is fused to a fluorescent protein such as GFP, and the cells monitored for fluorescence (Waldo et. al. Nat. Biotechnol. 17: 691 (1999)). In an alternate embodiment, the protein of interest is fused to the antibiotic resistance enzyme chloramphenicol transferase. If the protein expresses solubly, the enzyme will be functional, thereby allowing growth on media with increased concentration of the antibiotic chloramphenicol (Maxwell et. al. Protein Sci. 8: 1908 (1999)). In another embodiment, the protein of interest is expressed as a fusion with the alpha domain of the enzyme beta-galactosidase. If the protein expresses in soluble form, the alpha domain will complement the omega domain to yield a functional enzyme. This may be detected as blue rather than white colony formation when the cells are plated on media containing the indicator X-gal (Wigley et. al. Nat. Biotechnol. 19: 131 (2001)).

ASSAYING THE ACTIVITY OF THE VARIANTS

[095] In a preferred embodiment, the activity of the wild-type and variant proteins are analyzed using in vitro receptor binding assays, cell-based activity assays, or in vivo activity assays.

RECEPTOR BINDING ASSAYS

[096] In a preferred embodiment, the affinity of the variant BMPs for one or more BMP receptors is determined. In an especially preferred embodiment, affinities for ALK-2, ALK-3, ALK-6, ActRII, ActRIIb, and BMPRII are determined. Suitable binding assays include, but are not limited to, ELISA, fluorescence anisotropy and intensity, scintilation proximity assays (SPA) Biacore (Pearce et al., Biochemistry 38:81-89 (1999)), DELFIA assays, and AlphaScreen™ (commercially available from PerkinElmer; Bosse R., Illy C., and Chelsky D (2002)).

[097] In a preferred embodiment, Biacore or surface plasmon resonance assays (see for example McDonnell (2001) Curr. Opin. Chem. Biol. 5:572-577) are used to determine the affinity of one or more BMP variants for one or more BMP receptors. Biacore experiments may be performed, for example, by binding BMP receptor-Fc fusion proteins to a protein A derivitized chip or an NTA chip and testing each BMP variant as an analyte. It is also possible to bind an anti-BMP antibody to the chip, or to bind the BMP variant to the chip and test soluble receptor or Fc-receptor fusion proteins as analytes. Biacore experiments have been used previously to characterize binding of TGF-β isoforms to their receptors (De Crescenzo et. al. (2001) J. Biol. Chem. 276: 29632-29643, De Crescenzo et. al. (2003) J. Mol. Biol. 328: 1173-1183).

[098] In an alternate preferred embodiment, a plate-based Direct Binding Assay is used to determine the affinity of one or more BMP variants for one or more BMP receptors. This method is a modified sandwich ELISA in which BMP is captured using an anti-BMP monoclonal antibody and then detected using a BMP receptor/Fc fusion protein.

[099] In another preferred embodiment, AlphaScreen™ assays (Bosse R., Illy C., and Chelsky D (2002). Principles of AlphaScreen™ PerkinElmer Literaure Aplication Note Ref# s4069. http://lifesciences.perkinelmer.com/Notes/S4069-0802.pdf) are used to characterize receptor and inhibitor binding. AlphaScreen™ is a bead-based non-radioactive luminescent proximity assay where the donor beads are excited by a laser at 680 nm to release singlet oxygen. The singlet oxygen diffuses and reacts with the thioxene derivative on the surface of acceptor beads leading to fluorescence emission at ~600 nm. The fluorescence emission occurs only when the donor and acceptor beads are brought into close proximity by molecular interactions occurring when each is linked to ligand and receptor (or ligand and inhibitor) respectively. This interaction may be competed away by adding an appropriate amount of unlabeled BMP variant that binds the relevant receptor or inhibitor.

[0100] In one embodiment, AlphaScreen™ assays are performed using 1) BMP modified by the addition of a suitable tag or label; 2) donor beads capable of binding the tag or label used to modify the BMP; 3) a BMP receptor or inhibitor modified by the addition of a suitable tag or label; 4) acceptor beads capable of binding the tag or label used to modify the BMP receptor, and 5) varying amounts of an unlabeled variant BMP-7 molecule, which acts as a competitor. It is also possible to coat the donor or acceptor beads with antibodies that specifically recognize

the native BMP or BMP receptor, or to bind the receptor to the donor beads and the ligand to the acceptor beads. In an alternate embodiment, AlphaScreen™ assays are performed using 1) a type I BMP receptor modified by the addition of a suitable tag or label; 2) donor beads capable of binding the tag or label used to modify the type I BMP receptor; 3) a type II BMP receptor modified by the addition of a suitable tag or label; 4) acceptor beads capable of binding the tag or label used to modify the type II BMP receptor; 5) BMP, and 6) varying amounts of an unlabeled variant BMP-7 molecule, which acts as a competitor. It is also possible to bind the type I BMP receptor to the acceptor beads and the type II BMP receptor to the donor beads.

[0101] In another embodiment, fluorescence assays are used. Either BMP-7 or a BMP-7 receptor or inhibitor may be labeled with a fluorescent dye (for examples of suitable dyes, see the Molecular Probes catalog). As is known in the art, the fluorescence intensity or anisotropy of a labeled molecule may change upon binding to another molecule. Fluorescence assays may be performed using 1) fluorescently labeled BMP-7, 2) a BMP receptor or inhibitor, and 3) varying amounts of an unlabeled variant BMP-7 protein, which acts as a competitor.

[0102] In an additional embodiment, scintillation proximity assays (SPA) are used to determine receptor binding affinity. For example, BMP receptor-Fc fusions may be bound to protein A coated SPA beads or flash-plate and treated with S35-labeled BMP; the binding event results in production of light.

CELL-BASED ACTIVITY ASSAYS

[0103] BMPs promote the growth and differentiation of a number of types of cells. BMP activity may be monitored, for example, by measuring BMP-induced differentiation of MC3T3-E1 (an osteoblast-like cell derived from murine calvaria), C3H10T1/2 (a mouse mesenchymal stem cell line derived from embryonic connective tissue), ATDC5 (a mouse embryonal carcinoma cell), L-6 (a rat myoblast cell line) or C2C12 (a mouse myoblastic cell line) cells. Differentiation may be monitored using, for example, luminescence reporters for alkaline phosphatase or colorimetric reagents such as Alcian Blue or PNPP (Asahina et. al. (1996) Exp. Cell Res. 222: 38-47; Inada et. al. (1996) Biochem. Biophys. Res. Commun. 222: 317-322; Jortikka et. al. (1998) Life Sci. 62: 2359-2368; Cheng et. al. (2003) J. Bone Joint Surgery 95A: 1544-1552). The rat limb bud cartilage differentiation assay may also be used to monitor activity in primary cells. In an alternate embodiment, reporter gene or kinase assays may be used. BMPs activate the JAK-STAT signal transduction pathway. Accordingly, a BMP responsive cell line containing a STAT-responsive reporter such as GFP or luciferase may be used (Kusanagi et. al. (2000) Mol. Biol. Cell. 11: 555-565). In a preferred embodiment, BMP activity in kidney cells is determined using cell-based assays; see for example Wang and Hirschberg (2004) J. Biol. Chem. 279: 23200-23206.

ANIMAL MODELS OF BMP ACTIVITY

[0104] In the simplest embodiment, BMP activity in an animal is measured as bone induction following subcutaneous injection. In a preferred embodiment, the activity of one or more BMP variants is determined in an animal model of a BMP-responsive disease or condition. Animal models of renal disease include, but are not limited to, the rat nephrotoxic serum nephritis model

(Zeisberg et. al. 2003)), the rat chronic cyclosporine A-induced nephropathy model (Li et. al. (2004) Am. J. Physiol. Renal Physiol. 286: F46-57), the mouse unilateral uretreral obstruction model (Schanstra et. al. (2003) Thromb. Haemost. 89: 735-740), streptozotocin-induced diabetic nephropathy (Taneda et. al. (2003) J. Am. Soc. Nephrol. 14: 968-980), the anti-thy 1.1 mAb and Habu snake venom induced glomerulonephritis models (Dimmler et. al. (2003) Diagn. Mol. Pathol. 12: 108-117), and the rat 5/6 remnant kidney model (Romero et. al. (1999) Kidney Int. 55: 945-955). Animal models of liver disease include, but are not limited to, rat bile duct ligation/scission model (Park et. al. (2000) Pharmacol. Toxicol. 87: 261-268), CCl_4 plus ethanol-induced liver damage (Hall et. al. (1991) Hepatology 12: 815-819), dimethylnitrosamine-induced liver cirrhosis (Kondou et. al. (2003) J. Hepatol. 39: 742-748), and thioacetamide-induced liver damage (Muller et. al. (1988) Exp. Pathol. 34: 229-236). Animal models of lung disease include, but are not limited to, ovalbumin-induced airway fibrosis (Kenyon et. al. (2003) Toxicol. Appl. Pharmacol. 186: 90-100), bleomycin-induced lung fibrosis (Izbicki et. al. (2002) Int. J. Exp. Pathol. 83: 111-119), monocrotaline-induced pulmonary fibrosis (Hayashi et. al. (1995) Toxicol. Pathol. 23: 63-71), and selective irradiation (Pauluhn et. al. (2001) Toxicology 161: 153-163). Animal models of neurological disease include, but are not limited to, animal models for Parkinson's disease such as the 6-hydroxydopamine (6-OHDA) hemilesioned rat model and MPTP-induced Parkinson's disease, animal models of ALS such as rats or mice expressing mutant SOD1 (Shibata et. al. (2002) Neuropathology 22: 337-349), and animal models of stroke induced by intracortical microinjection of endothelin or quinolinic acid (Gilmour et. al. (2004) Behav. Brain Res. 150: 171-183) or cerebral artery occlusion (Merchenthaler et. al. (2003) Ann. NY Acad. Sci. 1007: 89-100).

ADMINISTRATION AND TREATMENT USING BMP VARIANTS

[0105] Once made, the BMP variants of the invention may be administered to a patient to treat a BMP related disorder. The BMP variants may be administered in a variety of ways, including, but not limited to orally, parenterally, subcutaneously, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonarally, vaginally, rectally, intranasally or intraocularly. In some instances, the variant BMP may be directly applied as a solution or spray.

[0106] The pharmaceutical compositions of the present invention comprise a BMP variant in a form suitable for administration to a patient. In the preferred embodiment, the pharmaceutical compositions are in a sterile, water-soluble form and may include pharmaceutically acceptable acid addition salts or pharmaceutically acceptable base addition salts. The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers such as NaOAc; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives that are "generally recognized as safe" (GRAS) are well known in the art, and are used in a variety of formulations.

[0107] Any of a number of drug delivery devices or sustained release formulations may be used. For example, a variant BMP may be administered as a pro-protein comprising a BMP pro-

domain and a BMP mature domain. BMPs may also be administered as BMP-impregnated matrix material (for example Geiger et. al. Adv. Drug Deliv. Rev. (2003) 55: 1613-1629; Hu et. al. J. Biomed. Mater. Res. (2003) 67A: 591-598; Peel et. al. J. Craniofac Surg. (2003) 14: 284-291); such a method of administration is especially preferred for promoting bone healing and growth. Furthermore, implants for bone repair may be coated with BMPs to promote bone healing and improve bone strength (Schmidmaier et. al. Bone (2002) 30: 816-822). In a further embodiment, the variant BMPs are added in a micellular formulation (U.S. Patent No. 5,833,948), liposomes (Matsuo et. al. J. Biomed. Mater. Res. (2003) 66A: 747-754), biodegradable polymers (Saito and Takaoka, Biomaterials (2003) 24: 2287-2293; Saito et. al. Bone (2003) 32: 381-386; Weber et. al. Int. J. Oral Maxillofac. Surg. (2002) 31: 60-65; Saito et. al. J. Bone Joint Surg. Am. (2001) 83-A: S92-S98), hydrogels (Yamamoto et. al. Biomaterials (2003) 24: 4375-4383), or the like.

[0108] Combinations of pharmaceutical compositions may be administered. Moreover, the compositions may be administered in combination with other therapeutics.

[0109] Nucleic acid encoding the variant BMPs may also be used in gene therapy. In gene therapy applications, genes are introduced into cells in order to achieve *in vivo* synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Any of a variety of techniques known in the art may be used to introduce nucleic acids to the relevant cells. The oligonucleotides may be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups. For review of gene marking and gene therapy protocols see Anderson et al., Science 256:808-813 (1992).

EXAMPLES

[0110] Example 1: ***Structural modeling***

[0111] Hexameric complexes comprising a BMP-7 dimer or a BMP-2 dimer bound to two ALK-3 receptors and two ActRIIa receptors was constructed using the structure of BMP-7 bound to ActRIIa (PDB code 1LX5) and the structure of BMP-2 bound to ALK-3 (PDB code 1ES7). Using InsightII (Accelrys), the BMP structures were superimposed as follows: BMP-2 residues 22-32 superimposed with BMP-7 residues 47-56, BMP-2 residues 49-71 superimposed with BMP-7 residues 73-95, and BMP-2 residues 101-106 superimposed with BMP-7 residues 126-131. This yielded a backbone atom RMSD of 0.77 Å. The superposition was repeated so that chain A in the BMP-7 structure was superimposed onto chains A and C of the BMP-2 structure. The sequence alignment between BMP-2 and BMP-7 is shown in Figure 2 and the structure of the hexameric complex is shown in Figure 3.

[0112] Homology modeling was used to generate structures of additional BMP receptors bound to BMP-2 and BMP-7. As shown in Figure 5, the sequences of ALK-2 and ALK-6 were aligned with ALK-3, and the sequences of ActRIIb and BMPRII were aligned with ActRIIa. The Modeler tool in InsightII (Accelrys) was used to generate the homology models. Disulfide pairs

were manually constrained as follows (using the crystallographic numbering from 1LX5 and 1ES7): Alk3: 238-259, 240-244, 253-277, 287-301, 302-307; ActRIIa: 11-41, 31-59, 66-85, 72-84, and 86-91. Three models were generated for each molecule; the model with the best energy and –lnPDF score was selected for subsequent PDA® technology calculations. Homology modeling was also used to generate structures of BMP-4, BMP-5, BMP-6, and BMP-8. BMP-4 was modeled using the BMP-2 structure while BMP-5, BMP-6, and BMP-8 were modeled using the BMP-7 structure. The BMP sequences were aligned as shown in Figure 3 PDA® technology calculations were used to model the BMP-4, BMP-5, BMP-6, and BMP-8 structures.

[0113] Example 2: ***Identification of exposed hydrophobic residues in BMPs***

[0114] Structures of BMP-7 dimer ("dimer") and BMP-7 dimer bound to ALK-3 and ActRIIa ("hexamer") were analyzed to identify solvent-exposed hydrophobic residues. The absolute and fractional solvent-exposed hydrophobic surface area of each residue was calculated using the method of Lee and Richards (J. Mol. Biol. 55: 379-400 (1971)) using an add-on radius of 1.4 Å (Angstroms). Each residue was also classified as core, boundary, or surface (see Dahiyat and Mayo Science 278: 82-87 (1997)).

[0115] Solvent exposed hydrophobic residues in BMP-7 were defined to be hydrophobic residues with at least 50 $Å^2$ (square Angstroms) exposed hydrophobic surface area in the BMP-7 dimer (PDB code 1LX5, chain A, plus symmetry-related BMP-7 molecule). Exposed hydrophobic surface area was also measured in the context of the BMP-7 / ALK-2 / ActRIIa hexamer, and RESCLASS was run to categorize each position as core, boundary, or surface.

Table 1 . Exposed hydrophobic residues in BMP-7.

#	wt	dimer RC	hexamer RC	dimer expH	hexamer expH
44	TYR	surface	surface	85.5	95.5
52	TRP	boundary	core	82.9	5.9
55	TRP	boundary	boundary	168.4	125.5
57	ILE	boundary	core	70.3	29.8
73	PHE	surface	core	70.5	64.6
78	TYR	surface	core	107.9	71.2
86	ILE	surface	core	60.7	4.3
90	LEU	boundary	core	51.7	10.6
93	PHE	boundary	core	113.1	10.9
94	ILE	boundary	core	79.0	37.3
115	LEU	surface	core	56.9	0.0
116	TYR	boundary	core	51.5	51.6
117	PHE	surface	boundary	97.5	25.4
123	VAL	surface	core	86.0	14.4
125	LEU	surface	core	88.4	60.9
128	TYR	boundary	core	64.9	17.4

[0116] Example 3: ***Identification of receptor and inhibitor interface residues in BMP-7***

[0117] Potential sites of interactions between BMP-7 and ALK-3, BMP-7 and ActRIIa, and BMP-7 and noggin were identified by examining the structure of the hexameric structure described in Example 1 and the co-crystal structure of BMP-7 and noggin (PDB code 1M4U). Next, distance measurements were used to identify residues that may participate in intermolecular interactions. Residues in BMP-7 that are within 5 Å (Angstroms) of the ALK-3, ActRIIa, or noggin interfaces (as measured by CA-CA distances) are shown below, along with the receptor or inhibitor positions that are contacted. Next, the receptor sequence alignments used for homology modeling were analyzed for polymorphisms. Information about receptor polymorphisms was used to design receptor-specific variants, described below. If the receptor positions are polymorphic, it is noted in Table 2; "na" indicates that the receptor positions were not sufficiently well-aligned to unambiguously identify the polymorphisms. However, receptor-specific BMP variants may be identified that contact such unaligned regions of the receptors.

Table 2. BMP-7 receptor and inhibitor contacts

#	Wt	Contacts: A, B=ALK-3; D, F = ActRIIa; and N = noggin	receptor polymorphisms
39	LYS	ASP A 246	246 (ALK6=E, ALK3=D, ALK2 na)
44	TYR	ASN D 65, ILE D 64, ASP D 63	65(ActRIIa, ActRIIb N, BMPRII na), 64(ActRIIb=F, ActRIIa=I, BMPRII na), 63(ActRIIa, ActRIIb D, BMPRII na)
47	PHE	PHE B 285	285(ALK6, ALK3=F, ALK2=M)
48	ARG	LYS D 76, GLN N 208, ARG N 209, ARG N 210	76(ActRIIb=E, ActRIIa=K, BMPRII=T)
49	ASP	LYS B 292	na
50	LEU	SER B 290, PHE B 285	290(ALK6=T, ALK3=S, ALK2=P), 285(ALK6, ALK3=F, ALK2=M)
51	GLY	PRO B 291, SER B 290, LYS B 292	291 P conserved, 290(ALK6=T, ALK3=S, ALK2=P), 292 na
52	TRP	PHE B 285, LYS B 288, SER B 290, PRO B 291, ILE N 33, ARG N 34, PRO N 35	285(ALK6, ALK3=F, ALK2=M), 288(ALK6=R, ALK3,ALK2=K), 290(ALK6=T, ALK3=S, ALK2=P), 291 P conserved
53	GLN	LYS D 76, ARG N 206	76 (ActRIIb=E, ActRIIa=K, BMPRII=T)
54	ASP	LYS B 288, GLU D 80, ARG N 206, GLN N 208	288(ALK6=R, ALK3,ALK2=K), 80(ActRIIb=Q, ActRIIa=E, BMPRII na)
55	TRP	LYS B 288, ARG N 34, PRO N 35	288(ALK6=R, ALK3,ALK2=K)

56	ILE	PHE B 285	285(ALK6, ALK3=F, ALK2=M)
57	ILE	PHE D 83, VAL D 81, THR D 44, ARG N 204, ARG N 206, ILE N 218	83 F conserved, 81(ActRIIa, ActRIIb V, BMPRII na), 44(ActRIIb=S, ActRIIa=T, BMPRII=L)
58	ALA	PHE D 83, TRP D 60, LEU N 46, GLU N 48, ARG N 204	60 W conserved, 83 F conserved
59	PRO	ASP D 63, ASN D 65, TRP D 60, PHE D 83, LEU N 46, ILE N 47	63(ActRIIa, ActRIIb D, BMPRII na), 65(ActRIIa, ActRIIb N, BMPRII na), 60 W conserved, 83 F conserved
60	GLU	LYS D 76, ASN D 65, GLU D 74, PHE N 54	76(ActRIIb=E, ActRIIa=K, BMPRII=T), 65(ActRIIa, ActRIIb N, BMPRII na), 74(ActRIIb=A, ActRIIa=E, BMPRII=V)
61	GLY	ASN D 65	65(ActRIIa, ActRIIb N, BMPRII na)
62	TYR	ASP D 63, ASN D 65, ILE D 64	63(ActRIIa, ActRIIb D, BMPRII na), 65(ActRIIa, ActRIIb N, BMPRII na), 64(ActRIIb=F, ActRIIa=I, BMPRII na)
63	ALA	ILE D 64	64(ActRIIb=F, ActRIIa=I, BMPRII na)
73	PHE	ARG A 297, GLU A 264, ILE N 33	297(ALK6,ALK3=R, ALK2=Q) 264(ALK6,ALK3=E, ALK2=S)
74	PRO	HIS A 243, ILE A 262, ILE A 299, PHE A 260, GLU A 264, GLN A 286, MET A 278, LEU N 31, ILE N 33	243(ALK3, ALK6 H, ALK2 na), 262(ALK6=M, ALK3=I, ALK2=S), 299(ALK6, ALK3=I ALK2=V), 260 F conserved, 264(ALK6, ALK3=E, ALK2=S), 286(ALK6, ALK3=Q ALK2=T), 278(ALK6=L, ALK3=M, ALK2=F)
75	LEU	GLN A 286, TYR N 30, LEU N 31, HIS N 32, ILE N 33	286(ALK6, ALK3=Q ALK2=T)
76	ASN	HIS A 243, PHE A 260, GLY A 276, MET A 278, PRO A 245, CYS A 277, TYR N 30, LEU N 31, HIS N 32	243(ALK3, ALK6 H, ALK2 na), 260 F conserved, 276 G conserved, 278(ALK6=L, ALK3=M, ALK2=F), 245(ALK3, ALK6 P, ALK2 na), 277 C conserved
77	SER	CYS A 277, CYS A 253, THR A 255, PHE A 260, LYS A 279, GLY A 276, MET A 278, PRO A 245,	277 C conserved, 253 C conserved, 255(ALK3, ALK6 T, ALK2 na), 260 F conserved, 279(ALK6=G, ALK3=K, ALK2 na), 276 G conserved,

		MET N 27, HIS N 29, TYR N 30, HIS N 32	278(ALK6=L, ALK3=M, ALK2=F), 245(ALK3, ALK6 P, ALK2 na)
78	TYR	ASP A 246, PRO A 245, ASP A 247	246(ALK6=E, ALK3=D, ALK2 na), 245(ALK3, ALK6 P, ALK2 na), 247(ALK3, ALK6 D, ALK2 na)
80	ASN	LYS A 279	279(ALK6=G, ALK3=K, ALK2 na)
83	ASN	GLU A 281, GLY A 282, PHE A 285, ARG N 34, PRO N 35, ALA N 36	281(ALK3, ALK6,282 ALK2 na), 282(ALK3, ALK6 G, ALK2 na), 285(ALK6,ALK3=F ALK2=M)
86	ILE	GLN A 286, GLY A 282, PHE A 285, HIS N 32, ILE N 33, ARG N 34, PRO N 35	286(ALK6,ALK3=Q ALK2=T), 282(ALK3, ALK6 G, ALK2 na), 285(ALK6,ALK3=F ALK2=M)
87	VAL	PRO N 35	
90	LEU	ASP A 289, SER A 290, PHE A 285, ARG A 297, GLN A 286, ILE N 33	289(ALK6,ALK3=D ALK2=T), 290(ALK6=T ALK3=S ALK2=P), 285(ALK6,ALK3=F ALK2=M), 297(ALK6,ALK3=R ALK2=Q), 296(ALK3, ALK6 R, ALK2 na)
93	PHE	ALA A 293, ARG A 297, ASP A 289, LEU A 295, SER A 290, GLN A 294, GLU A 264	293(na), 295(na), 294(na), 297(ALK6,ALK3=R ALK2=Q), 289(ALK6,ALK3=D ALK2=T), 290(ALK6=T ALK3=S ALK2=P), 264(ALK6,ALK3=E ALK2=S)
94	ILE	SER A 290, LYS A 292, ALA A 293	290(ALK6=T ALK3=S ALK2=P), 292(na), 293(na)
108	GLN	ASP D 36	36(ActRII, ActRIIb D, BMPRII N)
110	ASN	LYS D 37, ASP D 62, ASP D 36	36(ActRII, ActRIIb D, BMPRII N),37(ActRII, ActRIIa K, BMPRII na), 62 D conserved
111	ALA	LEU D 61, LYS D 37	61(ActRIIa, ActRIIb L, BMPRII G), 37(ActRII, ActRIIa K, BMPRII na)
112	ILE	LEU D 61	61(ActRIIa, ActRIIb L, BMPRII G)
113	SER	LEU D 61, TRP D 60, LEU N 43, VAL N 44, ASP N 45, LEU N 46	61(ActRIIa, ActRIIb L, BMPRII G), 60 W conserved
114	VAL	TRP D 60, LEU N 46	60 W conserved

115	LEU	PHE D 83, TRP D 60, PHE D 42, THR D 44, LYS D 56, LEU N 46, PHE N 168, ARG N 204, ILE N 220	83 F conserved, 60 W conserved, 56 K conserved, 42(ActRIIb, BMPRII=Y, ActRIIa=F), 44(ActRIIb=S, ActRIIa=T, BMPRII=L)
116	TYR	ASP B 284, PRO N 37	284(ALK6,ALK3=D ALK2=K)
117	PHE	VAL D 81, GLU D 80, ARG N 206, ILE N 218	81(ActRIIa, ActRIIb V, BMPRII na), 80(ActRIIb=Q, ActRIIa=E, BMPRII na)
119	ASP	HIS N 29	
122	ASN	ARG D 20, ASN D 17, GLN N 221	20(ActRIIb=L, ActRIIa=K, BMPRII na), 17(ActRIIa, ActRIIb N, BMPRII na)
123	VAL	VAL D 81, VAL D 55, LYS D 56, THR D 44, LYS D 46, ILE N 218, PRO N 219, ILE N 220, GLN N 221	81(ActRIIa, ActRIIb V, BMPRII na), 55 V conserved, 56 K conserved, 44(ActRIIb=S, ActRIIa=T, BMPRII=L), 46(ActRIIb=A, ActRIIa=K, BMPRII=E),
124	ILE	HIS N 199, GLN N 221	
125	LEU	TRP D 60, PHE D 42, LYS D 56, LEU N 43, ASP N 45, LEU N 46, GLN N 221, TYR N 222, PRO N 223	60 W conserved, 56 K conserved, 42(ActRIIb, BMPRII=Y, ActRIIa=F)
126	LYS	TYR B 280, GLU B 281, ASP B 284, PRO N 37, SER N 38, ASP N 39, LEU N 43, HIS N 199	280(ALK6=L ALK3=Y ALK2 na), 281(ALK2 na), 284(ALK6,ALK3=D ALK2=K)
127	LYS	LEU D 61, LYS D 37, ALA N 36, PRO N 37, SER N 38, ASN N 40, LEU N 41, PRO N 42, LEU N 43	37(ActRII, ActRIIa K, BMPRII na), 61(ActRIIa, ActRIIb L, BMPRII G), 36(ActRII, ActRIIb D, BMPRII N)
128	TYR	ASP B 284, PHE B 285, PRO N 35, ALA N 36, PRO N 37, SER N 38	284(ALK6,ALK3=D ALK2=K), 285(ALK6,ALK3=F ALK2=M)
129	ARG	GLU B 281, ASN E 83, ALA N 36, SER N 38, ASN N 40	281(ALK3, ALK6 E, ALK2 na)

130	ASN	GLU B 281	281(ALK3, ALK6 E, ALK2 na)
131	MET	PHE B 285, PRO N 35	285(ALK6,ALK3=F ALK2=M)
134	ARG	ASP D 36	36(ActRII, ActRIIb D, BMPRII N)

[0118] Example 4: ***Identification of regions of high electrostatic potential in BMP-7***

[0119] The electrostatic potential at each position in BMP-7 was determined using the Debye-Huckel equation in the context of the BMP-7 dimer. Positions with electrostatic potential greater than 0.5 or less than –0.5 are listed in the table below; modifications at these positions may confer increased stability or receptor binding specificity.

Table 3. Regions of high electrostatic potential in BMP-7

Residue number	Residue name	Electrostatic potential
46	SER	-0.72
67	CYS	0.73
68	GLU	0.50
69	GLY	0.58
70	GLU	0.55
71	CYS	0.50
72	ALA	0.62
82	THR	0.57
105	ALA	0.54
106	PRO	0.67
107	THR	0.65
108	GLN	0.68
109	LEU	0.79
110	ASN	1.00
111	ALA	1.51
113	SER	0.68
122	ASN	-0.64
133	VAL	0.68
135	ALA	0.53
136	CYS	0.62

[0120] Example 5: ***Identification of preferred substitutions to BMPs***

[0121] Analogous contact environment (ACE) calculations, were performed on BMP-7 using complete PFAM alignment for BMP-7. ACE calculations identify alternate residues for each position that are observed in structurally similar contexts in homologous proteins. The calculations were performed using a low stringency threshold of 0.8 and a high stringency threshold of 0.5.

[0122] **Table 4.** Residues observed in analogous structural contexts in BMP-7 homologs.

residue	Wt	ACE, low stringency	ACE, high stringency
36	GLN	E Q T	Q T
37	ALA	A G S V	A G
38	CYS	C	C
39	LYS	K R	K
40	LYS	K R T	K
41	HIS	H K R	H
42	GLU	E S	E
43	LEU	F L M P	L P
44	TYR	F Y	F Y
45	VAL	I R V	V
46	SER	D E N S	S
47	PHE	F L S	F
48	ARG	K Q R	K Q R
49	ASP	A D E Q	D
50	LEU	F I L M V	L V
51	GLY	D G N	G N
52	TRP	W	W
53	GLN	D H L N Q R S	D H L N Q S
54	ASP	D N R	D N
55	TRP	W	W
56	ILE	I V	I
57	ILE	I V	I
58	ALA	A K Q S Y	A
59	PRO	P	P
60	GLU	A E G H K M P Q R S T	A E K M P Q R S
61	GLY	G	G
62	TYR	F Y	Y
63	ALA	A D E G H M N Q S	A M Q S
64	ALA	A G	A
65	TYR	F N Y	F N Y
66	TYR	F Y	Y
67	CYS	C	C
68	GLU	A D E H K Q R	D E
69	GLY	G	G
70	GLU	E	E
71	CYS	C	C
72	ALA	A D N P S V	A N S V
73	PHE	F	F
74	PRO	P	P
75	LEU	L	L
76	ASN	A D N S	N
77	SER	A S	A S
78	TYR	C F H Y	C F H Y
79	MET	A M	M
80	ASN	N	N
81	ALA	A F G P S T	A
82	THR	T	T

[0122] **Table 4.** Residues observed in analogous structural contexts in BMP-7 homologs.

83	ASN	K N S	N
84	HIS	H	H
85	ALA	A	A
86	ILE	I L V	I
87	VAL	I L M V	V
88	GLN	K Q	Q
89	THR	L T	T
90	LEU	L	L
91	VAL	V	V
92	HIS	H N	H
93	PHE	A F L S	F S
94	ILE	F I	I
95	ASN	N	N
96	PRO	P	P
97	GLU	A D E G K N Q R S	D E
98	THR	A K R T	T Y
99	VAL	T V	V
100	PRO	G P	P
101	LYS	K L Q	K Q
102	PRO	A P S T V W	P
103	CYS	C K	C
104	CYS	C W	C
105	ALA	A G H I Q R S T V	A
106	PRO	N P	P
107	THR	T	T
108	GLN	K Q	K Q
109	LEU	L	L
110	ASN	H N	N
111	ALA	A G S	A
112	ILE	I L T	I
113	SER	P S T	P S
114	VAL	I L M V	L M V
115	LEU	L	L
116	TYR	F Y	Y
117	PHE	F I K L Q Y	F Y
118	ASP	D	D
119	ASP	D E N S	D E N S
120	SER	D E G H N S	N S
121	SER	A D E H K N R S	A D S
122	ASN	A N S	N
123	VAL	I L V	V
124	ILE	I V	I V
125	LEU	I K L Y	L Y
126	LYS	K N R Y	K R
127	LYS	K R	K
128	TYR	F Y	Y
129	ARG	R	R
130	ASN	D N	N
131	MET	M	M
132	VAL	V	V

[0122] **Table 4.** Residues observed in analogous structural contexts in BMP-7 homologs.

133	VAL	A V	V
134	ARG	Q R	Q R
135	ALA	A G S	A
136	CYS	C	C
137	GLY	A G	G
138	CYS	C	C
139	HIS	G H K L Q R	H L

[0123] PDA® technology calculations were performed to identify alternate residues that are compatible with the structure and function of BMP-7. At each variable position, energies were calculated for the wild type residue and alternate residues with decreased hydrophobic or increased polar character. First, point mutation calculations were run for each template. The energy of each alternate amino acid in its most favorable rotameric conformation was compared to the energy of the wild type residue in the crystallographically observed rotameric conformation; all reported energies in the table below are [E(wild type) – E(variant)]. For residues that are within 5 Å of at least one atom in the type I or type II receptor, calculations were also performed using templates consisting of the BMP-7 dimer bound to receptor.

[0124] **Table 5.** Energies of most favorable alternate residues in each variable position in BMP-7

#	Wt	dimer	ALK2	ALK3	ALK6	ActRIIa	ActRIIb
36	GLN	N:-4.8 D:-3.7 S:-3.0		N:0.4 Q:0.8 D:0.8		N:-6.6 Q:-5.6 D:-5.2	
39	LYS	E:-11.9 K:-10.5 Q:-10.0	T:0.0 E:0.2 S:0.8	T:1.4 A:1.6 S:2.6	S:2.7 E:3.3 T:4.1	E:-2.0 S:-0.4 K:-0.2	E:-1.8 K:-0.3 Q:0.4
42	GLU	Q:-3.2 E:-2.3 N:-0.9		Q:-5.0 E:-4.0 N:-2.9		Q:-6.2 R:-5.7 E:-5.5	
44	TYR	Q:-6.6 E:-5.6 N:-4.6	Q:-3.5 E:-2.6 N:-1.5	Q:-3.5 E:-2.6 D:-1.5	Q:-3.5 E:-2.6 N:-1.5	Q:0.8 R:1.1 E:1.9	Q:-3.1 R:-2.5 E:-2.2
48	ARG	E:-5.4 Q:-4.3 N:-3.8	N:-3.3 Q:-3.2 R:-2.6	Q:-3.9 N:-3.3 R:-2.9	N:-3.2 Q:-2.9 R:-2.5	N:-8.4 Q:-7.5 D:-7.1	N:-3.9 Q:-3.7 E:-2.9
49	ASP	S:-1.4 D:-0.6 N:-0.5		D:-0.3 R:0.0 Q:0.2		N:-3.4 D:-2.5 Q:-1.9	
52	TRP	Q:2.3 K:3.2 E:3.4	K:14.5 E:15.0 A:16.7	E:14.5 K:15.0 Q:16.7	E:14.5 K:15.0 Q:16.7	K:1.8 Q:2.3 E:3.5	K:0.6 Q:1.4 E:2.6
53	GLN	D:-6.1 A:-5.7 S:-5.7	D:6.2 A:6.3 T:6.6	A:7.0 H:7.3 T:7.8	D:5.9 A:6.4 H:6.6	E:-7.5 S:-6.3 Q:-6.2	D:-6.9 S:-6.4 Q:-6.4
54	ASP	D:-0.3 S:0.2 N:0.6	N:-6.4 D:-5.5 Q:-4.9	N:-6.4 D:-5.6 Q:-4.5	N:-6.3 D:-5.5 Q:-5.4	N:-3.6 Q:-3.4 D:-2.8	N:0.0 D:0.7 Q:1.4

55	TRP	N:-19.6 R:-19.3 D:-18.8	R:11.3 H:13.0 Q:13.9	H:11.3 S:13.0 T:13.9	H:11.3 Q:13.0 R:13.9	Q:-18.3 N:-16.7 E:-16.6	Q:-17.4 E:-15.9 N:-15.9
57	ILE	E:1.0 K:1.1 Q:2.2	T:-4.9 D:-3.1 R:-2.5	T:-4.9 D:-3.1 Q:-2.5	T:-4.9 D:-3.1 R:-2.5	E:9.0 T:11.6 D:12.0	E:8.8 D:9.3 T:9.4
60	GLU	Q:-1.9 N:-1.4 R:-1.3	E:-1.8 Q:3.9 N:4.5	E:0.6 Q:4.5 N:5.3	E:-1.7 Q:4.1 N:4.7	E:0.2 T:1.7 D:2.8	T:-0.3 E:0.2 K:0.8
63	ALA	R:-5.1 Q:-4.8 E:-3.9				A:0.0 S:0.8 T:2.2	E:-4.0 Q:-1.6 A:0.2
65	TYR	A: 7.9 E: 8.5 H: 8.5					
70	GLU	E:2.8 Q:4.7 D:4.9		D:-2.3 Q:-1.5 E:-0.7		E:-5.6 Q:-3.0 T:0.4	
73	PHE	R:0.4 Q:1.8 E:2.4	H:11.2 E:18.6 D:21.2	H:11.2 A:18.6 S:21.1	H:11.2 D:18.6 A:21.1	H:-6.5 D:-5.7 S:-4.0	H:-6.5 D:-5.7 A:-4.1
76	ASN	N:-6.6 Q:-6.4 D:-6.4	A:-3.9 T:-2.2 S:-1.1	A:-5.2 S:-2.4 T:3.5	N:-4.0 S:-1.2 T:1.6	D:-6.2 Q:-5.3 N:-5.2	S:-4.5 Q:-4.4 D:-4.2
77	SER	N:-5.7 D:-4.6 S:-3.9	S:-1.8 A:1.3 T:10.1	A:-4.5 S:-0.2 T:0.1	K:-3.0 D:-0.9 A:-0.3	N:-8.9 D:-7.9 S:-6.8	N:-6.9 D:-5.8 S:-4.8
78	TYR	N:-15.6 D:-14.6 S:-13.9	S:3.2 D:6.4 Q:6.5	S:3.2 A:6.4 H:6.5	S:3.2 T:6.4 A:6.5	N:-13.2 D:-12.8 S:-11.6	R:-12.8 N:-12.4 D:-12.1
86	ILE	K:-5.0 E:-4.3 Q:-4.0	T:9.9 D:10.9 A:11.6	T:9.9 D:10.9 A:11.6	D:9.9 A:10.9 T:11.6	E:-3.6 K:-2.2 Q:-1.9	K:-5.1 E:-3.6 Q:-2.1
88	GLN	E:-0.1 T: 1.6 Q: 2.4					
90	LEU	K:-3.3 E:-0.6 Q:0.5	E:6.3 D:9.4 T:9.5	E:6.3 T:9.4 D:9.5	E:6.3 T:9.4 D:9.5	E:-0.8 Q:-0.7 R:-0.6	E:-2.2 Q:-2.1 R:-0.1
93	PHE	S:-18.0 D:-17.4 R:-17.4	E:0.3 T:2.3 D:3.0	S:0.3 D:2.3 E:3.0	E:0.3 D:2.3 Q:3.0	D:-15.3 S:-15.1 N:-13.8	D:-15.1 S:-14.8 N:-13.6
94	ILE	K:-2.7 E:-2.0 Q:-0.8	E:4.1 D:4.3 A:4.7	T:4.1 N:4.3 D:4.7	H:4.1 D:4.7 E:5.9	E:-2.0 Q:-.06 K:-0.4	K:-2.0 E:-1.9 Q:-0.4
95	ASN	Q:3.4 D:3.9 S:4.4		N:5.9 D:6.6 Q:7.3		Q:-2.6 N:0.3 D:0.4	
97	GLU	N:-5.8 D:-4.9 S:-4.1		-		N:-3.6 D:-2.6 S:-2.6	
98	THR	D:-0.8 E:-0.7 K:-0.3		N:-2.1 D:-1.4 Q:-1.1		Q:-9.3 R:-8.3 E:-8.0	
108	GLN	N:-5.7 D:-5.2 Q:-5.1		Q:-2.6 D:-0.7 S:0.4		S:-6.8 N:-5.8 D:-4.8	

110	ASN	E:1.0 Q:4.0 S:8.3				A:-7.1 E:-5.7 D:-4.6	Q:-8.1 E:-6.7 A:-4.6
111	ALA	A:0.0 S:1.7 H:9.2				A:-4.7 S:-2.1 T:26.2	A:-6.4 S:-3.3 D:46.3
115	LEU	K:-3.4 E:-3.2 D:-1.3	K:-3.8 E:-3.3 D:-1.5	E:-3.8 K:-3.3 D:-1.5	K:-3.8 E:-3.3 D:-1.5	E:8.9 T:11.5 D:12.1	E:6.7 A:10.1 Q:10.4
116	TYR	H:4.5 S:6.0 T:7.5	H:4.6 T:8.8 A:9.3	H:4.6 T:8.8 A:9.3	H:4.6 A:8.8 S:9.3	H:1.7 T:4.3 S:6.4	H:1.5 T:4.6 S:6.7
117	PHE	Q:-7.3 R:-7.2 E:-6.9	K:-4.1 Q:-3.9 R:-3.7	R:-4.1 K:-3.9 Q:-3.7	R:-4.1 Q:-3.9 K:-3.7	K:6.7 E:9.1 H:10.3	H:7.1 K:8.0 E:8.1
119	ASP	R:-2.5 Q:-2.3 N:-1.9		N:-0.4 S:0.7 D:0.8		N:-5.2 S:-4.1 D:-3.8	
120	SER	N:-5.8 S:-4.6 D:-4.4		S:-1.7 N:-1.6 D:-0.1		N:-7.4 Q:-6.6 S:-5.9	
121	SER	N:-4.7 Q:-4.0 S:-3.7		N:-4.8 D:-3.4 Q:-3.2		Q:-7.0 E:-6.2 K:-5.9	
122	ASN	R:-2.6 N:-2.5 Q:-2.4		N:5.3 Q:6.3 D:7.6		Q:-6.0 E:-5.7 R:-5.6	R:0.0 Q:0.6 N:0.7
123	VAL	Q:-5.4 E:-4.5 S:-4.1	T:-9.2 R:-8.6 E:-8.3	T:-9.2 R:-8.6 E:-8.3	T:-9.2 R:-8.6 E:-8.3	D:4.8 T:5.1 A:6.4	T:4.8 A:6.0 D:7.6
125	LEU	Q:-10.7 E:-9.8 S:-9.3	Q:-9.0 E:-8.4 S:-7.3	Q:-9.0 E:-8.4 S:-7.3	Q:-9.0 E:-8.4 S:-7.3	H:-1.0 A:3.8 D:3.8	H:-0.6 E:3.2 K:3.2
126	LYS	T:-1.2 D:1.0 E:1.0	D:-10.8 S:-7.4 N:-5.9	Q:-2.2 R:-1.5 D:-1.3	D:-4.6 E:-4.6 K:-4.2	Q:-9.9 E:-9.3 R:-7.9	Q:-9.2 E:-8.5 R:-7.3
127	LYS	Q:-13.7 E:-12.8 R:-12.0	N:-17.3 D:-16.7 Q:-13.7	Q:-7.9 E:-7.2 S:-5.9	N:-17.8 D:-17.5 Q:-13.5	D:-12.5 T:-11.7 S:-10.2	T:-5.2 S:-2.5 D:-0.9
128	TYR	K:1.2 E:2.3 Q:4.1	E:4.9 A:7.1 H:9.9	H:4.9 D:9.9 K:10.5	H:4.9 K:9.9 D:10.5	E:2.5 D:3.9 K:5.9	E:0.7 D:3.6 K:5.2
129	ARG	Q:-21.5 E:-21.1 N:-19.1	D:-8.3 E:-6.6 N:-6.0	D:-4.3 S:-4.1 Q:-2.9	D:-6.6 S:-6.6 Q:-6.1	N:-10.3 D:-9.7 E:-9.4	E:-9.9 N:-8.9 Q:-8.3
130	ASN	E:-0.9 Q:-0.7 R:-0.4	D:-4.3 E:-3.5 Q:-3.3	D:-2.1 R:-1.7 E:-1.7	D:-2.0 R:-1.7 Q:-0.9		
134	ARG	Q:-7.6 E:-7.1 R:-6.5	Q:-6.7 E:-6.0 R:-5.2	Q:-4.0 D:-3.8 S:-3.7	S:-1.1 D:-1.0 Q:-1.0	R:-0.8 S:0.0 D:1.3	R:-1.0 S:0.5 D:1.7
135	ALA	E:-3.4 D:-3.4 Q:-3.0					

[0125] Next, combinatorial calculations were performed in which multiple variable positions located close in space were allowed to vary. The most favorable amino acid sequence was first

identified with DEE, and then Monte Carlo calculations were performed to identify 10,000 favorable energies. All residues that were selected for a given position in at least 500 of the top 10,000 sequences were noted, and the number of occurrences is given in the table below. For residues that are within 5 Å of at least one atom in the type I or type II receptor, calculations were also performed using templates consisting of the BMP-7 dimer bound to receptor.

[0126] **Table 6.** Preferred alternate residues identified using combinatorial PDA® calculations

#	wt	dimer	ALK2	ALK3	ALK6	ActRII	ActRIIb
36	GLN	Q:7809 N:1884		M:9996		Q:10000	
39	LYS	S:9842	S:8163 D:1118	S:9997	D:5917 M:3085 T:572	S:10000	S:10000
42	GLU	D:4594 N:4513 L:893		D:4405 F:3105 N:2490		L:9999	
48	ARG	H:9950	R:9994	Q:7431 E:2530	R:10000	E:9274 Q:649	R:6089 Q:1849 N:1072 K:936
49	ASP	S:5123 N:4554		R:10000		N:8591 M:766 R:589	
52	TRP	W:9937	W:10000	W:10000	W:10000	W:10000	W:10000
53	GLN	Q:5056 E:4894	D:9292 Q:695	W:6190 D:3771	D:9673	S:9247 Q:753	R:9596
54	ASP	D:9422 S:578		N:7578 D:1217 S:1205		N:9326 S:606	
55	TRP	F:9234	W:10000	W:10000	W:10000	K:7426 Q:2063	Q:4838 K:2656 N:1596
57	ILE	Q:4803 E:4362	V:7154 T:1771	V:9029 E:940	T:6435 V:2518 Q:928	I:10000	I:10000
60	GLU	K:4894 Q:4752	Q:6658 E:1911 N:1072	R:6190 Q:3243	Q:8619 E:878	E:10000	E:9727
70	GLU	E:10000		M:7656 E:1051 T:840		E:10000	
73	PHE	M:9998	F:9569	F:10000	F:9968		
76	ASN	N:7622 D:2269	A:9496 S:504	A:10000	A:9309 S:658	D:8520 K:1480	D:9528
77	SER	N:9226	A:9989	D:10000	D:10000	N:4869 S:2426 D:2247	N:5705 D:2067 S:1703
86	ILE	L:5322 F:4678	V:3799 M:2956 I:2197 D:757	I:10000	D:4757 I:3625 H:737 V:548		
90	LEU	I:7320 L:1971	L:7909 I:2091	I:10000	L:8453 I:1547		
93	PHE	R:10000	M:9971	F:10000	F:9422		

94	ILE	K:6521 I:3472	I:10000	I:8908 V:705	I:5328 V:3624 H:636		
95	ASN	N:10000		N:10000		E:6756 M:2423 R:821	
97	GLU	E:8909 N:565				R:6746 W:1317 F:968 E:681	
98	THR	K:9813		E:9849		R:8142 K:1037 Q:659	
108	GLN	W:10000		W:5051 L:4949		E:8219 M:1368	
115	LEU	E:9073	E:5297 K:1647 D:857 Q:682 L:611	E:9835	E:5464 K:2112 D:1598	I:9494 L:506	I:8877 L:865
116	TYR	S:7525 H:1972	T:8380 S:734	Y:10000	Y:10000	Y:9428	Y:6205 F:3186 T: 577
117	PHE	K:10000	R:5996 S:734	K:5592 Q:2504 E:1856	K:5038 E:4955	F:10000	F:9956
119	ASP	N:3258 R:2589 Q:1910 D:1614		N:4613 D:4434 S:665		N:9068 D:842	
120	SER	R:9814		Q:9548		E:10000	
121	SER	N:8165 Q:1581		N:8910 Q:791		W:10000	
122	ASN	Q:9767		R:7212 Q:2768		Q:10000	
123	VAL	R:4692 N:4665	Q:3725 K:3427 E:2351	R:10000	K:4955 Q:2595 E:1252 R:999	V:10000	V:9965
125	LEU	Q:2247 E:2234 N:2103 D:1840	Q:2148 E:2024 D:1558 N:925 R:854 T:681 S:553	Q:2688 E:2252 D:1815 R:1215 N:828	Q:2618 E:1838 T:1591 D:1522 R:897	L:10000	E:7583 L:1122 Q:540
126	LYS	E:10000	D:10000	E:7981 Q:2019	E:10000	Q:8882 E:904	Q:7360 R:2587
127	LYS	Q:10000	Q:7702 N:2120	Q:10000	Q:7301 N:1850 D:849	T:8654 D:1346	Q:10000
128	TYR	K:7798 M:2139	M:10000	M:10000	M:10000	W:10000	W:10000
129	ARG	D:9142 N:858	E:7628 D:1971	E:10000	E:7301 R:2699	N:8799 D:1201	N:7847 Q:1495 D:541
134	ARG	M:6832 E:3143	E:9900	E:10000	E:9997	E:9996	Q:5893 Y:2281 E:1787

[0127] PDA® technology calculations were also performed to identify mutations that are likely to either increase or substantially eliminate binding to the BMP inhibitor protein Noggin. At each position in BMP-7 that is within 5 A of at least one atom in Noggin (see table above), energies were calculated for alternate residues using a template comprising (1) BMP-7 only, and (2) BMP-7 bound to Noggin. Preferred substitutions include, but are not limited to, those listed in the tables below.

[0128] **Table 7.** Preferred substitutions to substantially eliminate Noggin binding

Residue number	Alternate amino acid	Energy (BMP-7 only)	Energy (BMP-7-noggin)	Δ(Energy)
55	ILE	5.38	109.30	103.91
55	LEU	4.01	421.61	417.60
55	LYS	0.94	2576.09	2575.15
55	ARG	-1.82	1012.10	1013.92
57	MET	4.30	219.47	215.17
57	TYR	7.74	42890.27	42882.53
57	GLU	-3.05	119.37	122.42
57	HIS	6.53	2905.10	2898.57
57	HSP	5.72	3035.01	3029.29
57	LYS	0.40	114.98	114.58
57	GLN	-4.66	150.57	155.23
57	ARG	-3.67	4086.31	4089.97
58	ILE	9.42	322.50	313.09
58	LEU	9.30	84822.06	84812.76
58	MET	8.88	754.69	745.81
58	TYR	4.94	1105.51	1100.57
58	VAL	6.66	226.71	220.05
58	GLU	-1.90	199.69	201.59
58	HIS	5.03	1385.50	1380.47
58	HSP	4.31	1951.88	1947.58
58	LYS	3.52	987.81	984.28
58	GLN	-3.02	181.68	184.70
58	ARG	-3.00	221.94	224.94
59	TYR	9.57	15040.62	15031.05
76	GLU	3.88	210.29	206.41
76	GLN	2.78	304.90	302.12
76	ARG	8.02	9204.62	9196.60

77	GLU	9.73	5019.33	5009.59
77	GLN	8.40	4804.74	4796.34
83	PHE	3.55	2730.52	2726.97
83	TRP	1.99	4103.04	4101.05
83	TYR	-3.56	1606.78	1610.35
83	HSP	-1.42	110.31	111.73
83	LYS	0.82	275.49	274.67
83	ARG	-4.14	114.90	119.04
86	LEU	6.41	177.93	171.52
86	MET	3.76	182.42	178.66
86	PHE	4.06	510.92	506.87
86	TYR	0.29	486.51	486.22
86	ARG	-1.29	360.80	362.09
87	HIS	4.35	722.22	717.87
87	HSP	9.18	650.44	641.27
113	ILE	9.08	220.54	211.47
113	LEU	4.06	1142.97	1138.91
113	MET	6.10	1203.71	1197.61
113	PHE	5.50	*****	
113	TYR	5.35	*****	
113	GLU	-2.87	199.31	202.17
113	HIS	-0.15	219.95	220.09
113	HSP	2.62	294.44	291.82
113	LYS	2.39	219.74	217.35
113	GLN	-3.68	419.42	423.10
113	ARG	-0.22	31582.79	31583.01
115	MET	0.59	433.33	432.74
115	LYS	-4.58	104.47	109.05
115	ARG	-2.89	629.82	632.71
123	MET	2.17	186.71	184.54
123	TYR	7.81	*****	
123	HIS	6.31	2238.61	2232.29
123	HSP	5.63	11040.76	11035.13
127	ILE	7.26	270.14	262.88
127	VAL	5.22	223.61	218.39
127	HIS	8.31	1012.09	1003.79
127	HSP	8.00	1708.17	1700.17
128	ILE	0.25	401.32	401.07

128	ARG	-3.86	124.31	128.17

[0129] **Table 8.** Preferred substitutions to increase Noggin binding affinity

Residue number	Alternate amino acid	Energy (BMP-7 only)	Energy (BMP-7-noggin)	Δ(Energy)
48	MET	8.38	-3.60	-11.99
57	VAL	-3.57	-14.50	-10.92
59	MET	7.34	-7.13	-14.47
60	ILE	14.56	-0.05	-14.61
60	LEU	13.50	2.97	-10.52
60	MET	15.34	2.70	-12.64
60	VAL	13.08	0.51	-12.58
74	MET	13.88	1.04	-12.84
76	ILE	13.60	-1.47	-15.07
76	VAL	11.19	-6.60	-17.79
76	ALA	9.97	-3.48	-13.45
77	ALA	13.76	-2.23	-15.99
77	HIS	16.02	2.66	-13.36
77	THR	12.53	-7.56	-20.09
86	VAL	4.41	-10.60	-15.02
113	ALA	2.89	-7.70	-10.58
119	ILE	28.27	18.21	-10.06
119	LEU	29.23	17.25	-11.99
124	VAL	-4.30	-14.52	-10.22
125	ILE	247.94	228.79	-19.16
125	MET	10.73	-17.18	-27.91
125	ALA	4.10	-7.22	-11.32
126	MET	8.64	-9.57	-18.21
126	TRP	64.98	35.09	-29.89
126	HIS	49.02	38.84	-10.18
126	HSP	47.75	37.16	-10.59
126	THR	6.31	-5.14	-11.45
127	MET	7.52	-11.44	-18.95
127	ALA	3.79	-6.37	-10.16

[0130] A number of alternate residues were selected for each variable position. In all cases, the alternate residues are predicted to be compatible with the structure of BMP-7 dimer. The alternate residues are predicted to interact with the receptors in a diverse manner, encompassing competitive inhibitor variants, receptor specific variants, and high affinity variants.

The table shown below indicates preferred substitutions that were identified using sequence alignment data, ACE calculations, and PDA® technology calculations. Note that "X" indicates a one-residue deletion.

Table 9. BMP-7 variants in Library 1.

Residue	wt	calculation	Library 1.1	Library 1.2	Library 1.3	# variants
21	LEU	expH	DKS			3
23	MET	expH	DKS			3
26	VAL	expH	DKS			3
36	GLN	adtl. surf			ENR	3
39	LYS	specificity		DERST		5
42	GLU	adtl. surf			DQRT	4
44	TYR	expH	AEHKQR			6
48	ARG	specificity		EKNQ		4
49	ASP	adtl. surf			ES	2
52	TRP	expH	AEKQ			4
53	GLN	specificity		ADERS	H	6
54	ASP	adtl. surf			KNRS	4
55	TRP	expH	AEHKNQ		R	7
57	ILE	expH	AEHKTV	D		7
60	GLU	specificity		KQRST		5
63	ALA	adtl. surf			EQRS	4
65	TYR	electrostatic			DEN	3
70	GLU	adtl. surf			AQ	2
73	PHE	expH	AEHQRS	D		7
76	ASN	specificity		ADST		4
77	SER	specificity		ADKQT		5
78	TYR	expH	DGHNST			6
80	ASN	glycosylation	DQST			4
82	THR	glycosylation	V			1
83	ASN	glycosylation	P			1
86	ILE	expH	EKQT	AD		6
88	GLN	electrostatic			E	1
90	LEU	expH	EKNQRST			7
93	PHE	expH	ADEQRST			7
94	ILE	expH	AEKQRT	H		7
95	ASN	adtl. surf			DKQR	4
97	GLU	adtl. surf			DKR	3
98	THR	adtl. surf			AEKRX	5
108	GLN	adtl. surf			DKS	3
110	ASN	electrostatic			DEH	3
111	ALA	electrostatic			DS	2
115	LEU	expH	EKT			3
116	TYR	expH	DEHKST	A		7
117	PHE	expH	ADEKQR	H		7
119	ASP	adtl. surf			ENST	4
120	SER	adtl. surf			DERN	4
121	SER	adtl. surf			DEKNT	5
122	ASN	adtl. surf			EQR	3
123	VAL	expH	ADNRT			5
125	LEU	expH	AEKQ		Y	5
126	LYS	specificity		DEQR		4
127	LYS	specificity		DQST	E	5
128	TYR	expH	DEHKQ			5
129	ARG	specificity		DES		3
130	ASN	electrostatic			D	1

134	ARG	specificity		EKQS	D	5
135	ALA	electrostatic			DES	3

[0131] As may easily be appreciated, many of these preferred substitutions may easily be incorporated into the analogous positions in other BMPs and TGF-β family members. A sequence alignment of human BMPs is given in Figure 3. In order to identify which substitutions may be incorporated into BMP-2, BMP-4, BMP-5, BMP-6, and BMP-8, the energy of each of the above substitutions was calculated in the context of each dimer structure. Substitutions with similar energies in two different structures are likely to produce similar effects in the two proteins.

[0132] **Table 10.** Energies of library mutations in the context of the BMP-2, 4, 5, 6, 7, and 8 structures.

Residue	substitution	BMP2 E(tot)	BMP4 E(tot)	BMP5 E(tot)	BMP6 E(tot)	BMP-7 E(tot)	BMP8 E(tot)
Q 36	GLU	5.6	3.4	6.0	6.4	5.8	5.6
Q 36	ASN	4.2	5.1	2.5	3.0	2.4	2.2
Q 36	ARG	8.0	5.2	5.4	5.7	5.3	5.0
A 37	ASP	-3.0	-3.1	-0.2	1.7	-0.3	-3.4
A 37	GLU	-1.8	-2.4	5.0	3.5	2.9	1.3
A 37	HIS	2.3	2.5	10.6	7.1	8.5	5.1
A 37	LYS	2.5	2.7	2.9	1.1	2.7	-0.8
A 37	ARG	1.0	1.1	4.2	2.8	4.1	4.7
K 39	ASP	1.7	1.7	3.6	1.7	2.2	3.9
K 39	GLU	0.7	0.6	-0.7	-2.7	-0.4	0.9
K 39	ARG	2.5	2.5	1.8	0.0	3.2	2.1
K 39	SER	-0.2	-0.3	2.0	0.0	1.8	2.9
K 39	THR	-0.7	-0.7	1.2	1.2	2.3	1.6
E 42	ASP	4.2	1.3	2.7	3.4	2.7	2.5
E 42	GLN	1.3	0.0	-0.8	1.4	-0.7	0.0
E 42	ARG	1.9	1.3	2.2	4.6	1.9	1.5
E 42	THR	4.4	3.3	4.2	5.0	4.1	3.7
Y 44	ALA	2.6	2.0	7.3	7.3	7.3	8.3
Y 44	GLU	-3.5	-4.1	-0.2	-0.2	-0.1	1.3
Y 44	HIS	-2.4	-2.6	3.0	2.9	3.0	3.9
Y 44	LYS	-2.6	-2.9	3.3	3.4	3.2	5.5
Y 44	GLN	-4.4	-4.9	-1.1	-1.1	-1.0	0.4
Y 44	ARG	-2.8	-2.9	2.6	2.7	2.6	4.5
R 48	GLU	2.0	2.0	-1.5	-1.7	-1.5	1.2
R 48	LYS	7.0	7.0	2.2	2.9	2.2	7.0
R 48	ASN	-0.6	-0.6	-0.1	-0.1	0.1	-1.7
R 48	GLN	0.3	0.4	-0.4	-0.4	-0.4	-0.2
D 49	GLU	4.3	4.3	2.2	2.2	2.1	1.8
D 49	SER	4.3	4.3	-0.7	-0.7	-0.9	-1.0
W 52	ALA	-4.1	-4.0	2.7	2.7	2.4	2.1
W 52	GLU	-6.4	-6.4	-2.3	-2.3	-2.5	-3.4
W 52	LYS	-5.1	-5.0	-2.6	-2.6	-2.8	-5.0
W 52	GLN	-5.2	-5.0	-3.5	-3.5	-3.7	-4.8
Q 53	ALA	2.0	1.9	0.1	-1.4	0.1	-3.9
Q 53	ASP	-1.2	-1.2	-0.3	-2.0	-0.3	-5.3
Q 53	GLU	0.3	0.3	9.1	-1.5	9.1	-6.8
Q 53	HIS	2.8	2.8	1.4	0.5	1.4	1.6
Q 53	ARG	-1.6	-1.5	3.5	0.7	3.3	-3.6
Q 53	SER	0.2	0.2	0.2	-1.8	0.2	-3.4
D 54	LYS	12.3	12.4	4.3	4.4	5.5	6.0
D 54	ASN	4.5	4.4	-1.9	-1.9	0.1	-1.6

D 54	ARG	8.6	8.7	-0.3	-0.3	1.6	1.2
D 54	SER	6.1	6.1	-0.2	-0.2	-0.3	0.1
W 55	ALA	1.9	2.0	1.9	1.9	1.8	2.2
W 55	GLU	-2.5	-2.4	-1.8	-1.8	-1.8	-1.7
W 55	HIS	0.6	1.4	4.0	4.0	3.7	4.4
W 55	LYS	0.2	0.3	2.7	2.7	2.6	3.0
W 55	ASN	-4.0	-3.9	-3.9	-3.9	-3.9	-3.6
W 55	GLN	-3.7	-3.7	-2.7	-2.7	-2.8	-2.5
W 55	ARG	-5.0	-4.8	-3.6	-3.6	-3.7	-3.2
I 57	VAL	-3.9	-3.6	-2.7	-3.9	-2.8	-2.2
I 57	ALA	1.1	1.5	-0.5	-1.3	-0.6	-0.7
I 57	ASP	-2.8	-2.7	-4.2	-5.3	-4.2	-4.3
I 57	GLU	-4.8	-4.5	-6.2	-7.1	-6.1	-5.4
I 57	HIS	2.7	2.9	4.2	2.8	4.3	3.5
I 57	LYS	-1.0	-0.9	-5.8	-6.5	-6.1	-2.0
I 57	THR	-7.3	-7.1	-1.2	-2.0	-1.2	-0.7
E 60	LYS	5.3	5.1	5.9	6.1	5.4	6.1
E 60	GLN	0.3	0.2	2.8	2.5	0.7	1.3
E 60	ARG	2.0	1.9	3.6	3.7	1.3	5.1
E 60	SER	2.2	2.1	3.5	2.7	2.9	3.2
E 60	THR	3.2	3.1	5.2	5.1	2.7	5.7
A 63	GLU	-2.0	-2.0	-0.9	-0.6	-2.2	-4.5
A 63	GLN	-2.7	-2.7	-1.7	-1.5	-3.1	-4.7
A 63	ARG	-2.3	-2.3	-1.0	-0.3	-3.4	-1.4
A 63	SER	-0.4	-0.4	0.5	1.1	1.4	1.4
Y 65	ASP	-4.1	-6.0	-3.4	-3.5	-3.4	-3.7
Y 65	GLU	21.7	19.5	-3.7	-3.8	-3.9	73.9
Y 65	ASN	-0.7	-2.4	-0.3	-0.3	-0.4	-0.7
E 70	ALA	4.3	2.2	2.6	4.6	2.6	1.9
E 70	GLN	-0.7	-0.9	0.4	1.1	-0.3	-0.3
A 72	ASP	10.7	-1.8	-1.3	-1.3	-1.1	-1.7
A 72	GLU	-0.1	-3.6	-1.3	-1.2	-3.1	-1.5
A 72	HIS	3.7	-0.6	2.9	2.9	2.4	2.6
A 72	LYS	1.9	-2.4	1.1	1.1	0.5	0.8
A 72	ASN	9.0	-0.3	2.3	2.3	2.4	1.9
A 72	ARG	-4.4	-2.2	-2.9	-2.9	-3.1	-3.1
A 72	SER	2.0	-1.0	-4.3	-4.3	-4.4	-4.6
F 73	ALA	0.2	0.3	4.0	4.0	6.0	4.0
F 73	ASP	-1.9	-1.8	3.4	3.4	4.0	3.6
F 73	GLU	-3.2	-3.0	1.1	1.1	1.2	1.3
F 73	HIS	-0.7	-0.6	3.7	3.7	5.6	2.7
F 73	GLN	-4.0	-3.9	0.3	0.3	0.6	0.6
F 73	ARG	-4.7	-4.5	-1.2	-1.2	-0.8	-1.0
F 73	SER	-1.2	-1.1	1.8	1.8	1.2	1.8
N 76	ALA	4.1	4.0	6.2	6.2	5.9	4.8
N 76	ASP	-0.3	-0.3	1.1	1.1	0.9	0.1
N 76	SER	0.4	0.4	2.5	2.5	2.3	1.1
N 76	THR	8.2	7.8	5.9	5.9	5.4	4.0
S 77	ALA	13.7	13.7	15.1	15.1	15.1	14.7
S 77	ASP	6.3	6.4	7.8	7.8	7.9	7.5
S 77	LYS	18.2	18.2	19.4	19.4	19.3	18.8
S 77	GLN	8.9	9.0	9.8	9.8	9.8	9.3
S 77	THR	12.6	12.8	13.7	13.7	13.6	12.0
Y 78	ASP	3.5	3.7	3.4	3.4	5.0	5.7
Y 78	HIS	11.4	10.4	11.7	11.7	12.5	11.2
Y 78	ASN	2.2	2.4	2.6	2.6	4.0	4.8

Y 78	SER	4.2	4.3	4.5	4.5	5.7	6.3
Y 78	THR	8.1	8.2	8.2	8.2	9.3	9.1
I 86	ALA	1.0	0.9	0.2	0.2	-0.1	-0.4
I 86	ASP	-1.1	-1.4	-4.8	-4.8	-4.9	-5.5
I 86	GLU	-4.2	-4.1	-6.5	-6.5	-6.5	-6.6
I 86	LYS	-0.8	-0.8	-7.0	-7.0	-7.2	-7.7
I 86	GLN	-2.7	-2.4	-6.2	-6.2	-6.3	-6.6
I 86	THR	2.2	2.3	-1.0	-1.0	-1.1	-1.4
Q 88	GLU	-11.9	-9.9	-10.8	-10.8	-10.9	-9.9
L 90	GLU	-7.9	-7.8	-6.4	-6.4	-7.1	-8.4
L 90	LYS	-4.4	-4.2	-7.0	-6.9	-9.8	-8.5
L 90	ASN	-1.7	-1.6	-3.6	-3.6	-2.1	-1.7
L 90	GLN	-6.0	-5.9	-5.4	-5.4	-6.0	-7.0
L 90	ARG	-1.4	-1.2	-4.7	-4.7	-5.6	-5.5
L 90	SER	-5.4	-5.4	-2.4	-2.4	-2.6	-2.7
L 90	THR	-4.0	-3.9	-1.5	-1.5	-2.6	-3.8
F 93	ALA	1.9	2.1	1.3	1.3	1.2	1.2
F 93	ASP	-2.1	-2.0	-2.9	-2.8	-3.0	-3.2
F 93	GLU	-4.5	-4.4	0.7	0.8	0.4	0.2
F 93	GLN	-4.9	-4.8	-0.6	-0.6	-0.9	-1.1
F 93	ARG	-3.4	-3.2	-2.6	-2.6	-3.0	-2.9
F 93	SER	-2.9	-2.8	-3.7	-3.7	-3.6	-3.6
F 93	THR	3.9	4.0	6.7	6.7	6.3	6.2
I 94	ALA	1.8	1.9	-1.9	0.5	0.6	-2.4
I 94	GLU	-4.9	-4.8	-7.6	-5.2	-5.0	-7.8
I 94	HIS	-1.7	-1.6	6.1	-0.2	2.9	5.8
I 94	LYS	-5.0	-5.0	-8.4	-5.2	-5.7	-8.4
I 94	GLN	-3.7	-3.6	-6.1	-4.0	-3.8	-6.2
I 94	ARG	-0.4	-0.2	-5.9	-3.4	-3.5	-4.2
I 94	THR	0.4	0.4	-1.7	0.5	1.0	-1.1
N 95	ASP	0.8	2.8	-4.1	2986.4	-1.7	-2.1
N 95	LYS	3.8	9.2	-1.2	4982.8	3.9	2.7
N 95	GLN	0.6	3.4	-4.9	3497.0	-2.2	0.0
N 95	ARG	2.6	6.3	-2.5	4116.5	3.7	2.4
E 97	ASP	3.2	3.6	6.6	3.3	3.5	6.1
E 97	LYS	3.7	3.9	15.6	12.9	13.1	15.9
E 97	ARG	4.3	4.2	11.4	8.0	8.2	11.1
T 98	ALA	8.5	8.2	0.4	-0.4	-0.4	-0.6
T 98	GLU	4.3	5.6	-5.5	-3.7	-2.8	-4.3
T 98	LYS	9.5	11.1	-5.8	-3.4	-2.4	-2.2
T 98	ARG	6.0	7.9	0.6	3.1	3.5	3.1
A 105	VAL	-11.4	-14.8	-4.2	-4.2	0.4	-4.2
Q 108	ASP	-1.6	-1.6	-3.3	-3.4	-3.3	-2.2
Q 108	LYS	4.4	4.4	2.8	3.0	2.8	3.3
Q 108	SER	-0.6	-0.7	-2.6	-2.6	-2.6	-1.3
N 110	ASP	-0.9	-0.7	7.5	7.5	12.8	-0.5
N 110	GLU	-0.6	-0.5	-1.6	-1.7	-1.4	0.0
N 110	HIS	10.3	10.8	11.8	11.8	12.4	8.9
A 111	ASP	266.0	263.9	1199.6	1199.6	1155.7	1201.4
A 111	SER	2.5	2.5	1.4	1.4	1.4	3.4
L 115	GLU	-7.9	-7.9	-7.8	-7.9	-7.7	-7.8
L 115	LYS	-7.2	-7.2	-7.6	-7.5	-7.8	-7.5
L 115	THR	-1.3	-2.6	-5.4	-5.4	-5.4	-5.4
Y 116	ASP	22.1	24.7	275.0	274.9	278.8	274.6
Y 116	GLU	76.1	65.5	125.3	125.2	126.5	122.8
Y 116	HIS	-9.2	-9.5	-7.5	-7.5	-7.1	-6.7

Y 116	LYS	4301.8	3766.9	1 98.7	198.7	211.7	203.6
Y 116	SER	-2.3	-2.3	–5.6	-5.6	-5.6	-4.3
Y 116	THR	-4.8	-4.8	–4.2	-4.3	-4.1	-2.5
F 117	ALA	-3.5	-2.5	–0.1	-0.1	-2.0	-2.7
F 117	ASP	-5.1	-4.7	–2.4	-2.5	-2.2	-4.7
F 117	GLU	-7.2	-7.1	–4.6	-4.7	-4.4	-6.8
F 117	LYS	-6.4	-7.6	–2.9	-2.8	-2.7	-5.5
F 117	GLN	-8.1	-8.0	–4.7	-4.7	-4.8	-8.0
F 117	ARG	-6.5	-5.8	–4.8	-4.7	-4.7	-5.8
D 119	GLU	10.2	12.8	6.8	5.6	6.9	6.7
D 119	ASN	4.5	7.3	6.0	4.9	6.1	6.0
D 119	SER	6.5	9.3	8.0	6.9	8.0	7.9
D 119	THR	12.2	14.3	10.4	10.3	10.5	10.4
S 120	ASP	1.9	1.8	1.5	1.5	1.6	1.0
S 120	GLU	1.1	1.0	3.5	3.5	3.6	2.9
S 120	ASN	0.5	0.3	0.1	0.1	0.2	0.0
S 120	ARG	1.6	1.4	5.3	5.3	5.4	5.0
S 121	ASP	3.2	3.4	3.2	3.2	3.2	2.5
S 121	GLU	4.2	4.4	3.9	3.8	3.9	3.2
S 121	LYS	9.7	9.8	7.9	8.0	7.8	8.1
S 121	ASN	1.8	1.9	1.7	1.7	1.7	1.1
S 121	THR	8.2	8.4	6.5	6.5	6.4	5.7
N 122	GLU	-0.3	-0.1	-1.0	-1.1	2.9	-1.1
N 122	GLN	-2.0	-1.8	-2.6	-2.6	1.4	-2.6
N 122	ARG	-0.8	-0.5	-2.6	-2.9	1.2	-2.6
V 123	ALA	0.7	0.9	1.9	1.9	1.7	1.7
V 123	ASP	-1.8	-1.8	-1.0	-1.1	-0.8	-1.2
V 123	ASN	-2.9	-2.8	0.1	0.1	0.3	0.0
V 123	ARG	-3.3	-2.9	-2.7	-2.6	-2.4	-3.0
V 123	THR	-2.6	-2.3	-2.2	-2.2	-2.4	-3.2
L 125	ALA	5.0	5.2	4.7	4.7	4.5	4.7
L 125	GLU	-0.8	-0.8	-1.2	-1.3	-1.3	-1.2
L 125	LYS	4.2	4.2	3.6	3.7	3.4	3.7
L 125	GLN	-1.8	-1.7	-2.2	-2.2	-2.3	-2.2
K 126	ASP	-1.6	-3.4	-5.5	-5.5	-5.5	-5.2
K 126	GLU	-1.4	-3.0	-3.3	-3.4	-5.4	-2.7
K 126	GLN	-2.4	-4.3	-1.4	-1.4	-3.6	-3.3
K 126	ARG	-2.7	-5.7	0.5	0.5	-0.7	9.9
K 127	ASP	5.9	4.0	0.7	0.7	0.6	0.9
K 127	GLN	2.0	0.2	-2.5	-2.5	-3.1	-2.3
K 127	SER	3.0	1.2	0.1	0.1	-0.1	0.1
K 127	THR	5.6	3.7	1.5	1.5	-0.5	1.7
Y 128	ASP	-6.4	-6.6	-5.5	-5.5	-5.6	-5.3
Y 128	GLU	-3.3	-3.0	-7.4	-7.5	-7.6	-8.9
Y 128	HIS	-10.0	-9.8	-4.3	-4.3	-4.4	-7.3
Y 128	LYS	8.1	10.0	-8.7	-8.6	-8.8	-5.9
Y 128	GLN	3.6	3.6	-5.7	-5.7	-5.8	-4.6
R 129	ASP	-0.5	-0.5	0.1	0.0	0.1	0.1
R 129	GLU	-0.3	1.6	-2.0	-2.0	-2.0	-1.9
R 129	SER	-0.4	1.5	1.5	1.5	1.5	1.5
N 130	ASP	-6.6	-4.6	-2.8	-2.8	-2.6	-2.7
R 134	GLU	-6.9	-6.9	-7.1	-7.1	-6.9	-6.9
R 134	LYS	12.3	18.6	-4.3	-4.1	-2.2	-4.1
R 134	GLN	-4.3	-4.2	-7.5	-7.4	-7.4	-6.6
R 134	SER	-2.4	-2.4	-3.8	-3.8	-3.9	-3.2
A 135	ASP	1.6	1.2	-5.1	-5.1	-5.9	-1.7

A 135	GLU	2.2	1.2	-4.8	-4.8	-5.9	-2.6
A 135	SER	3.7	2.4	-4.0	-4.0	-5.2	-2.1
H 139	ARG	-4.4	-4.4	-0.6	-0.6	-2.6	-0.6

[0133] Correlation coefficients (R^2) for energies calculated using the BMP-2 template versus other BMP templates are as follows: BMP2 vs BMP4 = 0.93, BMP2 vs BMP5 = 0.52, BMP2 vs BMP6 = 0.44, BMP2 vs BMP-7 = 0.64, and BMP2 vs BMP8 = 0.52. Correlation coefficients (R^2) for energies calculated using the BMP-7 template versus other BMP templates are as follows: BMP-7 vs BMP2 = 0.54, BMP-7 vs BMP4 = 0.54, BMP-7 vs BMP5 = 0.95, BMP-7 vs BMP6 = 0.71, and BMP-7 vs BMP8 = 0.76. These trends correlate with the sequence similarity of the pair. Modifications to BMP-7 have substantially similar effects on other BMPs if the energy difference between the modification in the BMP-7 template and another BMP template are less than 1 kcal/mol, or if both energies are higher than 50 kcal/mol. The following BMP-7 modifications have substantially similar effects in BMP-2: Q36E, A37K, K39D, E42R, E42T, R48N, R48Q, Q53D, Q53S, W55A, W55E, W55N, W55Q, E60K, E60Q, E60R, E60S, E60T, A63E, A63Q, Y65D, Y65N, E70Q, S77Q, S77T, Q88E, L90E, L90N, L90Q, F93A, F93D, F93R, F93S, I94E, I94K, I94Q, I94T, N95K, E97D, N110E, A111D, L115E, L115K, Y116D, Y116E, Y116K, Y116T, S120D, S120N, S121D, S121E, S121N, V123A, V123D, V123R, V123T, L125A, L125E, L125K, L125Q, Y128D, R129D, and R134E. The following BMP-7 modifications have substantially similar effects in BMP-4: Q36R, A37K, K39D, K39E, E42Q, E42R, E42T, R48N, R48Q, Q53D, Q53S, W55A, W55E, W55N, W55Q, I57V, E60K, E60Q, E60R, E60S, E60T, A63E, A63Q, E70A, E70Q, A72D, A72E, A72R, S77Q, S77T, Q88E, L90E, L90N, L90Q, F93A, F93D, F93R, F93S, I94E, I94K, I94Q, I94T, E97D, N110E, A111D, L115E, L115K, Y116D, Y116E, Y116K, Y116T, F117A, S120D, S120N, S121D, S121E, S121N, V123A, V123D, V123R, V123T, L125A, L125E, L125K, L125Q, K126Q, Y128D, R129D, R129S, and R134E. The following BMP-7 modifications have substantially similar effects in BMP-5: Q36E, Q36N, Q36R, A37D, A37K, A37R, K39E, K39S, E42D, E42Q, E42R, E42T, Y44A, Y44E, Y44H, Y44K, Y44Q, Y44R, R48E, R48K, R48N, R48Q, D49E, D49S, W52A, W52E, W52K, W52Q, Q53A, Q53D, Q53E, Q53H, Q53R, Q53S, D54S, W55A, W55E, W55H, W55K, W55N, W55Q, W55R, I57V, I57A, I57D, I57E, I57H, I57K, I57T, E60K, E60S, A63S, Y65D, Y65E, Y65N, E70A, E70Q, A72D, A72H, A72K, A72N, A72R, A72S, F73D, F73E, F73Q, F73R, F73S, N76A, N76D, N76S, N76T, S77A, S77D, S77K, S77Q, S77T, Y78H, I86A, I86D, I86E, I86K, I86T, I86T, Q88E, L90E, L90Q, L90R, L90S, F93A, F93D, F93E, F93Q, F93R, F93S, F93T, T98A, Q108D, Q108K, Q108S, N110E, N110H, A111D, A111S, L115E, L115K, L115T, Y116D, Y116E, Y116H, Y116K, Y116S, Y116T, F117D, F117E, F117K, F117Q, F117R, D119E, D119N, D119S, D119T, S120D, S120E, S120N, S120R, S121D, S121E, S121K, S121N, S121T, V123A, V123D, V123N, V123R, V123T, L125A, L125E, L125K, L125Q, K126D, K127D, K127Q, K127S, Y128D, Y128E, Y128H, Y128K, Y128Q, R129D, R129E, R129S, N130D, R134E, R134Q, R134S, and A135D. The following BMP-7 modifications have substantially similar effects in BMP-6: Q36E, Q36N, Q36R, A37E, K39D, E42D, E42T, Y44A, Y44E, Y44H, Y44K, Y44Q, Y44R, R48E, R48K, R48N, R48Q, D49E, D49S, W52A, W52E, W52K, W52Q, Q53H, D54S, W55A, W55E, W55H, W55K,

W55N, W55Q, W55R, I57A, I57E, I57K, I57T, E60K, E60S, Y65D, Y65E, Y65N, A72D, A72H, A72K, A72N, A72R, A72S, F73D, F73E, F73Q, F73R, F73S, N76A, N76D, N76S, N76T, S77A, S77D, S77K, S77Q, S77T, Y78H, I86A, I86D, I86E, I86K, I86T, I86T, Q88E, L90E, L90Q, L90R, L90S, F93A, F93D, F93E, F93Q, F93R, F93S, F93T, I94A, I94E, I94K, I94Q, I94R, I94T, E97D, E97K, E97R, T98A, T98E, T98K, T98R, Q108D, Q108K, Q108S, N110E, N110H, A111D, A111S, L115E, L115K, L115T, Y116D, Y116E, Y116H, Y116K, Y116S, Y116T, F117D, F117E, F117K, F117Q, F117R, D119T, S120D, S120E, S120N, S120R, S121D, S121E, S121K, S121N, S121T, V123A, V123D, V123N, V123R, V123T, L125A, L125E, L125K, L125Q, K126D, K127D, K127Q, K127S, Y128D, Y128E, Y128H, Y128K, Y128Q, R129D, R129E, R129S, N130D, R134E, R134Q, R134S, and A135D. The following BMP-7 modifications have substantially similar effects in BMP-8: Q36E, Q36N, Q36R, A37R, K39T, E42D, E42Q, E42R, E42T, Y44A, Y44H, R48Q, D49E, D49S, W52A, W52E, Q53H, D54K, D54R, D54S, W55A, W55E, W55H, W55K, W55N, W55Q, W55R, I57V, I57A, I57D, I57E, I57H, I57T, E60K, E60Q, E60S, A63S, Y65D, Y65N, E70A, E70Q, A72D, A72H, A72K, A72N, A72R, A72S, F73D, F73E, F73Q, F73R, F73S, N76D, S77A, S77D, S77K, S77Q, Y78D, Y78N, Y78S, Y78T, I86A, I86D, I86E, I86K, I86T, I86T, Q88E, L90N, L90Q, L90R, L90S, F93A, F93D, F93E, F93Q, F93R, F93S, F93T, I94R, N95D, T98A, T98K, T98R, Q108K, A111D, L115E, L115K, L115T, Y116D, Y116E, Y116H, Y116K, F117A, D119E, D119N, D119S, D119T, S120D, S120E, S120N, S120R, S121D, S121E, S121K, S121N, S121T, V123A, V123D, V123N, V123R, V123T, L125A, L125E, L125K, L125Q, K126D, K126Q, K127D, K127Q, K127S, Y128D, R129D, R129E, R129S, N130D, R134E, R134Q, and R134S.

[0134] ACE calculations were also performed to assess the similarity of the structural environment at each variable position in BMP-7 vs. BMP-2, BMP-4, BMP-5, BMP-6, and BMP-8. At positions with an ACE similarity score of 0.4 or higher in the table below, mutations will have similar effects in BMP-7 vs. the other BMP. ACE similarity scores between 0.6 and 0.8 indicate that the effects of mutations are highly likely to have similar effects, and ACE similarity scores greater than 0.8 indicate that the effects of mutations should be substantially identical.

[0135] **Table 11.** ACE similarity scores for BMP-7 versus selected additional human TGF-β proteins

	BMP-2	BMP-3	BMP-3B	BMP-4	BMP-5	BMP-6	BMP-8	BMP-9	BMP-10	BMP-15	GDF-1	GDF-3	GDF-5	GDF-8	GDF-9	TGF-β1	TGF-β2	TGF-β3	TGF-β4
36	0.58	0.11	0.14	0.23	0.85	0.81	0.60	0.10	0.10	0.14	0.42	0.41	0.10	0.36	0.24	0.10	0.10	0.10	0.09
37	0.12	0.02	0.02	0.07	0.72	0.27	0.30	0.02	0.05	0.02	0.02	0.03	0.03	0.01	0.02	0.01	0.01	0.01	0.00
39	0.35	0.03	0.04	0.24	0.75	0.67	0.38	0.02	0.02	0.19	0.07	0.23	0.04	0.06	0.19	0.02	0.02	0.03	0.02
42	0.30	0.37	0.37	0.33	0.56	0.27	0.69	0.36	0.36	0.11	0.11	0.15	0.21	0.11	0.09	0.09	0.09	0.09	0.34
44	0.19	0.04	0.04	0.22	0.95	0.82	0.59	0.04	0.21	0.03	0.29	0.19	0.04	0.03	0.03	0.18	0.17	0.17	0.12
48	0.17	0.17	0.17	0.17	1.00	0.74	0.26	0.19	0.16	0.24	0.28	0.21	0.15	0.16	0.22	0.21	0.21	0.21	0.15
49	0.11	0.09	0.09	0.12	0.56	0.50	0.40	0.07	0.07	0.40	0.27	0.20	0.12	0.06	0.12	0.09	0.15	0.14	0.06
52	0.37	0.32	0.34	0.37	0.99	0.98	0.46	0.41	0.39	0.62	0.26	0.49	0.42	0.08	0.15	0.36	0.37	0.34	0.01
53	0.28	0.11	0.11	0.28	1.00	0.49	0.48	0.09	0.12	0.18	0.14	0.17	0.45	0.03	0.01	0.07	0.04	0.06	0.01
54	0.29	0.30	0.29	0.29	1.00	0.96	0.19	0.29	0.30	0.30	0.31	0.30	0.30	0.30	0.09	0.39	0.38	0.38	0.12
55	0.59	0.30	0.26	0.58	1.00	0.99	0.52	0.26	0.17	0.10	0.14	0.16	0.45	0.08	0.11	0.07	0.07	0.07	0.12
57	0.20	0.45	0.11	0.20	1.00	0.96	0.43	0.05	0.08	0.12	0.73	0.10	0.17	0.62	0.11	0.01	0.03	0.03	0.00

60	0.48	0.18	0.18	0.48	1.00	0.74	0.65	0.21	0.61	0.32	0.90	0.72	0.27	0.20	0.22	0.51	0.41	0.50	0.31
63	0.19	0.04	0.03	0.21	0.35	0.10	0.81	0.06	0.31	0.00	0.03	0.02	0.06	0.01	0.00	0.05	0.04	0.04	0.18
65	0.04	0.01	0.01	0.05	0.96	0.99	0.20	0.03	0.02	0.01	0.02	0.05	0.03	0.02	0.01	0.02	0.01	0.01	0.02
70	0.04	0.02	0.02	0.04	0.38	0.12	0.22	0.03	0.02	0.02	0.05	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.00
72	0.23	0.01	0.01	0.08	0.93	0.80	0.23	0.03	0.01	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
73	0.18	0.02	0.02	0.17	0.41	0.41	0.12	0.18	0.05	0.02	0.04	0.02	0.12	0.00	0.01	0.00	0.00	0.00	0.00
76	0.21	0.12	0.11	0.17	0.58	0.58	0.50	0.10	0.15	0.01	0.05	0.02	0.25	0.01	0.01	0.00	0.00	0.00	0.05
77	0.26	0.12	0.12	0.26	0.76	0.76	0.37	0.12	0.16	0.22	0.11	0.24	0.20	0.07	0.13	0.11	0.11	0.11	0.14
78	0.34	0.13	0.13	0.28	0.77	0.75	0.61	0.14	0.17	0.23	0.11	0.24	0.21	0.08	0.10	0.08	0.12	0.10	0.14
80	0.09	0.01	0.01	0.09	0.32	0.46	0.37	0.04	0.07	0.00	0.01	0.04	0.11	0.03	0.00	0.00	0.00	0.00	0.01
82	0.02	0.01	0.01	0.01	0.99	0.99	0.68	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
83	0.41	0.14	0.14	0.36	1.00	1.00	0.44	0.26	0.25	0.13	0.10	0.08	0.26	0.01	0.08	0.02	0.02	0.03	0.01
86	0.36	0.09	0.09	0.36	0.97	0.97	0.39	0.10	0.04	0.09	0.04	0.11	0.21	0.00	0.01	0.00	0.00	0.00	0.00
88	0.04	0.15	0.15	0.04	0.82	0.82	0.05	0.01	0.00	0.07	0.02	0.00	0.01	0.00	0.04	0.00	0.00	0.00	0.00
90	0.18	0.11	0.11	0.18	0.33	0.33	0.18	0.11	0.04	0.05	0.02	0.10	0.09	0.01	0.01	0.02	0.06	0.04	0.00
93	0.37	0.39	0.39	0.38	0.59	0.63	0.54	0.32	0.14	0.14	0.22	0.62	0.40	0.34	0.06	0.15	0.43	0.30	0.07
94	0.11	0.07	0.07	0.12	0.18	0.34	0.22	0.15	0.16	0.07	0.07	0.11	0.09	0.12	0.04	0.08	0.10	0.10	0.01
95	0.00	0.01	0.01	0.00	0.05	0.11	0.05	0.02	0.00	0.01	0.02	0.01	0.14	0.02	0.01	0.01	0.11	0.09	0.00
97	0.02	0.05	0.05	0.02	0.06	0.59	0.15	0.06	0.02	0.01	0.08	0.18	0.15	0.50	0.01	0.43	0.44	0.44	0.01
98	0.20	0.02	0.02	0.20	0.05	0.88	0.11	0.02	0.07	0.01	0.01	0.05	0.03	0.11	0.01	0.03	0.06	0.05	0.00
105	0.03	0.01	0.01	0.03	0.95	0.98	0.69	0.03	0.03	0.00	0.02	0.00	0.03	0.02	0.00	0.00	0.00	0.00	0.00
108	0.09	0.03	0.02	0.09	0.37	0.98	0.45	0.05	0.04	0.02	0.02	0.05	0.10	0.03	0.01	0.05	0.03	0.04	0.03
110	0.11	0.03	0.01	0.10	0.69	0.70	0.30	0.01	0.02	0.03	0.03	0.03	0.05	0.02	0.02	0.14	0.06	0.08	0.01
111	0.02	0.00	0.00	0.01	1.00	1.00	0.23	0.00	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00
115	0.45	0.39	0.30	0.45	1.00	0.99	0.82	0.08	0.07	0.15	0.73	0.55	0.23	0.22	0.09	0.00	0.00	0.00	0.01
116	0.38	0.65	0.55	0.38	1.00	0.98	0.38	0.09	0.04	0.22	0.42	0.31	0.46	0.07	0.10	0.01	0.01	0.01	0.01
117	0.36	0.43	0.37	0.35	1.00	0.98	0.61	0.20	0.14	0.23	0.43	0.51	0.51	0.29	0.16	0.01	0.01	0.01	0.04
119	0.53	0.58	0.54	0.37	1.00	0.69	0.89	0.38	0.41	0.34	0.84	0.57	0.58	0.29	0.29	0.16	0.19	0.17	0.21
120	0.07	0.37	0.32	0.07	1.00	1.00	0.33	0.04	0.01	0.02	0.33	0.32	0.31	0.01	0.02	0.00	0.00	0.00	0.01
121	0.13	0.62	0.25	0.10	1.00	0.72	0.53	0.05	0.07	0.09	0.83	0.16	0.24	0.17	0.08	0.04	0.04	0.04	0.05
122	0.21	0.22	0.18	0.09	1.00	0.42	0.62	0.08	0.07	0.04	0.52	0.21	0.21	0.03	0.02	0.00	0.00	0.00	0.01
123	0.16	0.64	0.21	0.15	1.00	0.99	0.52	0.07	0.09	0.11	0.67	0.17	0.21	0.43	0.06	0.01	0.03	0.03	0.00
125	0.69	0.62	0.27	0.69	1.00	1.00	0.84	0.50	0.43	0.62	0.67	0.65	0.71	0.32	0.47	0.05	0.14	0.14	0.01
126	0.60	0.33	0.29	0.60	1.00	1.00	0.28	0.46	0.38	0.15	0.39	0.61	0.31	0.07	0.08	0.03	0.03	0.03	0.01
127	0.37	0.07	0.03	0.37	1.00	1.00	0.61	0.04	0.03	0.05	0.05	0.05	0.05	0.01	0.04	0.01	0.02	0.01	0.01
128	0.55	0.38	0.38	0.55	1.00	1.00	0.30	0.07	0.05	0.45	0.25	0.20	0.49	0.03	0.15	0.05	0.06	0.06	0.02
129	0.10	0.05	0.06	0.09	0.99	0.99	0.61	0.01	0.03	0.01	0.02	0.01	0.02	0.02	0.01	0.03	0.03	0.03	0.01
130	0.26	0.02	0.03	0.26	0.98	0.98	0.77	0.06	0.07	0.01	0.03	0.04	0.14	0.01	0.00	0.01	0.01	0.01	0.00
134	0.07	0.05	0.12	0.07	0.36	0.38	0.12	0.04	0.03	0.01	0.03	0.04	0.07	0.04	0.02	0.02	0.02	0.02	0.01
135	0.10	0.00	0.00	0.10	0.23	0.22	0.10	0.04	0.03	0.00	0.00	0.05	0.03	0.02	0.00	0.00	0.00	0.00	0.01
139	0.19	0.01	0.00	0.18	0.94	0.97	0.80	0.10	0.10	0.00	0.01	0.03	0.11	0.02	0.00	0.00	0.00	0.00	0.00

[0136] Based on the above ACE analysis, BMP-7 variants at the following positions are transferable to BMP-2: 36, 55, 60, 83, 115, 119, 125, 126, and 128. BMP-7 variants at the following positions are transferable to BMP-3: 57, 116, 117, 119, 121, 123, and 125. BMP-7 variants at the following positions are transferable to BMP-3b: 116 and 119. BMP-7 variants at

the following positions are transferable to BMP-4: 55, 60, 115, 125, 126, and 128. BMP-7 variants at the following positions are transferable to BMP-5: 36, 37, 39, 42, 44, 48, 49, 52, 53, 54, 55, 57, 60, 65, 72, 73, 76, 77, 78, 82, 83, 86, 88, 93, 105, 110, 111, 115, 116, 117, 119, 120, 121, 122, 123, 125, 126, 127, 128, 129, 130, and 139. BMP-7 variants at the following positions are transferable to BMP-6: 36, 39, 44, 48, 49, 52, 53, 54, 55, 57, 60, 65, 72, 73, 76, 77, 78, 80, 82, 83, 86, 88, 93, 97, 98, 105, 108, 110, 111, 115, 116, 117, 119, 120, 121, 122, 123, 125, 126, 127, 128, 129, 130, and 139. BMP-7 variants at the following positions are transferable to BMP-9: 36, 42, 44, 49, 52, 53, 55, 57, 60, 63, 75, 78, 82, 83, 93, 105, 108, 115, 117, 119, 121, 122, 123, 125, 127, 129, 130, and 139. BMP-7 variants at the following positions are transferable to BMP-9: 52, 125, and 126. BMP-7 variants at the following positions are transferable to BMP-10: 60, 119, and 125. BMP-7 variants at the following positions are transferable to BMP-15: 49, 52, 125, and 128. BMP-7 variants at the following positions are transferable to GDF-1: 36, 57, 60, 115, 116, 117, 119, 121, 123, and 125. BMP-7 variants at the following positions are transferable to GDF-3: 36, 52, 60, 93, 115, 117, 119, 125, and 126. BMP–7 variants at the following positions are transferable to GDF-5: 52, 53, 55, 93, 116, 117, 119, 125, and 128. BMP-7 variants at the following positions are transferable to GDF-8: 57, 97, and 123. BMP-7 variants at the following positions are transferable to GDF-9: 125. BMP-7 variants at the following positions are transferable to TGF-β1: 60 and 97. BMP-7 variants at the following positions are transferable to TGF-β2: 60, 93 and 97. BMP-7 variants at the following positions are transferable to TGF-β3: 60 and 97.

[0137] **Example 6:** *Generation of DNA encoding the parent BMP-7*

[0138] A number of constructs for the expression of wild type human BMP-7 were made and tested: (1) native BMP-7 Image clone in pSport6 vector (obtained from ATCC), (2) FLAG-tagged BMP-7, with G4S linker and FLAG tag located just after the RXXR cleavage site in pCMVTnT vector (Promega), (3) FLAG-tagged BMP-7 with a perfect Kozak sequence (MAV rather than MHV) in pCMVTnT vector, (4) FLAG-tagged BMP-7 using native BMP-2 Kozak sequence, signal sequence, and pro-domain in pCMVTnT vector, (5) FLAG-tagged BMP-7 using native MIC-1 Kozak sequence, signal sequence, and pro-domain in pCMVTnT vector, and (6) native BMP-7 Image clone in pSport6 vector (obtained from ATCC) with 6x His tag at the N-terminus of the pro-domain. The untagged native Image clone in the pSport6 vector was found to produce the highest expression yields and was used as the template for variant construction.

[0139] Additional constructs were prepared for yeast expression: (1) pYES vector negative control, (2) BMP-7 mature domain in pYES vector, (3) E-BMP-7 mature domain in pYES vector, (4) AEAE-BMP-7 mature domain in pYES vector, (5) full length BMP-7 in pYES vector, and (6) REKR-full length BMP-7 in pYES vector.

[0140] **Example 7:** *Generation of DNA encoding BMP variants*

[0141] Constructs for the BMP-7 variants shown in the above table were prepared by site directed mutagenesis. The pSport6 Image clone (ATCC) was used as a template for all of the variants. DNA corresponding to each desired construct was prepared using Qiagen Miniprep or Maxiprep kits. As some of the BMP-7 variants were constructed using degenerate oligos,

variants in addition to those explicitly included in Library 1 above were generated. Such variants include the following: L21E, L21G, L21N, L21R, M23G, M23N, M23R, V26E, V26G, V26N, K39A, K39G, K39N, Y44D, Y44G, Y44N, Y44P, Y44S, Y44T, R48D, R48H, W52P, W52T, Q53G, Q53K, Q53T, W55P, W55T, I57L, I57P, I57Q, E60H, E60N, E60P, F73G, F73K, F73N, F73T, N76Y, S77E, S77H, S77N, S77P, Y78P, Y78R, I86P, L90G, L90H, L90P, F93G, F93H, F93P, L115A, L115Q, Y116A, Y116Q, F117S, F117Y, V123G, I125P, I125T, K126G, K127A, K127H, K127N, K127P, K127Y, R129K, R129N, R134L, and R134P. Furthermore, silent mutations (that is, changes in DNA codon sequence that do not change the corresponding amino acid sequence) were introduced at I57, N76, Y116, V123, and R134.

[0142] **Example 8:** *In vitro translation of wild type BMP-7*

[0143] The following constructs were generated and used for in vitro translation experiments: (1) full length BMP-7 with FLAG tag at N-terminus of mature domain, (2) full length MIC-1 with FLAG tag at the N-terminus of the mature domain, (3) BMP-7 pro-domain with N-terminal FLAG tag, (4) MIC-1 pro-domain with N-terminal FLAG tag, (5) BMP-7 mature domain with N-terminal FLAG tag, (6) MIC-1 mature domain with N-terminal FLAG tag, (7) full length BMP-7 with C-terminal FLAG tag, (8) full length MIC-1 with C-terminal FLAG tag, (9) BMP-7 pro-domain with C-terminal FLAG tag, (10) MIC-1 pro-domain with C-terminal FLAG tag, (11) BMP-7 mature domain with C-terminal FLAG tag, and (12) MIC-1 mature domain with C-terminal FLAG tag. The 5' end of each construct had a spacer, T7 promoter, globin UTR, and optimized ribosomal binding site preceeding the gene. Luciferase was used as a positive control. A coupled transcription/translation off the PCR product was used (TnT, Promega). Protein expression comparable to luciferase was obtained for all of the BMP-7 constructs other than (11) above.

[0144] **Example 9:** *Expression of wild type and variant BMP-7*

[0145] The following small-scale expression protocol was used for initial library screening. 293T cells were plated into 6-well dishes (1-5 x 10^5 cells/mL in 4mL DMEM 10% FBS). The next day, the cells were transfected using 5 ug DNA / well. As an internal control for transformation efficiency, several mini-preps of the native Image clone were used. After three days, the conditioned media was harvested and screened. Expression yields were determined using ELISA (all variants, not corrected for changes in antibody binding affinity, using R&D Systems ELISA Duoset Cat#DY354) and Western blotting (selected variants).

[0146] **Table 12.** Expression yields of Library 1 variants in 293T cells

Variant name	ELISA concentration (ng/mL)	Fold change relative to Image clone	Western blot band intensity
L21D	999	1.34	
L21E	332	0.45	
L21G	4917	0.78	++
L21K	495	0.50	
L21N	414	2.97	-
L21R	682	0.92	

[0146] **Table 12.** Expression yields of Library 1 variants in 293T cells

Variant name	ELISA concentration (ng/mL)	Fold change relative to Image clone	Western blot band intensity
M23D	565	0.56	+
M23G	4	0.03	+++
M23N	600	0.81	+
M23R	3	0.02	-
M23S	15	0.11	-
V26D	747	0.75	+
V26E	1021	1.37	+
V26G	605	0.81	-
V26K	0	0.00	-
V26N	976	1.31	+
V26S	6	0.01	-
Q36E	7107	1.13	++
Q36N	1	0.00	-
K39A	22724	3.61	+
K39D	19	0.01	
K39E	1	0.00	
K39G	1	0.00	
K39N	69	0.04	
K39R	8376	1.33	+++
K39S	23063	3.67	+
K39T	19	0.01	
E42D	7307	1.16	+++
E42Q	6702	1.07	+++
E42R	583	0.58	-
E42T	449	0.45	-
Y44A	259	0.04	-
Y44D	1	0.00	
Y44E	42	0.04	-
Y44G	0	0.00	
Y44H	1	0.01	
Y44K	2	0.00	-
Y44N	14	0.10	-
Y44P	0	0.00	
Y44Q	19	0.02	-
Y44S	1	0.00	-
Y44T	21	0.15	
R48D	1	0.00	
R48E	74	0.04	
R48H	6063	0.96	-
R48N	7100	1.13	-
R48Q	743	0.42	
D49S	24	0.02	-
W52A	38	0.27	
W52E	1	0.00	
W52K	0	0.00	
W52P	407	0.55	
W52Q	6	0.01	-
W52T	5	0.03	
Q53A	251	0.04	-
Q53D	6876	3.87	++

[0146] **Table 12.** Expression yields of Library 1 variants in 293T cells

Variant name	ELISA concentration (ng/mL)	Fold change relative to Image clone	Western blot band intensity
Q53E	1237	0.70	
Q53G	83	0.05	+
Q53H	645	0.29	++
Q53K	6413	1.02	+
Q53R	149	0.08	
Q53S	8530	1.36	+++
Q53T	13237	2.10	++
D54K	10	0.01	-
D54N	458	0.46	-
D54S	38	0.01	-
W55A	26	0.04	
W55E	2	0.00	
W55H	6	0.01	
W55K	10	0.07	
W55N	11	0.08	
W55P	0	0.00	
W55Q	10	0.07	
W55R	48	0.34	-
W55T	0	0.00	
I57A	47	0.01	-
I57D	0	0.00	
I57E	0	0.00	
I57H	8	0.06	
I57I	526	0.71	-
I57K	9	0.06	
I57L	16106	2.56	0.63
I57P	0	0.00	
I57Q	0	0.00	
I57T	1	0.00	-
I57V	6	0.01	
E60H	129	0.07	
E60K	755	0.76	++
E60N	5	0.00	
E60P	145	0.08	
E60Q	3172	3.17	+
E60R	2069	0.33	+
E60S	97	0.05	
E60T	410	0.41	-
A63E	4068	4.07	+
A63Q	8577	8.58	++
A63R	3183	3.18	+
A63S	8448	8.45	++
Y65D	18743	18.74	+++
Y65E	382	0.38	-
Y65N	11678	11.68	++
E70A	501	0.50	-
E70Q	679	0.68	-
A72D	5462	5.46	+
A72E	3822	3.82	-
A72H	3308	3.31	+

[0146] **Table 12.** Expression yields of Library 1 variants in 293T cells

Variant name	ELISA concentration (ng/mL)	Fold change relative to Image clone	Western blot band intensity
A72K	3863	3.86	+
A72N	1927	0.88	+
A72R	491	0.49	-
A72S	4784	2.17	++
F73A	153	1.10	
F73D	27	0.19	
F73E	138	0.99	
F73G	173	1.24	
F73H	5783	5.78	++
F73K	25	0.18	
F73N	321	0.43	
F73Q	75	0.54	
F73S	5159	0.82	++
F73T	193	1.38	-
N76A	5570	3.14	
N76D	7534	4.24	
N76S	8816	4.97	
N76T	4576	2.58	
N76Y	814	0.46	
S77A	1194	0.67	
S77D	1	0.00	
S77E	46	0.03	
S77H	49	0.03	
S77K	1	0.00	
S77N	1	0.00	
S77P	1302	0.73	
S77Q	645	0.64	-
S77T	1048	0.59	
Y78D	517	3.71	-
Y78G	2	0.00	
Y78H	10102	1.61	+
Y78N	5795	5.80	-
Y78P	1249	1.68	
Y78R	264	1.89	-
Y78S	9648	1.53	++
Y78T	714	5.12	-
I86A	5635	5.64	++
I86D	3480	3.48	++
I86E	2556	3.43	
I86K	2214	2.97	
I86P	5	0.01	
I86Q	3008	4.04	
I86T	532	0.53	+
Q88E	47	0.05	-
L90E	235	0.32	
L90H	62	0.08	
L90H	62	0.08	
L90K	3	0.00	+
L90N	200	0.09	+++
L90P	109	0.15	

[0146] **Table 12.** Expression yields of Library 1 variants in 293T cells

Variant name	ELISA concentration (ng/mL)	Fold change relative to Image clone	Western blot band intensity
L90R	356	0.48	
L90S	5463	0.87	+++
L90T	647	0.87	
F93A	12	0.02	
F93D	2346	0.37	++
F93E	646	0.87	++
F93G	24	0.03	++
F93H	16307	2.59	+++
F93P	15	0.01	+++
F93Q	279	0.13	+++
F93R	38	0.02	++
F93S	2836	1.29	++
F93T	287	0.39	+
I94A	2	0.00	+++
I94E	1	0.00	-
I94H	70	0.03	-
I94K	0	0.00	-
I94P	0	0.00	
I94Q	854	1.15	
I94R	0	0.00	+
I94T	10	0.01	-
N95D	18	0.02	-
N95K	2	0.00	-
N95Q	53	0.05	-
N95R	1	0.00	-
E97D	4784	2.17	-
E97K	2936	1.33	-
E97R	2113	0.96	-
T98A	4208	1.91	-
T98E	3064	1.39	
T98K	5464	2.48	-
T98X	927	0.93	-
A105V	440	0.44	-
Q108D	4052	1.84	-
Q108K	1	0.00	-
Q108S	3529	1.60	-
N110D	8591	3.91	-
N110E	3020	1.37	-
N110H	331	0.33	-
A111D	2317	0.37	++
A111S	4358	4.36	-
L115A	24	0.03	
L115E	1	0.00	-
L115K	1	0.00	-
L115Q	1	0.00	-
L115T	0	0.00	
Y116A	0	0.00	
Y116D	1	0.00	-
Y116E	0	0.00	
Y116H	16724	2.66	+

[0146] **Table 12.** Expression yields of Library 1 variants in 293T cells

Variant name	ELISA concentration (ng/mL)	Fold change relative to Image clone	Western blot band intensity
Y116K	0	0.00	
Y116Q	0	0.00	
Y116S	34	0.01	-
Y116T	2	0.00	-
Y116Y	1922	2.58	
F117A	2	0.00	-
F117D	0	0.00	
F117E	2407	0.38	-
F117H	18720	2.98	++
F117K	329	0.44	
F117Q	3730	0.59	+
F117R	604	0.81	
F117S	42	0.06	
F117Y	1682	2.26	
D119E	9669	1.54	++
D119N	2870	0.46	-
D119S	4824	0.77	+
D119T	2022	0.32	-
S120D	21370	3.40	+++
S120E	9259	1.47	++
S120N	11035	1.75	++
S120R	10544	1.68	++
S121D	9202	1.46	++
S121E	6098	0.97	+
S121K	4793	0.76	-
S121N	5161	0.82	-
S121T	2204	0.35	-
N122E	1	0.00	-
N122Q	6	0.00	-
N122R	63	0.01	-
V123A	0	0.00	
V123D	1	0.00	-
V123G	305	0.41	
V123N	0	0.00	
V123R	1	0.00	-
V123T	1	0.00	
V123V	6	0.01	
L125A	1	0.00	
L125E	0	0.00	
L125K	1	0.00	-
L125P	0	0.00	
L125Q	4	0.00	-
L125T	0	0.00	
L125Y	3306	0.53	-
K126D	4393	0.70	-
K126E	24	0.01	
K126G	55	0.03	
K126Q	145	0.08	
K126R	10478	1.67	+
K127A	1	0.00	

[0146] **Table 12.** Expression yields of Library 1 variants in 293T cells

Variant name	ELISA concentration (ng/mL)	Fold change relative to Image clone	Western blot band intensity
K127D	1	0.00	
K127E	3974	0.63	++
K127H	1	0.00	
K127N	1	0.00	
K127P	1	0.00	
K127Q	1	0.00	
K127S	1	0.00	-
K127T	3	0.00	
K127Y	1	0.00	
Y128D	8948	1.42	++
Y128E	0	0.00	
Y128H	10887	1.73	++
Y128K	452	0.25	
Y128Q	11415	1.81	++
R129D	3093	0.49	+
R129E	15209	2.42	+
R129K	411	0.23	
R129N	573	0.32	+
R129S	46	0.03	
N130D	18652	2.96	+
R134D	1998	0.32	-
R134E	21604	3.43	++
R134K	830	0.47	
R134L	9016	5.08	+
R134P	125	0.07	
R134Q	1	0.00	-
R134S	4314	2.43	++
A135D	5	0.00	-
A135E	28974	4.61	++
A135S	29539	4.70	++
H139R	27483	4.37	+++

[0147] Preferred modifications include those modifications that increase the expression yield in 293T cells by at least 2-fold, including but not limited to L21N, K39A, K39S, Q53D, Q53T, I57L, E60Q, A63E, A63Q, A63R, A63S, Y65D, Y65N, A72D, A72E, A72H, A72K, A72S, F73H, N76A, N76D, N76S, N76T, Y78D, Y78N, Y78T, I86A, I86D, I86E, I86K, I86Q, F93H, E97D, T98K, N110D, A111S, Y116H, F117H, F117Y, S120D, R129D, N130D, R134E, R134L, R134S, A135E, A135S, and H139R. Especially preferred modifications include those modifications that increase the expression yield in 293T cells by at least 5-fold, including but not limited to A63Q, A63S, Y65D, Y65N, A72D, F73H, Y78N, Y78T, I86A, and R134L. Furthermore, a silent mutation at Y116 from codon TCA to codon TAT was observed to increase the expression yield in 293T cells by 2.6-fold.

[0148] Additional expression protocols were used for different scales (96-, 48-, 24-, 12-, or 6-well dishes as well as 10 cm or 15 cm plates), serum-free expression, and expression in alternate hosts (CHO and BRK-21).

[0149] Expression yields of selected Library 1 variants were determined for 293T, CHO, and BRK21 cells, as shown below. Expression of the wild type Image clone does vary between expression hosts. However, the relative expression yields for the different variants tend to be improved across hosts, indicating that a variant that improves expression yield in one host tends to improve expression yield in other hosts.

[0150] **Table 13**. Expression yields of selected Library 1 variants in different expression hosts

Yield vs. Image in	Image clone	Variant 23: L21G	Variant 154: Q53T	Variant 249: Y65N	Variant 117: Y78H	Variant 80: F93H
293T	1	3.8	1.9	7.2	2.3	8.5
CHO	1	0.9	1.4	6.8	1.1	6.5
BRK21	1	1.0	1.4	10.2	1.3	5.8

[0151] Relative expression yield was also tested as a function of DNA dose (5.0, 2.5, and 1.0 ug were tested) and was found to be dose-independent.

[0152] **Example 10:** *Characterization of the receptor binding affinity of the BMP-7 Library 1 variants*

[0153] The affinity of human and variant BMP-7 for several BMP receptors (ActRIa, BMPRIb, ActRIIa, and BMPRII) was measured using an ELISA-like assay, described below. 96-well plates were coated with a capture antibody (R&D Systems BMP-7 ELISA Duoset DY354, part # 840971) by diluting the antibody to 2 µg/mL in PBS, applying 50 µL/well, and incubating overnight or over the weekend at 4 °C in a humidified chamber. Excess liquid was removed from each plate. The plates were blocked by adding 175 µL blocking solution (1% BSA and 5% sucrose in PBS) to each well and incubating 1-2 hours at room temperature in a humidified chamber. The plates were then washed using an automated plate washer. 50 µL of BMP-7 containing solution (for example, diluted conditioned media obtained from the BMP-7 expression protocol above, or purified recombinant human BMP-7 of a known concentration) was added to each well and incubated 1.5-2 hours at room temperature in a humidified chamber. The plates were then washed using an automated plate washer. 50 µL of 5 µg/mL BMP receptor–Fc fusion in PBS was added to each well and incubated 1.5 hours at room temperature in a humidified chamber. The plates were then washed using an automated plate washer. 50 µL of 1:10,000 diluted anti-human IgG-HRP conjugate in secondary antibody dilution buffer (1% BSA in PBS, filtered through a 0.2 50 µm filter) was added to each well and incubated 30 minutes at room temperature in a humidified chamber. The plates were then washed using an automated plate washer. 50 µL of pre-mixed TMB substrate (BD Pharmingen # 555214) was added to each well and incubated for 10-20 minutes at room temperature in the dark. 25 µL of 2N H_2SO_4 was added to each well. Absorbance readings at 450 nm were taken using a 540 nm wavelength correction.

[0154] The table below shows the receptor binding affinity of a selection of BMP-7 variants relative to the wild type protein (normalized to 1.0). Note that the assays were performed using a fixed volume of conditioned media rather than a specific concentration of protein, so differences in expression levels as well as differences in receptor binding affinity may affect the results. Data is shown only for variants with expression yields greater than 10.0 ng/mL.

[0155] **Table 14.** Receptor binding affinity of BMP-7 Library 1 variants.

wt	res#	variant	ELISA Conc. (ng/mL)	ActRIIa binding	BMPRII binding	BMPRIa binding	BMPRIb binding
M	23	S	15.20	2.20	2.41	0.88	1.09
W	52	A	37.78	1.69	1.25	0.92	0.33
W	55	N	10.93	0.41	0.82	0.35	0.00
F	73	S	15.45	2.54	2.84	0.90	0.25
F	73	D	26.89	2.50	3.05	0.33	0.64
F	73	Q	74.76	1.87	2.85	1.05	1.71
F	73	E	138.00	1.87	1.77	0.55	1.23
F	73	A	153.25	1.13	0.82	0.55	1.05
F	73	A	84.87	0.56	0.25	0.00	0.53
Y	78	D	517.04	1.93	2.72	1.05	2.01
Y	78	T	714.26	1.63	2.66	0.87	2.40
Y	78	S	789.68	1.58	1.77	1.29	2.75
L	21	N	414.21	1.03	1.73	1.57	2.81
L	21	G	12.36	1.50	2.30	2.53	3.65
Y	44	N	13.63	0.45	1.46	2.05	1.81
F	73	T	192.64	2.29	3.41	2.04	2.86
Y	44	T	21.46	0.36	0.25	1.09	1.33
W	55	R	47.80	1.20	1.52	1.75	2.17
F	73	G	22.42	1.41	1.55	1.36	1.64
F	73	K	24.61	0.61	0.38	0.69	1.14
F	73	G	172.77	1.73	1.26	1.16	1.53
F	73	T	154.57	1.92	1.93	1.12	1.44
Y	78	R	263.91	2.26	3.13	1.36	2.39
Y	78	P	1249.23	0.92	0.85	0.75	0.69
I	86	K	2214.38	0.97	1.02	0.81	0.69
I	86	E	2555.62	1.00	1.13	0.66	0.69
I	86	Q	3007.62	0.96	1.07	0.66	0.82
L	90	R	338.46	0.47	0.13	0.29	0.32
L	90	T	647.00	0.66	0.29	0.48	0.64
L	90	E	234.92	0.24	0.18	0.20	0.27
L	90	R	356.38	0.47	0.12	0.22	0.48
F	93	A	11.61	0.11	0.09	0.10	0.24
F	93	E	645.77	0.79	0.62	0.83	0.99
F	93	D	52.77	0.80	0.62	0.96	0.91
F	93	T	287.00	0.79	0.81	0.77	0.88
L	115	A	24.24	0.01	-0.01	-0.02	0.17
F	117	R	603.69	0.35	0.72	0.47	0.71
F	117	K	329.23	0.34	0.20	0.43	0.62
L	90	H	61.98	0.47	0.20	0.36	0.55
L	90	P	109.40	0.75	0.17	0.46	0.52
L	90	G	91.95	0.87	0.28	0.55	0.65
F	93	H	233.85	0.98	1.20	1.03	1.04
F	93	G	23.58	0.63	0.35	0.60	0.53
Y	116	Y	1921.77	1.02	1.12	1.07	0.79
F	117	S	42.22	0.11	-0.04	0.15	-0.08
F	117	Y	1682.15	1.03	0.96	1.07	0.73
V	123	G	304.85	0.53	0.22	0.67	0.17

L	21	R	681.69	0.84	0.66	0.83	1.01
L	21	E	332.00	0.25	0.07	0.12	0.16
L	21	D	999.08	0.94	0.94	1.01	1.52
M	23	N	600.08	0.94	0.90	0.98	1.36
V	26	G	604.77	1.07	1.00	1.08	1.66
V	26	N	976.38	1.00	0.97	1.14	1.43
V	26	E	1020.54	1.02	0.73	1.02	1.38
W	52	P	407.15	0.07	0.01	0.03	0.14
W	55	A	26.22	0.09	0.03	0.10	0.23
I	57	I	525.77	1.05	1.20	1.01	1.53
I	57	L	23.04	0.90	0.40	0.92	1.17
F	73	N	321.23	0.89	0.85	0.51	0.95
Y	78	H	1489.38	1.07	1.47	1.36	1.34
I	86	D	1703.85	1.09	1.31	1.03	1.18
I	94	Q	854.46	1.10	1.32	0.74	0.74
Y	128	K	452.40	1.11	1.78	1.40	1.57
K	39	N	69.00	1.08	0.99	1.98	1.63
K	39	A	8898.00	1.06	1.51	3.48	2.86
K	39	S	7148.00	1.04	1.37	2.77	2.23
R	48	E	73.90	0.34	-0.13	0.45	-0.51
R	48	Q	743.20	0.80	0.15	1.06	0.85
R	48	N	410.20	0.93	0.13	1.87	0.43
R	48	H	42.82	1.02	0.58	3.15	3.58
Q	53	G	82.88	0.99	1.37	3.70	3.95
Q	53	R	149.28	0.81	0.96	1.26	1.41
Q	53	E	1236.80	0.98	0.72	2.30	2.78
Q	53	D	6876.00	0.89	1.01	2.20	3.74
Q	53	K	485.40	0.73	0.14	0.70	2.03
Q	53	T	5296.00	1.24	1.28	0.80	0.90
Q	53	T	4072.00	1.14	1.23	0.73	0.85
Q	53	S	5978.00	1.04	1.26	0.66	0.68
E	60	R	122.78	0.68	0.77	0.35	0.23
E	60	P	145.10	0.94	1.01	0.47	0.53
E	60	H	128.78	0.86	1.25	0.34	0.42
E	60	R	148.04	0.80	0.40	0.43	0.50
N	76	T	4576.00	1.11	0.97	0.94	0.98
N	76	D	7534.00	1.13	0.87	0.84	0.80
N	76	Y	813.80	0.92	0.60	0.46	0.46
N	76	N	4998.00	1.05	1.12	0.72	0.78
N	76	A	5570.00	0.96	0.95	0.79	0.81
N	76	S	8816.00	0.99	0.98	0.76	0.85
S	77	A	24.84	0.91	0.80	0.55	0.76
S	77	T	15.45	1.15	0.97	0.77	0.79
S	77	E	45.54	1.17	0.98	0.81	0.94
S	77	P	691.00	1.00	0.91	0.58	0.79
S	77	A	1194.00	1.05	0.84	0.85	0.92
S	77	H	48.80	0.42	0.08	0.29	0.30
K	126	E	24.45	0.26	0.00	0.23	0.22
K	126	Q	145.46	0.81	0.28	0.46	0.57
R	129	N	573.40	1.10	1.40	0.89	1.03
R	129	D	1305.00	1.13	1.86	0.93	1.16
R	129	S	45.52	0.62	0.26	0.32	0.37
R	129	K	410.80	1.12	0.78	0.69	0.77
R	134	K	830.00	1.18	0.91	1.05	1.11
R	134	R	249.28	1.09	0.81	0.87	0.90
R	134	S	4314.00	1.17	1.07	1.10	1.15
K	39	T	19.26	0.98	0.75	0.74	0.86
K	39	D	18.87	0.88	0.72	0.67	0.82

Q	53	A	27.33	0.84	0.26	0.54	0.65
E	60	S	97.16	1.02	0.88	0.74	0.87
S	77	T	1047.80	0.99	0.93	0.94	0.90
S	77	P	1302.20	0.92	0.85	0.68	0.95
K	126	G	55.32	0.53	0.12	0.30	0.35
R	134	P	125.14	0.89	0.50	0.54	0.60
R	134	R	927.00	1.11	1.02	0.86	0.98
R	134	L	9016.00	0.99	1.00	1.03	1.02

[0156] To further characterize receptor binding, dose-response binding assays, using 12-point serial dilutions from conditioned media, were conducted for selected Library 1 variants.

[0157] Next, dissociation constants (K_D below) were calculated for each variant using the nonlinear regression – one site hyperbolic binding model in Prism. Note that the experiment was repeated for the wild type protein. The relative binding constants may be compared to determine whether the specificity of each variant is appreciably different from wild type.

Table 16. Dissociation constants for BMP-7 Library 1 variants

variant	ActRIIa K_D	BMPRII K_D	BMPRIa K_D	BMPRIb K_D
WT	0.40	1.28	1.61	1.77
WT	0.28	0.98	1.53	0.33
L21G	0.43	2.01	1.65	0.77
L21K	0.84	3.09	2.92	1.75
L21N	3.38	5.01	7.48	5.02
L21R	0.31	2.14	3.32	1.60
M23G	0.31	0.88	2.89	2.53
M23N	0.06	0.99	1.79	1.07
M23R	0.00	2.16	1.69	1.75
M23S	0.58	3.03	2.77	1.39
V26E	2.39	15.80	9.89	6.13
V26G	1.71	3.93	5.76	12.74
V26K	2.42	18.29	7.02	6.98
V26N	1.88	9.33	7.94	5.45
K39A	0.08	0.68	1.97	1.13
K39S	0.12	0.88	1.65	1.11
Y44A	1.57	4.27	5.54	2.39
Y44D	2.08	44.04	3.71	2.18
Y44G	1.10	21.39	4.98	2.56
Y44N	0.80	3.34	2.92	2.63
Y44P	0.80	11.56	6.46	1.67
Y44S	0.53	6.26	3.48	1.82
R48H	0.23	2.38	2.57	1.04
R48N	0.26	3.14	2.78	1.10
R48Q	1.42	5.37	5.17	5.16
W52A	0.45	8.16	5.00	1.61
Q53A	0.52	2.04	2.92	2.01
Q53D	1.32	5.37	5.22	4.45
Q53G	0.06	0.82	0.73	0.72
Q53H	0.53	7.97	3.38	2.13
Q53K	0.21	1.31	1.57	1.46
Q53S	0.13	1.12	1.36	1.07

Table 16. Dissociation constants for BMP-7 Library 1 variants

variant	ActRIIa K_D	BMPRII K_D	BMPRIa K_D	BMPRIb K_D
Q53T	0.09	0.80	1.36	1.13
W55N	0.55	5.52	5.99	1.99
I57H	0.56	5.34	3.70	2.47
I57I	0.13	1.23	1.36	0.67
I57L	0.09	0.71	1.32	0.81
E60K	0.70	3.71	4.03	3.25
E60Q	0.49	2.32	2.93	2.11
E60R	0.41	1.01	2.76	2.58
A63Q	0.51	0.98	2.20	1.09
A63S	0.61	2.29	2.90	1.30
Y65D	0.45	1.11	2.19	1.11
A72D	0.46	1.56	2.68	1.30
A72H	0.54	2.55	1.98	1.71
A72N	0.81	3.21	4.02	1.25
A72S	0.35	1.50	2.48	1.04
F73E	0.72	34.63	4.25	1.79
F73S	0.18	1.72	2.33	1.08
Y78H	0.10	1.05	1.46	1.12
Y78R	0.94	11.65	3.90	1.68
Y78S	0.19	1.10	1.40	0.98
N83P	0.18	0.77	1.39	1.78
I86A	0.16	1.12	3.55	1.12
I86D	0.85	11.19	24.59	8.03
Q88E	1.33	8.96	9.65	7.32
L90N	1.11	12.59	7.26	5.23
F93D	0.40	3.02	1.18	1.20
F93E	0.73	17.88	3.57	1.33
F93G	0.43	1.21	6.15	5.35
F93H	0.14	0.84	1.35	0.71
F93Q	0.94	4.44	6.25	3.46
F93R	1.11	8.96	9.30	5.95
F93S	0.61	1.89	1.11	1.37
F93T	0.74	1.85	3.94	1.34
I94E	3.02	9.73	14.20	8.08
I94H	0.93	9.03	7.10	4.62
I94K	1.07	1.07	4.74	3.05
I94R	0.68	1.07	3.70	1.78
N95K	2.13	1.49	8.31	1.46
N95R	5.35	14.15	14.18	11.13
E97D	0.38	1.27	3.35	1.40
E97K	0.65	2.36	7.88	2.72
E97R	0.24	1.38	2.22	0.31
T98A	0.20	1.17	2.09	0.27
T98E	0.14	1.41	1.95	0.35
T98K	0.18	1.04	1.72	0.33
T98X	0.15	1.32	1.60	0.34
A105V	0.15	1.01	1.11	0.41
Q108D	0.14	0.84	2.01	0.42
Q108S	0.15	1.25	0.95	0.46
N110D	0.20	0.35	0.62	0.34
N110E	0.28	0.68	0.98	0.39
Y116H	0.28	1.96	2.12	0.41

Table 16. Dissociation constants for BMP-7 Library 1 variants

variant	ActRIIa K_D	BMPRII K_D	BMPRIa K_D	BMPRIb K_D
Y116Y	0.25	1.06	2.20	0.93
F117H	0.16	0.84	1.43	0.28
F117R	0.65	5.54	4.17	1.17
F117Y	1.26	5.15	5.11	6.81
S120D	0.10	1.07	0.89	0.30
R129D	0.26	0.43	2.22	1.40
R129N	2.66	1.78	7.85	8.90
R134E	0.31	1.07	2.43	0.55
R134L	0.29	1.75	12.33	1.23
R134R	0.39	6.16	4.26	2.72
R134S	1.48	3.19	5.21	4.56
A135E	0.08	1.22	0.99	0.38
A135S	0.17	1.31	0.67	0.44
H139R	0.15	1.41	1.00	0.37

[0158] Based on the above-described results, the vast majority of the variants have receptor binding affinities that are similar to wild type BMP-7. The following variants appear to have altered specificity for the type II receptors: M23N, Q53G, Q53H, and I86D. Q53H and I86D bind to ActRIIa with similar affinity to wild type, but bind BMPRII with approximately 10-fold reduced affinity relative to wild type. M23N and Q53G bind to ActRIIa with approximately 10-fold increased affinity relative to wild type, but bind BMPRII with similar affinity to wild type.

[0159] **Example 11:** *Characterization of Library 1 BMP variants using the C2C12 bioassay*

[0160] The biological activity of human and variant BMP-7 molecules was measured using the C2C12 bioassay. C2C12 cells are a mouse myoblastic cell line that differentiates in response to BMPs such as BMP-7. C2C12 cells were trypsinized and diluted to approximately 60,000 cells/mL in C2C12 media (DMEM, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10% FBS, and antibiotics). 50 µL (3000 cells) were dispensed into each well of a 96-well plate and incubated overnight at 37 °C. The next day, 50 µL of BMP-7 containing solution (for example, diluted conditioned media obtained from the BMP-7 expression protocol above, or purified recombinant human BMP-7 of a known concentration) was added to each well; each sample was tested in duplicate. The plates were incubated for 3 days at 37 °C. The plates were then washed twice with 150 µL TBS (50 mM Tris pH 7.5, 150 mM NaCl). 25 µL TBS with 1% Triton-X100 was added to each well and the plate was incubated for 10-20 minutes at 4 °C. 100-150 µL CSPD Sapphire II luminescent alkaline phosphatase substrate (Applied Biosystems #T2210) was added to each well and incubated at room temperature in the dark. Luminescence readings were obtained for each well using the TopCount plate reader. Luminescence of the BMP-7 variants were compared to the luminescence of known quantities of recombinant human BMP-7 in order to determine the relative biological activity of the variants.

[0161] The table below shows the bioactivity of a selection of BMP-7 variants relative to the wild type protein (normalized to 1.0). Note that the assays were performed using a fixed volume of conditioned media rather than a specific concentration of protein, so differences in expression levels as well as differences in receptor binding affinity may affect the results. Data is shown only for variants with expression yields greater than 10.0 ng/mL.

[0162] **Table 17.** Bioactivity of BMP-7 Library 1 variants

var#	wt	res #	var	ELISA Conc. (ng/mL)	C2C12 Bioactivity
1	M	23	S	15.20	8.65
6	W	52	A	37.78	0.11
9	W	55	N	10.93	0.06
13	F	73	S	15.45	0.11
14	F	73	D	26.89	0.03
15	F	73	Q	74.76	0.20
16	F	73	E	138.00	0.09
17	F	73	A	153.25	0.18
18	F	73	A	84.87	-0.08

19	Y	78	D	517.04	0.13
20	Y	78	T	714.26	0.17
21	Y	78	S	789.68	0.09
22	L	21	N	414.21	1.21
23	L	21	G	12.36	4.34
28	Y	44	N	13.63	0.11
30	F	73	T	192.64	0.16
31	Y	44	T	21.46	0.13
33	W	55	R	47.80	0.51
37	F	73	G	22.42	0.07
39	F	73	K	24.61	0.11
40	F	73	G	172.77	0.13
41	F	73	T	154.57	0.17
42	Y	78	R	263.91	2.50
43	Y	78	P	1249.23	0.00
49	I	86	K	2214.38	0.01
50	I	86	E	2555.62	0.02
51	I	86	Q	3007.62	0.05
52	L	90	R	338.46	0.04
53	L	90	T	647.00	0.01
54	L	90	E	234.92	0.00
55	L	90	R	356.38	0.00
56	F	93	A	11.61	0.05
57	F	93	E	645.77	6.14
58	F	93	D	52.77	6.05
59	F	93	T	287.00	5.61
63	L	115	A	24.24	-0.01
66	F	117	R	603.69	0.06
67	F	117	K	329.23	0.08
76	L	90	H	61.98	0.01
77	L	90	P	109.40	-0.01
78	L	90	G	91.95	-0.01
80	F	93	H	233.85	5.78
82	F	93	G	23.58	5.38
85	Y	116	Y	1921.77	2.34
86	F	117	S	42.22	0.00
87	F	117	Y	1682.15	1.84
88	V	123	G	304.85	0.31
97	L	21	R	681.69	1.20

98	L	21	E	332.00	0.00
99	L	21	D	999.08	0.89
100	M	23	N	600.08	1.54
103	V	26	G	604.77	2.13
104	V	26	N	976.38	2.75
105	V	26	E	1020.54	0.97
106	W	52	P	407.15	0.00
109	W	55	A	26.22	-0.01
111	I	57	I	525.77	1.66
112	I	57	L	23.04	0.05
116	F	73	N	321.23	-0.01
117	Y	78	H	1489.38	2.67
119	I	86	D	1703.85	0.20
120	I	94	Q	854.46	0.00
125	Y	128	K	452.40	0.12
137	K	39	N	69.00	0.13
140	K	39	A	8898.00	1.27
141	K	39	S	7148.00	0.96
143	R	48	E	73.90	0.00
144	R	48	Q	743.20	0.10
145	R	48	N	410.20	0.01
147	R	48	H	42.82	0.33
148	Q	53	G	82.88	1.00
149	Q	53	R	149.28	0.02
150	Q	53	E	1236.80	0.08
151	Q	53	D	6876.00	1.67
152	Q	53	K	485.40	0.04
153	Q	53	T	5296.00	0.72
154	Q	53	T	4072.00	1.20
155	Q	53	S	5978.00	1.52
156	E	60	R	122.78	0.17
159	E	60	P	145.10	0.73
160	E	60	H	128.78	0.32
161	E	60	R	148.04	0.28
162	N	76	T	4576.00	0.12
163	N	76	D	7534.00	0.01
164	N	76	Y	813.80	0.00
165	N	76	N	4998.00	1.18
166	N	76	A	5570.00	0.05

167	N	76	S	8816.00	0.20
168	S	77	A	24.84	0.32
169	S	77	T	15.45	0.00
170	S	77	E	45.54	-0.01
171	S	77	P	691.00	-0.01
172	S	77	A	1194.00	0.60
173	S	77	H	48.80	0.01
174	K	126	E	24.45	0.01
175	K	126	Q	145.46	0.21
182	R	129	N	573.40	1.50
183	R	129	D	1305.00	3.96
184	R	129	S	45.52	0.05
185	R	129	K	410.80	0.01
186	R	134	K	830.00	0.87
187	R	134	R	249.28	0.50
188	R	134	S	4314.00	3.76
190	K	39	T	19.26	0.06
191	K	39	D	18.87	0.01
192	Q	53	A	27.33	0.02
193	E	60	S	97.16	0.44
197	S	77	T	1047.80	0.01
199	S	77	P	1302.20	0.00
200	K	126	G	55.32	0.00
204	R	134	P	125.14	0.17
205	R	134	R	927.00	3.57
206	R	134	L	9016.00	3.76

[0163] Interestingly, a number of the library 1 variants have significantly greater bioactivity than the wild type Image clone. This observed increase in activity is likely due to increased expression yield, although additional factors including but not limited to altered stability, solubility, or receptor binding affinity may also influence the observed bioactivity. Substitutions that increase bioactivity by at least 2-fold relative to wild type include, but are not limited to, L21G, M23S, V26G, V26N, Y78H, Y78R, F93D, F93E, F93G, F93H, F93T, R129D, R134L, and R134S.

[0164] **Example 12:** *Double mutant variants: BMP-7 Library 2.*

[0165] The point mutations from selected Library 1 variants were combined to yield a library of double mutants, referred to as Library 2. Methods for making and screening the Library 2 variants are as for the Library 1 variants described above. C2C12 bioassay data was determined at a single point by diluting conditioned media 1:66; due to the low expression yield of the Image clone, its bioassay signal is at background at this dilution.

[0166] **Table 18.** Expression yield and bioassay data, BMP-7 Library 2 variants.

Variant Name	293T ELISA (ng/mL)	Fold change : 293T	CHO ELISA (ng/mL)	Fold change : CHO	C2C12 @ 1:66	Western Blot Band Intensity
L21G-Y65N	4261.8	2.1	73.7	3.6	0.09	++
L21G-F93H	23923.5	12.0	20.8	1.1	0.23	++
L21R-Y65N	4371.2	2.2	59.9	2.9	0.16	++
M23R-Y65N	5468.8	2.7	182.3	8.8	0.20	++
K39A-F93H	37258.8	18.6	222.5	10.8	0.66	++
K39S-Y65N	5259.4	2.6	39.4	1.9	0.13	++
K39S-A72D	3703.5	1.9	0.7	0.0	0.05	++
K39S-Y78H	18617.6	9.3	173.8	8.4	0.02	++
K39S-I86A	4231.2	2.1	16.3	0.8	0.05	++
K39S-I94R	4.9	0.0	0.3	0.0	0.04	++
K39S-F93S	3256.5	1.6	45.7	2.2	1.23	+++
K39S-Q108D	5472.9	2.7	42.4	2.1	0.07	+++
K39S-N110D	19964.7	10.0	171.8	8.3	0.49	++
K39S-S120D	21147.1	10.6	134.3	6.5	0.46	+++
K39S-R129D	2048.8	1.0	34.0	1.6	0.28	++
K39S-N130D	3918.2	2.0	284.5	13.8	0.20	++
K39S-R134E	20564.7	10.3	6.2	0.3	0.28	++
K39S-R134S	18847.1	9.4	35.1	1.7	0.20	++
K39S-A135E	17694.1	8.8	159.9	7.8	0.03	++
K39S-A135S	2783.5	1.4	22.8	1.1	0.03	++
K39S-H139R	2868.8	1.4	37.2	1.8	0.03	++
Q53D-Y65N	22552.9	11.3	88.7	4.3	0.02	++
I57L-Y65N	18982.4	9.5	158.7	7.7	0.02	++
Y65N-Y78H	27923.5	14.0	197.3	9.6	0.13	++
Y65N-Y78R	20076.5	10.0	185.7	9.0	0.06	++
Y65N-S120D	23794.1	11.9	218.3	10.6	0.66	+++
Y65N-A135S	22023.5	11.0	51.5	2.5	0.12	++
A72D-F93H	23329.4	11.7	369.2	17.9	0.17	++
Y78H-F93H	18117.6	9.1	395.7	20.5	0.36	++
Y78H-Q108D	35176.5	17.6	0.7	0.0	0.27	++
Y78H-Y116H	23688.2	11.8	70.8	3.2	0.01	+
Y78H-F117Y	21794.1	10.9	62.3	5.5	0.07	++
Y78H-S120D	27864.7	13.9	179.6	12.5	0.34	++
Y78H-R134E	40129.4	20.1	410.1	21.6	0.72	++

Y78H-R134S	49617.6	24.8	36.3	2.4	0.68	++
Y78H-A135E	49729.4	24.9	167.6	9.6	0.03	++
Y78H-A135S	34458.8	17.2	43.4	2.2	0.05	++
Y78H-H139R	37770.6	18.9	40.5	2.1	0.14	++
F93H-F117Y	24747.1	12.4	296.0	14.4	0.40	++
F93H-S120D	35758.8	17.9	276.1	13.4	1.14	++
F93H-R134S	33800.0	16.9	378.7	18.4	1.21	++
F93H-H139R	39929.4	20.0	279.5	13.6	0.53	++

[0167] As may be seen above, a number of the Library 2 variants have significantly increased expression yield, in both 293T and CHO cells, relative to the wild type Image clone. Preferred variants show at least a 10-fold increase in at least one expression host; examples of such variants include but are not limited to L21G/F93H, K39A/F93H, K39S/N110D, K39S/S120D, K39S/N130D, K39S/R134E, Q53D/Y65N, Y65N/Y78H, Y65N/Y78R, Y65N/S120D, Y65N/A135S, A72D/F93H, Y78H/F93H, Y78H/Q108D, Y78H/Y116H, Y78H/F117Y, Y78H/S120D, Y78H/R134E, Y78H/R134S, Y78H/A135E, Y78H/A135S, Y78H/H139R, F93H/F117Y, F93H/S120D, F93H/R134S, F93H/H139R. Especially preferred variants show at least a 10-fold increase in expression yield in both 293T and CHO cells; examples of such variants include but are not limited to K39A/F93H, Y65N/S120D, A72D/F93H, Y78H/S120D, Y78H/R134E, Y78H/A135E, F93H/F117Y, F93H/S120D, F93H/R134S, and F93H/H139R.

[0168] **Example 13.** *Expression yield of triple, quadruple, and higher-order mutants.*

[0169] Triple, quadruple, and higher order mutants of BMP-7 were made and tested as described above. A number of these variants exhibit significantly increased expression yield or significantly increased bioactivity. Note that ELISA substantially underestimates the protein concentration of a substantial fraction of these variants due to decreased antibody binding affinity.

[0170] **Table 19.** Expression yield in 293T cells and bioactivity data for selected triple variants with high expression yield in 293T cells

Variant Name	ELISA Conc. (ng/mL)	Fold Increase Expression Yield	Fold Increase C2C12 Bioactivity	Western Blot Band Intensity
K39S/S120D/Y78H	50100.0	96.8	7.1	+++
Y78H/F93H/F117H	43490.0	84.0	23.0	+++
Y78H/F93H/Q108D	42880.0	82.8	21.6	+++
Y78H/F93H/A72D	42590.0	82.3	14.6	+++
K39S/S120D/Q108D	41170.0	79.5	15.6	+++
Y78H/F93H/Y65N	34830.0	79.4	21.3	+++
Y78H/F93H/S120D	40260.0	77.8	26.6	+++

Y78H/R134E/Y65N	40210.0	77.7	42.2	++++
K39S/S120D/Y65N	34260.0	66.2	17.8	+++
K39S/S120D/A72D	34070.0	65.8	25.9	+++
K39S/S120D/R134E	32320.0	62.4	37.9	+++
K39S/S120D/M23R	32060.0	61.9	14.3	+++
K39S/S120D/H139R	29930.0	57.8	21.2	++
K39S/S120D/R129D	5910.0	11.4	22.7	+++
Y78H/R134E/M23R	4775.0	9.2	27.7	++
Y78H/R134E/L21G	4763.0	9.2	28.4	++
Y78H/R134E/A72D	3269.0	6.3	43.6	++++
K39S/S120D/F93H	2950.0	5.7	2.1	+
K39S/S120D/F93S	2031.0	4.6	45.3	+++

[0171] All of the above triples have expression yields that are at least 50-fold higher than wild type, and at least 2-fold higher than the best doubles. Furthermore, the majority of the above triples have significantly increased bioactivity relative to wild type and the best doubles. Triple variants with especially high bioactivity include, but are not limited to, K39S/F93S/S120D, K39S/S120D/R134E, Y65N/Y78H/R134E, and A72D/Y78H/R134E.

[0172] **Table 20.** Expression yield in CHO-K1 cells and bioactivity data for selected triple variants with high expression yield in CHO-K1 cells

Variant Name	ELISA Conc. (ng/mL)	Fold Increase Expression Yield	Fold Increase C2C12 Bioactivity	Western Blot Band Intensity
Y78H/R134E/Y65N	316.2	17.1	3.6	–
Y78H/F93H/S120D	316.1	17.1	2.8	–
K39S/S120D/R134E	245.7	13.3	21.8	+
Y78H/R134E/A72D	232.1	12.5	14.7	+
K39S/S120D/Q108D	219.8	11.9	2.4	–
Y78H/F93H/Y65N	200.0	10.8	1.6	–
Y78H/R134E/L21G	198.0	10.7	2.8	–
Y78H/F93H/Q108D	115.3	6.2	2.9	–
Y78H/F93H/A72D	107.1	5.8	2.0	–
Y78H/R134E/M23R	78.8	4.3	6.4	–
Y78H/F93H/F117H	58.7	3.2	1.0	–
K39S/S120D/A72D	46.8	2.5	1.6	–
K39S/S120D/R129D	42.9	2.3	28.0	+
K39S/S120D/F93S	38.4	2.1	78.7	+
K39S/S120D/H139R	33.8	1.8	1.5	–

[0173] Preferred variants with dramatic increases in CHO-K1 expression yield or C2C12 bioactivity include but are not limited to K39S/F93S/S120D, K39S/S120D/R129D, K39S/S120D/R134E, Y65N/Y78H/R134E, and Y78H/F93H/S120D.

[0174] Additional triple mutants were generated to determine the impact of different substitutions on receptor and inhibitor binding specificity. These variants included the Y65N and S120D substitutions, which confer increased expression yield and do not significantly affect receptor or inhibitor binding, and one additional substitution that may alter binding specificity. Triples comprising Y65N/S120D, and one of the following substitutions were made: M23R, R48H, R48N, R48Q, Q53G, Q53H, Q53K, Q53T, E60R, F73S, F73T, Y78D, Y78S, Y78T, I86D, K126R, Y128D, Y128H, and Y128Q.

[0175] **Example 14**. *Purification of BMP-7 variants*

[0176] Y65N/S120D was partially purified using conventional chromatography. Heparin-sulphate sepharose (17-0407-01) was equilibrated in PBS. Conditioned media containing the Y65N/S120D variant was diluted 1:1 with 40 mM phosphate pH 6.5 filtered through a 0.45 micron filter, loaded onto the column, washed with 2-3 column volumes of PBS, and eluted in a single isocratic step with PBS/1M NaCl. The heparin bound fractions were dialyzed into 20 mM phosphate, 50 mM NaCl pH 7.0, loaded onto a SP-sepharose column, and eluted with a linear gradient (0 – 100% PBS/1M NaCl). A second purification protocol was used for larger scale purification of variants 457 (K39S/F93S), 471 (K39S/N130D), 492 (Y65N/Y78H), 504 (Y65N/S120D), 526 (K39S/S120D/R134E), and 565 (Y65N/F93T/R129D). Conditioned media was diluted 1:1 at neutral pH to lower the salt concentration to ~75 mM and loaded onto a SP-sepharose column. The column was then washed in 75 mM salt and then 300 mM salt, and BMP-7 was eluted with 1M salt.

[0177] **Example 15.** *Receptor, antibody, and inhibitor binding of selected variants*

[0178] The binding of selected variants to BMP receptors, antibodies, and inhibitors was characterized using a fluorescence binding assay and the AlphaScreen™ assay. BMP-7 variant Y65N/S120D, partially purified as described above, was labeled with the dye AlexaFluor 568 (Molecular Probes). Small-scale (25 uL) reactions were performed using 15 uM BMP-7 and dye concentrations ranging from 0.3 uM to 1000 uM. Reactions using 333 uM and 10 uM dye then were performed using 750 uL protein. The reaction was quenched with Tris pH 8.0 and cleaned up using a PD-10 desalt column; the second fraction was used in the experiments described below. BMP-7 was also labeled with C6-FXS, a FITC-derived fluorophore with a 6-carbon spacer between the fluor and the NHS group. Labeling conditions with various ratios of protein to dye were tested, in 20 mM PO4, 500 mM NaCl, pH 7 solution. After establishing labeling conditions, 500 ug Y65N/S120D was labeled. Excess dye was removed by centrifugation and a PD-10 desalt column.

[0179] The flourescently labeled BMP-7 was added to serial dilutions of receptor/Fc fusions of the BMP-7 receptors ActRIa, BMPRIa, BMPRIb, ActRIIa, ActRIIb, and BMPRII (R&D Systems). Experiments were also performed in which the fluorescently labeled BMP-7 was added to serial dilutions of noggin/Fc or gremlin (R&D Systems). The labeled BMP-7 and

receptor or inhibitor was allowed to incubate, and the fluorescence polarization and intensity was measured using a TopCount plate reader. Significant changes in intensity or anisotropy were observed for all of the receptors and inhibitors tested, for at least one of the labeled BMP-7 molecules.

[0180] Binding affinity of different BMP variants to these receptors and inhibitors may be determined by performing competition experiments. To perform these experiments, labeled BMP-7 and receptor or inhibitor are combined in amounts that yield an appreciable change in anisotropy or intensity relative to free labeled BMP-7. Then, varying amounts of a second, unlabeled BMP-7 molecule are added and the change in anisotropy or polarization is measured. The EC50 is then given by the concentration of competitor when half of the labeled BMP-7 is bound and half is free.

[0181] The Y65N/S120D variant of BMP-7 was also biotinylated for use in AlphaScreen assays using NHS-biotin. Bioactivity of the biotinylated protein was confirmed. AlphaScreen assays were performed to determine the binding affinity of selected BMP-7 variants for Fc fusions of the receptors BMPRIa, BMPRIb, ActRIIa, and BMPRII and the inhibitor noggin (R&D Systems). AlphaScreen assays were also performed to determine the affinity of selected variants for an anti-BMP-7 monoclonal antibody (R&D Systems mAb 3541). In all cases, 12-point binding curves were obtained in triplicate. Each data point corresponds to the luminescence produced from a solution comprising 10 uL of serially diluted BMP-7 variant, 10 uL receptor, inhibitor, or antibody, 10 uL biotinylated BMP-7, 10 uL AlphaScreen™ acceptor beads, and 10 uL AlphaScreen™ donor beads. Prism was used to calculate EC50 values for selected experiments.

Table 21. EC50 of wild type human BMP-7 (R&D Systems) and BMP-7 variants 504, 526, and 565 for the BMP receptors BMPRIb, ActRIIa, and BMPRII and the BMP inhibitor noggin, as determined using AlphaScreen™ assays.

BMP-7 variant	Receptor or inhibitor	EC(50) (ug/mL)	EC50 (ng/mL)	log(EC50) (ug/mL)	log(EC50) (ng/mL)	std. error (logEC50) ug/mL	Fold change vs. wt
wt	BMPRIb	0.0499	49.9	-1.30	1.70	0.136	
wt	ActRIIa	0.107	107	-0.971	2.03	0.218	
wt	BMPRII	0.230	230	-0.639	2.36	0.259	
wt	noggin	2.85	2850	0.455	3.46	0.894	
v504	BMPRIb	0.0185	18.5	-1.73	1.27	0.190	0.371
v504	ActRIIa	0.0475	47.5	-1.32	1.68	0.180	0.445
v504	BMPRII	0.222	222	-0.653	2.35	0.567	0.969
v504	noggin	3.40	3400	0.532	3.53	2.32	1.19
v526	BMPRIb	0.0363	36.3	-1.44	1.56	0.172	0.727
v526	ActRIIa	0.0445	44.5	-1.35	1.65	0.148	0.416
v526	BMPRII	0.139	139.4	-0.856	2.14	0.394	0.607
v526	noggin	16.7	16700	1.22	4.22	11.5	5.83
v565	BMPRIb	0.0224	22.4	-1.65	1.35	0.121	0.448
v565	ActRIIa	0.0424	42.4	-1.37	1.63	0.272	0.397
v565	BMPRII	0.409	409	-0.388	2.61	1.02	1.78
v565	noggin	0.392	392	-0.407	2.59	0.471	0.137

[0182] Overall, these three variants have binding affinities that are similar to wild type. Potentially significant differences include, but are not limited to, decreased noggin affinity of v526 and increased noggin affinity of v565.

[0183] **Example 16.** *Concentration determination*

[0184] Some of the BMP-7 variants, especially those variants with two or more mutations, exhibit reduced antibody binding affinity. As a result, ELISA concentration determination systematically underestimates the concentration of these variants. In order to obtain more accurate concentrations for these variants, as well as correction factors for the concentrations determined using ELISA, multiple concentration determination measurements were performed. Following purification, the concentration of variants 457, 471, 492, 504, 526, and 565 was assessed using the BCA assay, densitometry analysis of Coomasie blue stained mature domain following SDS-PAGE, and Western blotting using a polyclonal antibody. Wild type BMP-7 (R&D Systems) was used as a standard.

[0185] **Example 17.** *Specific activity determination*

[0186] The specific activity of five especially preferred BMP-7 variants was determined and compared with the specific activity of recombinant human BMP-7 purchased from R&D Systems. Equal concentrations of each protein, as determined above, were tested in the C2C12 bioassay three times.

[0187] **Table 22.** Specific activity of selected BMP-7 variants.

Name	EC50 : Exp #1	EC50: Exp #2	EC50 : Exp #3	Avg EC50 (ug/mL)	Std. dev.
Wild type (R&D Systems)	2.99	3.53	2.06	2.86	0.74
565-Y65N/F93T/R129D	0.1	0.3	0.09	0.16	0.12
526-K39S/S120D/R134E	0.58	1.69	0.49	0.92	0.67
504-Y65N/S120D	2.91	nd	4.85	3.88	1.37
492-Y65N/Y87H	5.91	nd	6.18	6.04	0.19
471-K39S/N130D	1.38	nd	3.99	2.69	1.85
457-K39S/F93S	0.37	1.1	nd	0.74	0.52

[0188] Variants with the F93S and F93T substitutions were found to have increased specific activity relative to the wild type protein.

[0189] **Example 18.** *Specific variant designs*

[0190] Quadruple mutant containing variants were designed to improve the proteins expression yields and ensure the highest biological activities. The mutant substitutions chosen for these variants comprise a subset of total variants that either singly, or in combination improve the properties of BMP-7. The stability and yield variants were chosen a subset of mutants that show beneficial protein properties and are located at amino acid residues that do

not make receptor contacts. Furin optimization is defined as a set of mutant variants, built in either the native or Y65N/S120D background that have an engineered consensus site for the furin protease required for normal processing and secretion of BMP-7. Glycosylation removal variants are mutant BMP-7 proteins, built in either the native or F93H/R134S background that contain mutations in the consensus glycosylation site, these variants are predicted to be aglycosylated. The following table summaries specific variant BMP-7 proteins created to have the listed properties:

Table 23

Variant	Property
K39S_S120D_Q108D_F93S	High activity and yield
K39S_S120D_R129D_F93S	High activity and yield
K39S_S120D_Y65N_F93S	High activity and yield
K39S_S120D_A72D_F93S	High activity and yield
Y78H_R134E_Y65N_F93S	High activity and yield
Y78H_R134E_A72D_F93S	High activity and yield
K39S_S120D_Q108D_Q108D	High activity and yield
K39S_S120D_R129D_Q108D	High activity and yield
K39S_S120D_Y78H_Q108D	High activity and yield
K39S_S120D_R134E_Q108D	High activity and yield
K39S_S120D_A72D_Q108D	High activity and yield
Y78H_R134E_A72D_Q108D	High activity and yield
Y65N_R129D_M23R_Q108D	High activity and yield
K39S_S120D_Y65N_R129D	High activity and yield
K39S_S120D_Y78H_R129D	High activity and yield
K39S_S120D_A72D_R129D	High activity and yield
Y65N_R129D_M23R_S120D	High activity and yield
Y65N_R129D_Q108D_S120D	High activity and yield
K39S_S120D_Q108D_Y65N	High activity and yield
K39S_S120D_R129D_Y65N	High activity and yield
K39S_S120D_R134E_Y78H	High activity and yield
K39S_S120D_Q108D_R134E	High activity and yield
K39S_S120D_Y65N_R134E	High activity and yield
K39S_S120D_Y78H_R134E	High activity and yield
K39S_S120D_A72D_R134E	High activity and yield
K39S_S120D_Q108D_M23R	High activity and yield
K39S_S120D_R129D_M23R	High activity and yield
K39S_S120D_Y78H_M23R	High activity and yield
K39S_S120D_R134E_M23R	High activity and yield
K39S_S120D_A72D_M23R	High activity and yield
Y78H_R134E_Y65N_M23R	High activity and yield
Y78H_R134E_A72D_M23R	High activity and yield
Y65N_R129D_Q108D_M23R	High activity and yield
Q108D_A72D	Stability and yield
S120D_A72D	Stability and yield
R129D_A72D	Stability and yield
A135E_A72D	Stability and yield
A72D_Q108D	Stability and yield
S120D_Q108D	Stability and yield
Q108D_S120D	Stability and yield
A135E_S120D	Stability and yield
A72D_R129D	Stability and yield
Q108D_R129D	Stability and yield
S120D_R129D	Stability and yield
Q108D_A135E	Stability and yield
S120D_A135E	Stability and yield

Table 23	
Variant	**Property**
A72D_F93S	Stability and yield
A72D_A105V	Stability and yield
A72D_N110D	Stability and yield
A72D_A135S	Stability and yield
A72D_H139R	Stability and yield
F93S_A105V	Stability and yield
F93S_Q108D	Stability and yield
F93S_N110D	Stability and yield
F93S_S120D	Stability and yield
F93S_R129D	Stability and yield
F93S_R134E	Stability and yield
F93S_A135S	Stability and yield
F93S_H139R	Stability and yield
A105V_S120D	Stability and yield
A105V_R129D	Stability and yield
A105V_R134E	Stability and yield
A105V_A135S	Stability and yield
A105V_H139R	Stability and yield
N110D_S120D	Stability and yield
N110D_R129D	Stability and yield
N110D_R134E	Stability and yield
N110D_A135S	Stability and yield
N110D_H139R	Stability and yield
Q108D_R134E	Stability and yield
Q108D_A135S	Stability and yield
Q108D_H139R	Stability and yield
F117Y_R129D	Stability and yield
F117Y_R134E	Stability and yield
S120D_R134E	Stability and yield
S120D_A135S	Stability and yield
S120D_H139R	Stability and yield
L21G_E42D	Stability and yield
L21G_T98K	Stability and yield
L21G_A105V	Stability and yield
L21G_S120D	Stability and yield
L21G_A135S	Stability and yield
L21G_A135E	Stability and yield
L21G_H139R	Stability and yield
M23R_E42D	Stability and yield
M23R_T98K	Stability and yield
M23R_A105V	Stability and yield
M23R_S120D	Stability and yield
M23R_A135S	Stability and yield
M23R_A135E	Stability and yield
M23R_H139R	Stability and yield
E42D_T98K	Stability and yield
E42D_A105V	Stability and yield
E42D_S120D	Stability and yield
E42D_A135S	Stability and yield
E42D_A135E	Stability and yield
E42D_H139R	Stability and yield
T98K_A105V	Stability and yield
T98K_S120D	Stability and yield
T98K_A135S	Stability and yield
T98K_A135E	Stability and yield
T98K_H139R	Stability and yield

Table 23	
Variant	**Property**
A105V_S120D	Stability and yield
A105V_A135S	Stability and yield
A105V_A135E	Stability and yield
A105V_H139R	Stability and yield
S120D_A135S	Stability and yield
S120D_A135E	Stability and yield
S120D_H139R	Stability and yield
WT_P1_QVKKRSKR	Furin optimization
WT_P2_QVKKRSRR	Furin optimization
WT_P3_QVRKRSKR	Furin optimization
WT_P4_QVRKRSRR	Furin optimization
WT_P5_KVKKRSKR	Furin optimization
WT_P6_KVKKRSRR	Furin optimization
WT_P7_KVRKRSKR	Furin optimization
WT_P8_KVRKRSRR	Furin optimization
WT_P9_EVKLRSKR	Furin optimization
WT_P10_EVKLRSRR	Furin optimization
WT_P11_EVRLRSKR	Furin optimization
WT_P12_EVRLRSRR	Furin optimization
Y65N_S120D_QVKKRSKR	Furin optimization
Y65N_S120D_QVKKRSRR	Furin optimization
Y65N_S120D_QVRKRSKR	Furin optimization
Y65N_S120D_QVRKRSRR	Furin optimization
Y65N_S120D_KVKKRSKR	Furin optimization
Y65N_S120D_KVKKRSRR	Furin optimization
Y65N_S120D_KVRKRSKR	Furin optimization
Y65N_S120D_KVRKRSRR	Furin optimization
Y65N_S120D_EVKLRSKR	Furin optimization
Y65N_S120D_EVKLRSRR	Furin optimization
Y65N_S120D_EVRLRSKR	Furin optimization
Y65N_S120D_EVRLRSRR	Furin optimization
N80A	Glycosylation removal
N80C	Glycosylation removal
N80D	Glycosylation removal
N80E	Glycosylation removal
N80F	Glycosylation removal
N80G	Glycosylation removal
N80H	Glycosylation removal
N80I	Glycosylation removal
N80K	Glycosylation removal
N80L	Glycosylation removal
N80M	Glycosylation removal
N80P	Glycosylation removal
N80Q	Glycosylation removal
N80R	Glycosylation removal
N80S	Glycosylation removal
N80T	Glycosylation removal
N80V	Glycosylation removal
N80W	Glycosylation removal
N80Y	Glycosylation removal
A81P	Glycosylation removal
T82A	Glycosylation removal
T82D	Glycosylation removal
T82E	Glycosylation removal
T82F	Glycosylation removal
T82G	Glycosylation removal

Table 23	
Variant	**Property**
T82H	Glycosylation removal
T82I	Glycosylation removal
T82K	Glycosylation removal
T82L	Glycosylation removal
T82M	Glycosylation removal
T82N	Glycosylation removal
T82P	Glycosylation removal
T82Q	Glycosylation removal
T82R	Glycosylation removal
T82V	Glycosylation removal
T82W	Glycosylation removal
T82Y	Glycosylation removal
F93H/R134S/N80A	Glycosylation removal
F93H/R134S/N80C	Glycosylation removal
F93H/R134S/N80D	Glycosylation removal
F93H/R134S/N80E	Glycosylation removal
F93H/R134S/N80F	Glycosylation removal
F93H/R134S/N80G	Glycosylation removal
F93H/R134S/N80H	Glycosylation removal
F93H/R134S/N80I	Glycosylation removal
F93H/R134S/N80K	Glycosylation removal
F93H/R134S/N80L	Glycosylation removal
F93H/R134S/N80M	Glycosylation removal
F93H/R134S/N80P	Glycosylation removal
F93H/R134S/N80Q	Glycosylation removal
F93H/R134S/N80R	Glycosylation removal
F93H/R134S/N80S	Glycosylation removal
F93H/R134S/N80T	Glycosylation removal
F93H/R134S/N80V	Glycosylation removal
F93H/R134S/N80W	Glycosylation removal
F93H/R134S/N80Y	Glycosylation removal
F93H/R134S/A81P	Glycosylation removal
F93H/R134S/T82A	Glycosylation removal
F93H/R134S/T82D	Glycosylation removal
F93H/R134S/T82E	Glycosylation removal
F93H/R134S/T82F	Glycosylation removal
F93H/R134S/T82G	Glycosylation removal
F93H/R134S/T82H	Glycosylation removal
F93H/R134S/T82I	Glycosylation removal
F93H/R134S/T82K	Glycosylation removal
F93H/R134S/T82L	Glycosylation removal
F93H/R134S/T82M	Glycosylation removal
F93H/R134S/T82N	Glycosylation removal
F93H/R134S/T82P	Glycosylation removal
F93H/R134S/T82Q	Glycosylation removal
F93H/R134S/T82R	Glycosylation removal
F93H/R134S/T82V	Glycosylation removal
F93H/R134S/T82W	Glycosylation removal
F93H/R134S/T82Y	Glycosylation removal

[0191] **Example 18.** *Variant designs*

[0192] In addition to the preferred embodiments disclosed in Table 23 above, the following variants are also preferred embodiments of the present invention:

Table 24
L21G_V26G
L21G_V26N
L21G_M23G
L21N_M23G
M23G_V26G
M23G_V26N
21N_23G_26G
A105V
A111D
A111S
A135D
A135E
A135S
A37E
A63E
A63Q
A63R
A63S
A72D
A72E
A72H
A72K
A72N
A72R
A72S
A105V
D119E
D119N
D119S
D119T
D49S
D54K
D54N
D54S
E42D
E42Q
E42R
E42T
E60H
E60K
E60N
E60P
E60Q
E60R
E60R
E60R
E60S
E60T

E70A
E70Q
E97D
E97K
E97R
F117A
F117D
F117E
F117H
F117K
F117Q
F117R
F117S
F117Y
F73A
F73A
F73D
F73E
F73G
F73G
F73H
F73K
F73N
F73Q
F73R
F73S
F73T
F73T
F93A
F93D
F93E
F93G
F93H
F93H_A105V
F93H_A135E
F93H_A72D
F93H_F117Y
F93H_H139R
F93H_I57L
F93H_I94R
F93H_K127E
F93H_K39A
F93H_K39S
F93H_L21G
F93H_L21R
F93H_N130D
F93H_Q108D
F93H_R129D
F93H_R134E

F93H_R134S
F93H_R48N
F93H_S120D
F93H_Y128D
F93H_Y78R
F93K
F93P
F93Q
F93R
F93S
F93T
H139R
I124A
I124D
I124E
I124K
I124N
I124Q
I124R
I124S
I124T
I124V
I57A
I57D
I57E
I57H
I57I
I57K
I57L
I57N
I57P
I57Q
I57T
I57V
I86A
I86D
I86D
I86E
I86K
I86P
I86Q
I86T
I94A
I94E
I94H
I94K
I94K
I94P
I94Q

I94R
I94T
K126D
K126E
K126G
K126Q
K126R
K127A
K127D
K127E
K127E
K127H
K127N
K127P
K127Q
K127S
K127T
K127Y
K39A
K39A
K39D
K39E
K39G
K39N
K39R
K39S
K39S_A135E
K39S_A135S
K39S_A72D
K39S_F117H
K39S_F117Y
K39S_F93S
K39S_H139R
K39S_I57L
K39S_I86A
K39S_I94R
K39S_L21G
K39S_L21R
K39S_M23R
K39S_N110D
K39S_N130D
K39S_Q108D
K39S_Q53G
K39S_Q53T
K39S_R129D
K39S_R134E
K39S_R134S
K39S_R48N
K39S_S120D

K39S_S120D_A72D
K39S_S120D_F93H
K39S_S120D_F93S
K39S_S120D_H139R
K39S_S120D_L21G
K39S_S120D_M23R
K39S_S120D_Q108D
K39S_S120D_R129D
K39S_S120D_R134E
K39S_S120D_Y65N
K39S_S120D_Y78H
K39S_Y116H
K39S_Y128D
K39S_Y65N
K39S_Y78R
K39T
L115A
L115E
L115K
L115Q
L115T
L125A
L125E
L125K
L125P
L125Q
L125T
L125Y
L21D
L21E
L21G
L21G
L21H
L21K
L21N
L21N_M23G_V26N
L21R
L21S
L90A
L90E
L90G
L90H
L90K
L90L
L90N
L90P
L90P
L90Q
L90R

L90R
L90R
L90S
L90T
M23D
M23G
M23K
M23N
M23R
M23S
N110D
N110E
N110H
N122E
N122Q
N122R
N130D
N76A
N76D
N76N
N76S
N76T
N76Y
N83P
N95D
N95K
N95Q
N95R
Q108D
Q108K
Q108S
Q36E
Q36N
Q53A
Q53D
Q53E
Q53G
Q53H
Q53K
Q53R
Q53S
Q53T
Q53T
Q88E
R129D
R129E
R129K
R129N
R129S

R134D
R134E
R134K
R134L
R134P
R134P
R134Q
R134R
R134R
R134S
R48D
R48E
R48H
R48N
R48Q
S113D
S113E
S120D
S120E
S120N
S120R
S121D
S121E
S121K
S121N
S121T
S77A
S77A
S77A
S77D
S77E
S77H
S77K
S77N
S77P
S77P
S77Q
S77T
S77T
T107D
T107E
T98A
T98Del
T98E
T98E
T98K
V123A
V123A
V123D

V123G
V123G
V123N
V123N
V123R
V123T
V123V
V26D
V26E
V26G
V26K
V26K
V26N
V26S
V26V
W52A
W52E
W52K
W52P
W52Q
W52T
W55A
W55A
W55E
W55H
W55K
W55N
W55P
W55Q
W55R
W55T
Y116A
Y116D
Y116E
Y116H
Y116K
Y116Q
Y116S
Y116T
Y116Y
Y128D
Y128E
Y128H
Y128K
Y128Q
Y44A
Y44D
Y44E
Y44G

Y44H
Y44K
Y44N
Y44P
Y44Q
Y44R
Y44S
Y44T
Y65D
Y65E
Y65N
Y65N_A105V
Y65N_A135E
Y65N_A135S
Y65N_F117Y
Y65N_F93H
Y65N_F93T
Y65N_H139R
Y65N_I57L
Y65N_I94R
Y65N_K127E
Y65N_K39A
Y65N_K39S
Y65N_L21G
Y65N_L21R
Y65N_M23N
Y65N_M23R
Y65N_Q108D
Y65N_Q53D
Y65N_Q53G
Y65N_Q53S
Y65N_Q53T
Y65N_R129D
Y65N_R129D_F117H
Y65N_R129D_F117Y
Y65N_R129D_F93H
Y65N_R129D_F93S
Y65N_R129D_F93T
Y65N_R129D_K39A
Y65N_R129D_K39S
Y65N_R129D_L21G
Y65N_R129D_M23R
Y65N_R129D_Q108D
Y65N_R129D_S120D
Y65N_R129D_Y78H
Y65N_R134E
Y65N_R134S
Y65N_S120D
Y65N_Y128D

Y65N_Y78H
Y65N_Y78R
Y78A
Y78D
Y78G
Y78H
Y78H_A105V
Y78H_A135E
Y78H_A135S
Y78H_A63S
Y78H_A72D
Y78H_F117H
Y78H_F117Y
Y78H_F93H
Y78H_F93H_A72D
Y78H_F93H_F117H
Y78H_F93H_F117Y
Y78H_F93H_H139R
Y78H_F93H_K39A
Y78H_F93H_K39S
Y78H_F93H_L21G
Y78H_F93H_M23R
Y78H_F93H_Q108D
Y78H_F93H_R129D
Y78H_F93H_R134S
Y78H_F93H_S120D
Y78H_F93H_Y65N
Y78H_F93T
Y78H_H139R
Y78H_I57L
Y78H_I94R
Y78H_K127E
Y78H_K39S
Y78H_L21R
Y78H_N110D
Y78H_N130D
Y78H_Q108D
Y78H_Q53G
Y78H_Q53S
Y78H_R129D
Y78H_R134E
Y78H_R134E_A72D
Y78H_R134E_F117Y
Y78H_R134E_F93H
Y78H_R134E_F93S
Y78H_R134E_F93T
Y78H_R134E_K39A
Y78H_R134E_K39S
Y78H_R134E_L21G

Y78H_R134E_M23R
Y78H_R134E_Q108D
Y78H_R134E_S120D
Y78H_R134E_Y65N
Y78H_R134S
Y78H_R48N
Y78H_S120D
Y78H_Y116H
Y78H_Y128D
Y78N
Y78P
Y78R
Y78S
Y78T
Y78Y

[0193] While the foregoing invention has been described above, it will be clear to one skilled in the art that various changes and additional embodiments made be made without departing from the scope of the invention. All publications, patents, patent applications (provisional, utility and PCT) or other documents cited herein are incorporated by references in their entirety.

CLAIMS

We Claim:

1. A variant BMP-7 protein comprising the sequence:

Fx(1-20)-Vb(21)-Fx(22-38)-Vb(39)-Fx(40-64)-Vb(65)-Fx(66-71)-Vb(72)-Fx(73-77)-Vb(78)-Fx(79-92)-Vb(93)-Fx(94-119)-Vb(120)-Fx(121-134)-Vb(135)

wherein

Fx1(1-20) corresponds to amino acid residues 1-20 of human BMP-7 (SEQ ID NO:5);

Vb(21) is selected from the group consisting of L and G;

Fx(22-38) corresponds to amino acid residues 22-38 of human BMP-7 (SEQ ID NO:5);

Vb(39) is selected from the group consisting of K, A and S;

Fx(40-64) corresponds to amino acid residues 40-64 of human BMP-7 (SEQ ID NO:5);

Vb(65) is selected from the group consisting of Y and N;

Fx(66-71) corresponds to amino acid residues 66-71 of human BMP-7 (SEQ ID NO:5);

Vb(72) is selected from the group consisting of A and D;

Fx(73-77) corresponds to amino acid residues 73-77 of human BMP-7 (SEQ ID NO:5);

Vb(78) is selected from the group consisting of Y and H;

Fx(79-92) corresponds to amino acid residues 79-92 of human BMP-7 (SEQ ID NO:5);

Vb(93) is selected from the group consisting of F, H, S and T;

Fx(94-119) corresponds to amino acid residues 94-119 of human BMP-7 (SEQ ID NO:5);

Vb(120) is selected from the group consisting of S and D;

Fx(121-134) corresponds to amino acid residues 121-134 of human BMP-7 (SEQ ID NO:5);

Vb(135) is selected from the group consisting of A and E;

wherein said variant comprises an amino acid substitution as compared to human BMP-7 (SEQ ID NO:5).

2. The BMP-7 variant protein of claim 1, wherein a substitution is L21G.

3. The BMP-7 variant protein of claim 1, wherein a substitution is K39A.

4. The BMP-7 variant protein of claim 1, wherein a substitution is K39S.

5. The BMP-7 variant protein of claim 1, wherein a substitution is Y65N.

6. The BMP-7 variant protein of claim 1, wherein a substitution is A72D.

7. The BMP-7 variant protein of claim 1, wherein a substitution is Y78H.

8. The BMP-7 variant protein of claim 1, wherein a substitution is F93H.

9. The BMP-7 variant protein of claim 1, wherein a substitution is F93S.

10. The BMP-7 variant protein of claim 1, wherein a substitution is F93T.

11. The BMP-7 variant protein of claim 1, wherein a substitution is S120D.

12. The BMP-7 variant protein of claim 1, wherein a substitution is A135E.

13. The BMP-7 variant protein of claim 1 comprising at least one set of substitutions selected from the group consisting of: K39S-F93S; K39S-S120D; K39S-S120D-Y65N; K39S-S120D-A72D; K39S-S120D-Y78H; K39S-S120D-F93H; K39S-S120D-F93S; Y65N-L21G; Y65N-L21R; Y65N-K39S; Y65N-Y78H; Y65N-S120D; Y78H–A72D; Y78H-F93H-Y65N; Y78H-F93H-A72D; Y78H-F93H-S120D; Y78H-S120D and F93H-K39S.

14. A variant BMP-7 protein comprising a substitution as compared to human BMP-7 (SEQ ID NO: 5) selected from the group consisting of: L21G, K39A, K39S, Y65N, A72D, Y78H, F93H, F93S, F93T, S120D and A135E.

15. The BMP-7 variant protein of claim 1, wherein a substitution is L21G.

16. The BMP-7 variant protein of claim 1, wherein a substitution is K39A.

17. The BMP-7 variant protein of claim 1, wherein a substitution is K39S.

18. The BMP-7 variant protein of claim 1, wherein a substitution is Y65N.

19. The BMP-7 variant protein of claim 1, wherein a substitution is A72D.

20. The BMP-7 variant protein of claim 1, wherein a substitution is Y78H.

21. The BMP-7 variant protein of claim 1, wherein a substitution is F93H.

22. The BMP-7 variant protein of claim 1, wherein a substitution is F93S.

23. The BMP-7 variant protein of claim 1, wherein a substitution is F93T.

24. The BMP-7 variant protein of claim 1, wherein a substitution is S120D.

25. The BMP-7 variant protein of claim 1, wherein a substitution is A135E.

26. The BMP-7 variant protein of claim 14 comprising at least one set of substitutions selected from the group consisting of: K39S-F93S; K39S-S120D; K39S-S120D-M23R; K39S-S120D-Y65N; K39S-S120D-A72D; K39S-S120D-Y78H; K39S-S120D-F93H; K39S-S120D-F93S; K39S-S120D-Q108D; K39S-S120D-R129D; K39S-S120D-R134E; K39S-S120D-H139R; K39S-N130D; K39S-R134S; Y65N-L21G; Y65N-L21R; Y65N-K39S; Y65N-Y78H; Y65N-A105V; Y65N-S120D; Y78H-A72D; Y78H-F93H-Y65N; Y78H-F93H-A72D; Y78H-F93H-Q108D; Y78H-F93H-F117H; Y78H-F93H-S120D; Y78H-S120D; Y78H-R134E-L21G; Y78H-R134E-M23R; Y78H-R134E-Y65N; Y78H-R134E-A72D; F93H-K39S; and, F93H-R134S.

27. A variant BMP-7 protein having altered receptor binding affinity compared to wild-type BMP-7 (SEQ ID NO:5), said variant BMP-7 protein comprising a substitution selected from the group consisting of: M23N, Q53G, Q53H and I86D.

Figure 1

```
Cons:    CxxxxLYVxFxDxGWxDWIIAPxGYxAxYCxGxCxFPLxxxxN----xTNHAIxQ
BMP-2:   CKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLADHLN----STNHAIVQ
BMP-3:   CARRYLKVDFADIGWSEWIISPKSFDAYYCSGACQFPMPKSLK----PSNHATIQ
BMP-3b:  CSRRYLKVDFADIGWNEWIISPKSFDAYYCAGACEFPMPKIVR----PSNHATIQ
BMP-4:   CRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLN----STNHAIVQ
BMP-5:   CKKHELYVSFRDLGWQDWIIAPEGYAAFYCDGECSFPLNAHMN----ATNHAIVQ
BMP-6:   CRKHELYVSFQDLGWQDWIIAPKGYAANYCDGECSFPLNAHMN----ATNHAIVQ
BMP-7:   CKKHELYVSFRDLGWQDWIIAPEGYAAYYCEGECAFPLNSYMN----ATNHAIVQ
BMP-8:   CRRHELYVSFQDLGWLDWVIAPQGYSAYYCEGECSFPLDSCMN----ATNHAILQ
BMP-9:   CQKTSLRVNFEDIGWDSWIIAPKEYEAYECKGGCFFPLADDVT----PTKHAIVQ
BMP-10:  CKRTPLYIDFKEIGWDSWIIAPPGYEAYECRGVCNYPLAEHLT----PTKHAIIQ
GDF-1:   CRARRLYVSFREVGWHRWVIAPRGFLANYCQGQCALPVALSGSGGPPALNHAVLR
GDF-3:   CHRHQLFINFRDLGWHKWIIAPKGFMANYCHGECPFSLTISLN----SSNYAFMQ
GDF-5:   CSRKALHVNFKDMGWDDWIIAPLEYEAFHCEGLCEFPLRSHLE----PTNHAVIQ
GDF-7:   CSRKPLHVDFKELGWDDWIIAPLDYEAYHCEGLCDFPLRSHLE----PTNHAIIQ
GDF-8:   CCRYPLTVDFEAFGWD-WIIAPKRYKANYCSGECEFVFLQKY-----PHTHLVHQ
BMP-11:  CCRYPLTVDFEAFGWD-WIIAPKRYKANYCSGQCEYMFMQKY-----PHTHLVQQ
BMP-15:  CSLHPFQISFRQLGWDHWIIAPPFYTPNYCKGTCLRVLRDGLN----SPNHAIIQ
BMP-16:  CRKVKFQVDFNLIGWGSWIIYPKQYNAYRCEGECPNPVGEEFH----PTNHAYIQ

Cons:    TLVxxxxxxxxxPKxCCxPTxLxAxSxLYxDxxxxVxLx-xYxxMxVxxCGCx
BMP-2:   TLVNSVN--SKIPKACCVPTELSAISMLYLDENEKVVLK-NYQDMVVEGCGCR
BMP-3:   SIVRAVGVVPGIPEPCCVPEKMSSLSILFFDENKNVVLK-VYPNMTVESCACR
BMP-3b:  SIVRAVGIIPGIPEPCCVPDKMNSLGVLFLDENRNVVLK-VYPNMSVDTCACR
BMP-4:   TLVNSVN--SSIPKACCVPTELSAISMLYLDEYDKVVLK-NYQEMVVEGCGCR
BMP-5:   TLVHLMFP-DHVPKPCCAPTKLNAISVLYFDDSSNVILK-KYRNMVVRSCGCH
BMP-6:   TLVHLMNP-EYVPKPCCAPTKLNAISVLYFDDNSNVILK-KYRNMVVRACGCH
BMP-7:   TLVHFINP-DTVPKPCCAPTQLNAISVLYFDDSSNVILK-KYRNMVVRACGCH
BMP-8:   SLVHLMKP-NAVPKACCAPTKLSATSVLYYDSSNNVILR-KHRNMVVKACGCH
BMP-9:   TLVHLKFP-TKVGKACCVPTKLSPISVLYKDDMGVPTLKYHYEGMSVAECGCR
BMP-10:  ALVHLKNS-QKASKACCVPTKLEPISILYL-DKGVVTYKFKYEGMAVSECGCR
GDF-1:   ALMHAAAP-GAADLPCCVPARLSPISVLFFDNSDNVVLR-QYEDMVVDECGCR
GDF-3:   ALMHAVDP-E-IPQAVCIPTKLSPISMLYQDNNDNVILR-HYEDMVVDECGCG
GDF-5:   TLMNSMDP-ESTPPTCCVPTRLSPISILFIDSANNVVYK-QYEDMVVESCGCR
GDF-7:   TLLNSMAP-DAAPASCCVPARLSPISILYIDAANNVVYK-QYEDMVVEACGCR
GDF-8:   A--NPRG--SAGP--CCTPTKMSPINMLYFNGKEQIIYG-KIPAMVVDRCGCS
BMP-11:  A--NPRG--SAGP--CCTPTKMSPINMLYFNDKQQIIYG-KIPGMVVDRCGCS
BMP-15:  NLINQLVD-QSVPRPSCVPYKYVPISVLMIEANGSILYK-EYEGMIAESCTCR
BMP-16:  SLLKRYQP-HRVPSTCCAPVKTKPLSMLYVDNG-RVLLDH-HKDMIVEECGCL
```

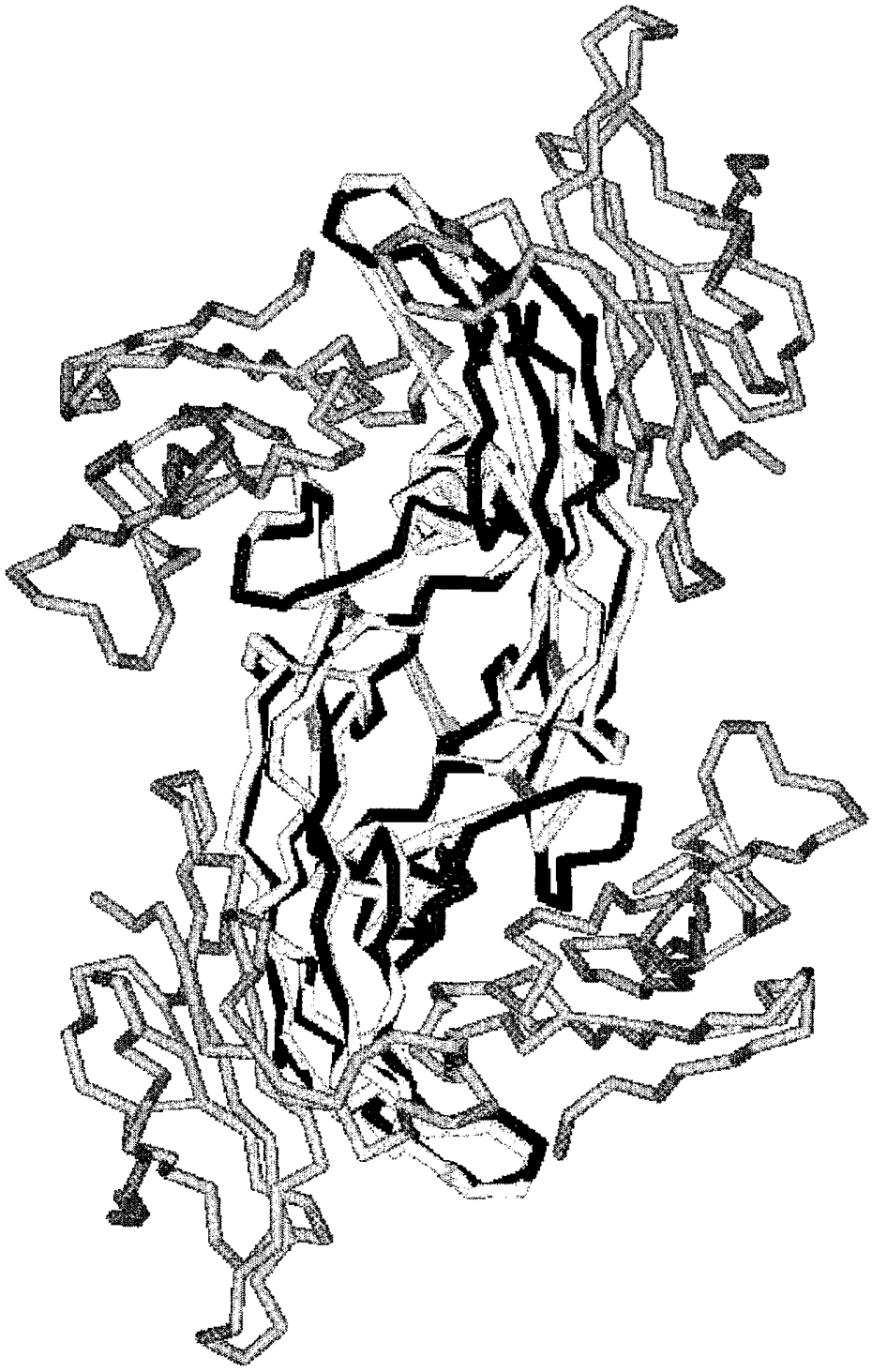

Figure 2

Figure 3

```
ALK2   : lymcvceglscgned---hcegq-qcfsslsindgfh-vyqkgcfqvyeqgkmtc
ALK3   : FLKCYCSG-HCPDDAINNTCITNGHCFAIIEEDDQGETTLASGCLGLEGS-DFQC
ALK6   : vlrckchh-hcpedsvnnicstdgycftmieeddsglpvvtsgCMKYEGS-DFQC

ALK2   : ktppspg--qaveccqg-dwcnrnitaql
ALK3   : KDSPKAQLRRTIECCRT-NLCNQYLQPTL
ALK6   : rdtpiphqrrsieccternecnkdlhptl

ActRIIa : ETQECLFFNANWEKDRTNQTGVEPCYGDKDKRRHCFATWKNISGSIEIVKQGCW
ActRIIb : ETRECIYYNANWELERTNQsGlErCeGeqDKRlHCyAsWaNsSGtIElVKkGCW
BMPRII  : fkdpyqqdlgigesrishengtilcskgst----cyglwekskgdinlvkqgcw

ActRIIa : ---LDDINCYDRTDCvEKKDSP-----EVYFCCCEGNMCNEKFSYFP
ActRIIb : ---LDDfNCYDRqeCvateenP-----qVYFCCCEGNfCNErFthlP
BMPRII  : shigdpqechy-eecvvtttppsiqngtyrfcccstdlcnvnftenf
```

Figure 4

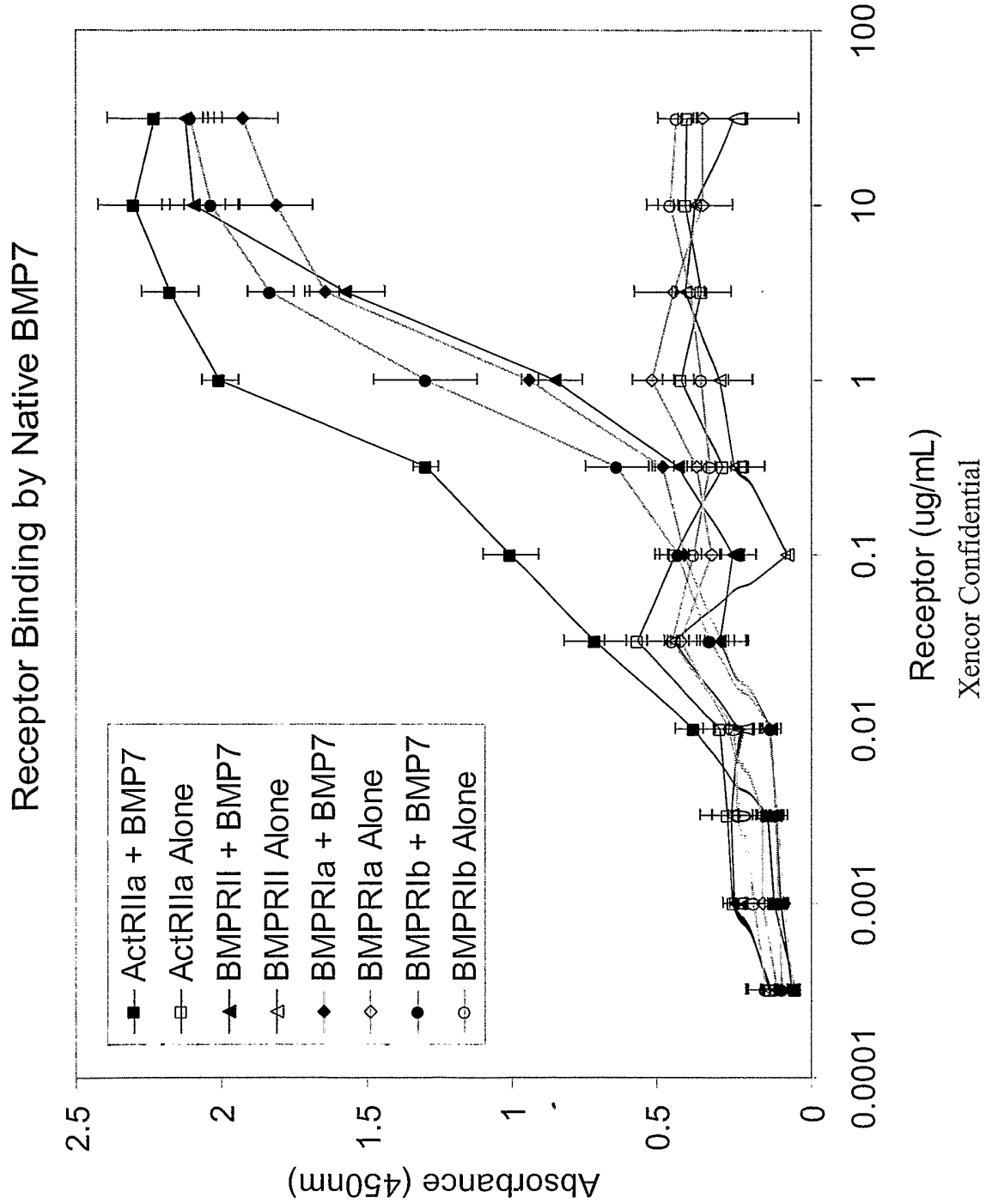

Figure 5

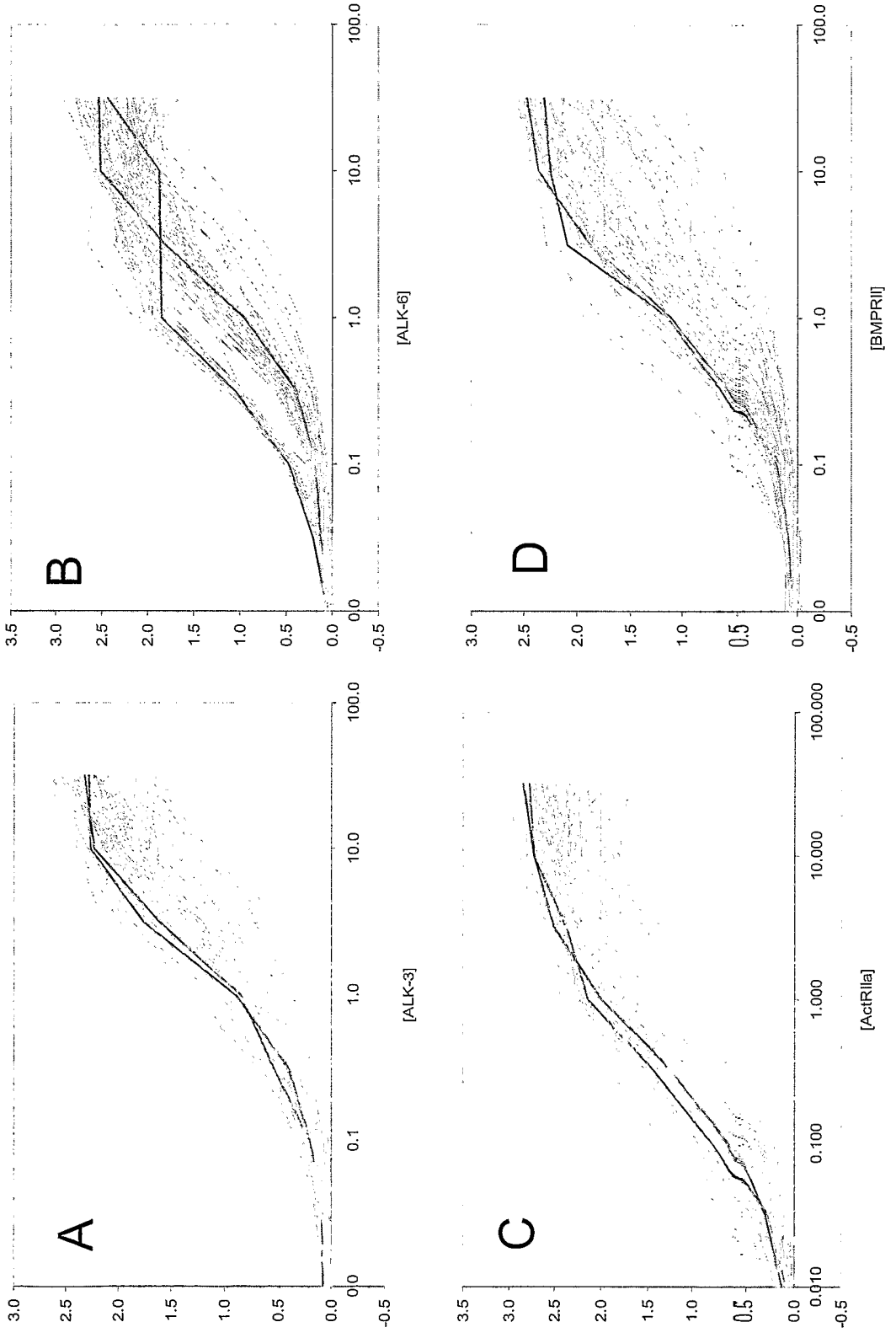

Figure 6

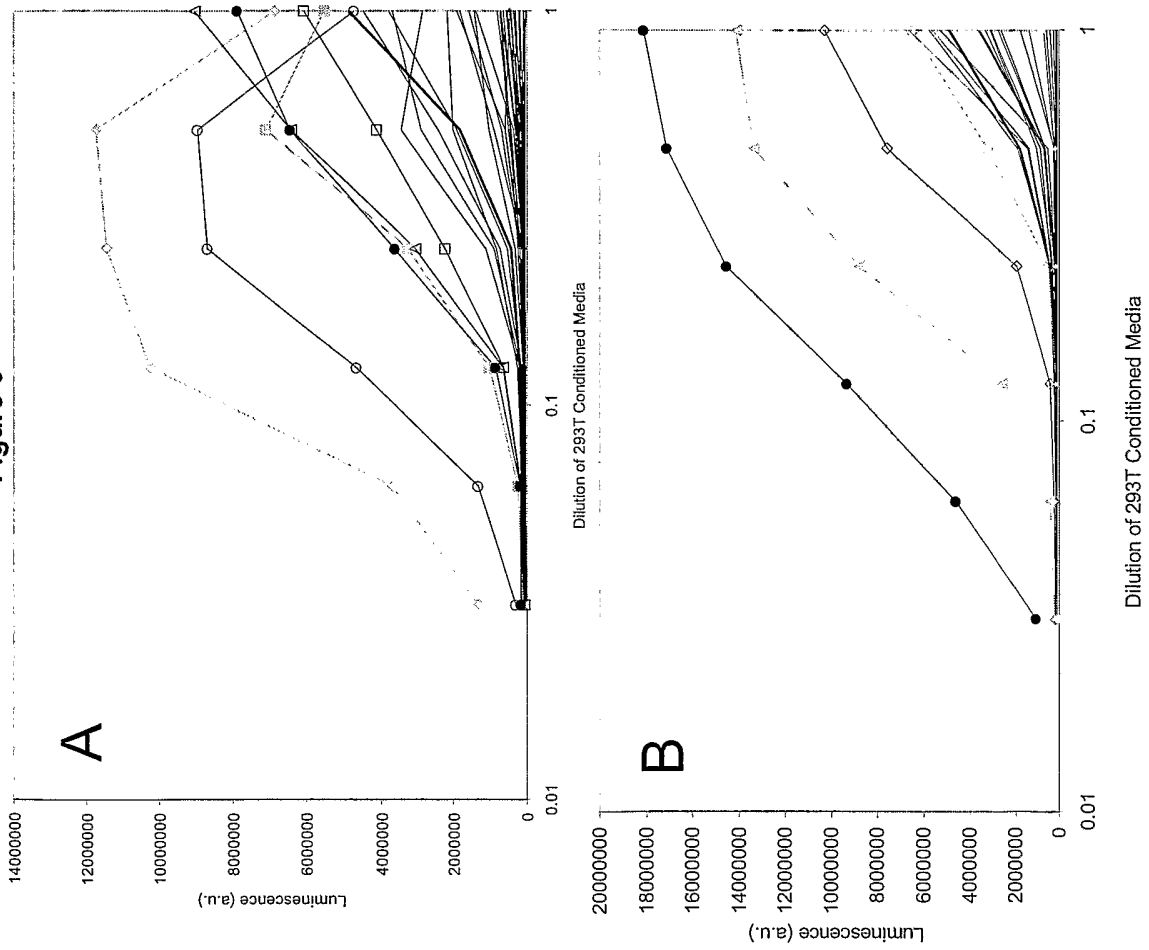

Figure 7

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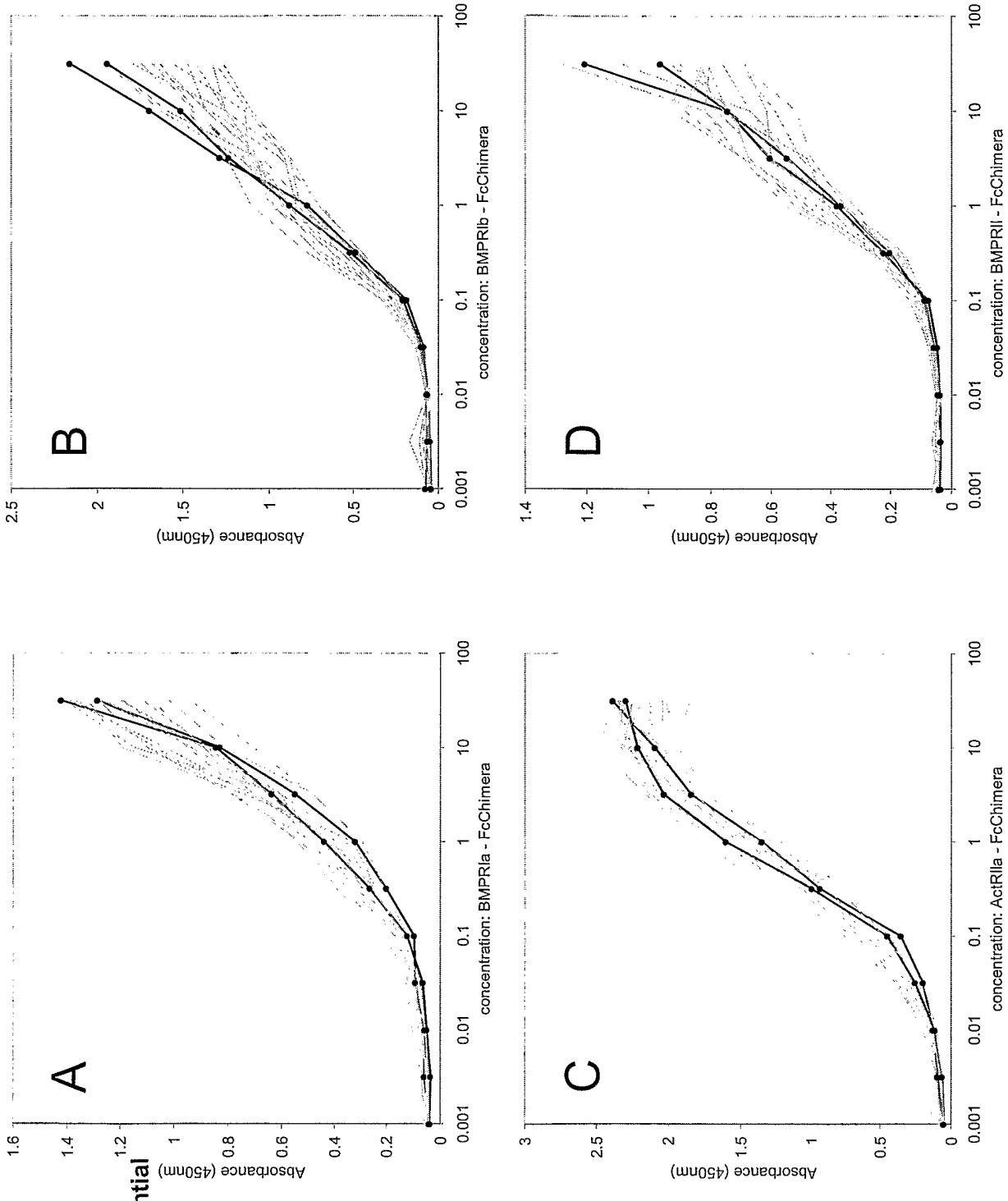


Figure 8

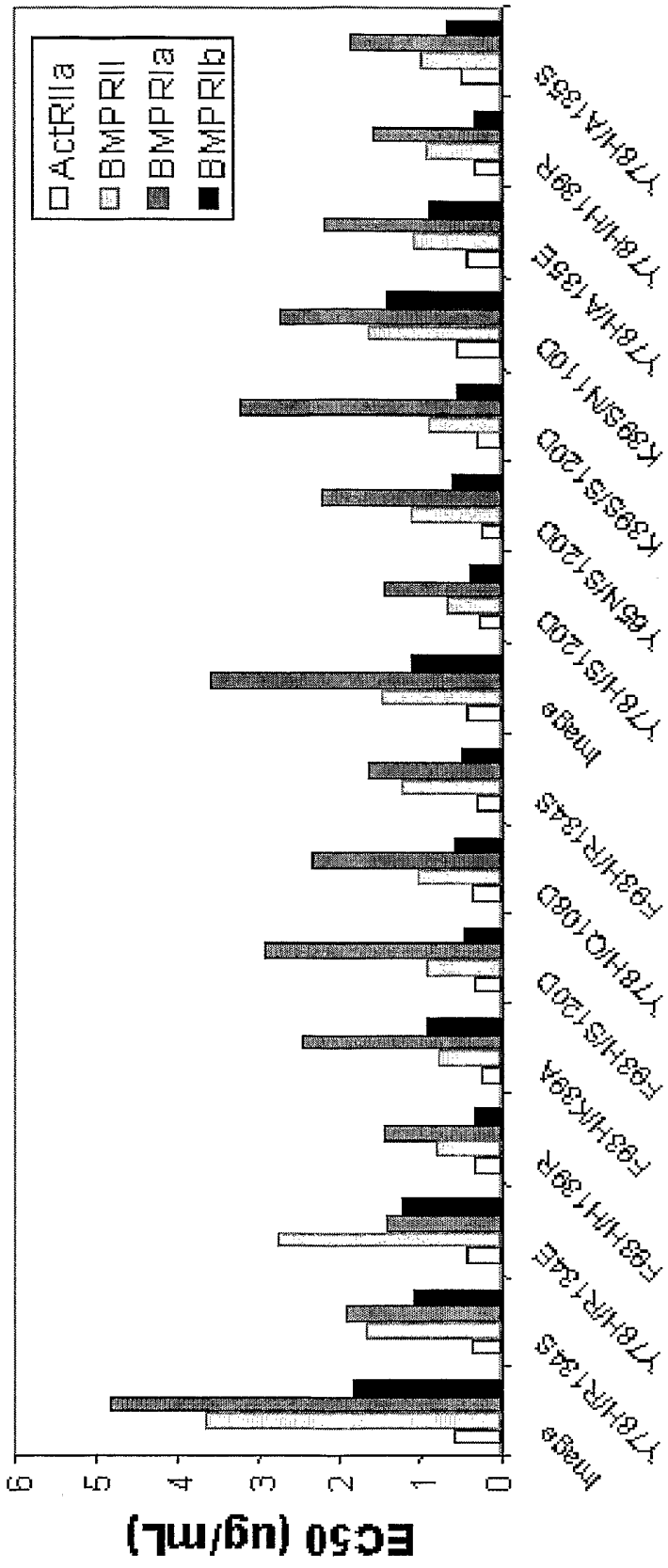


Figure 9

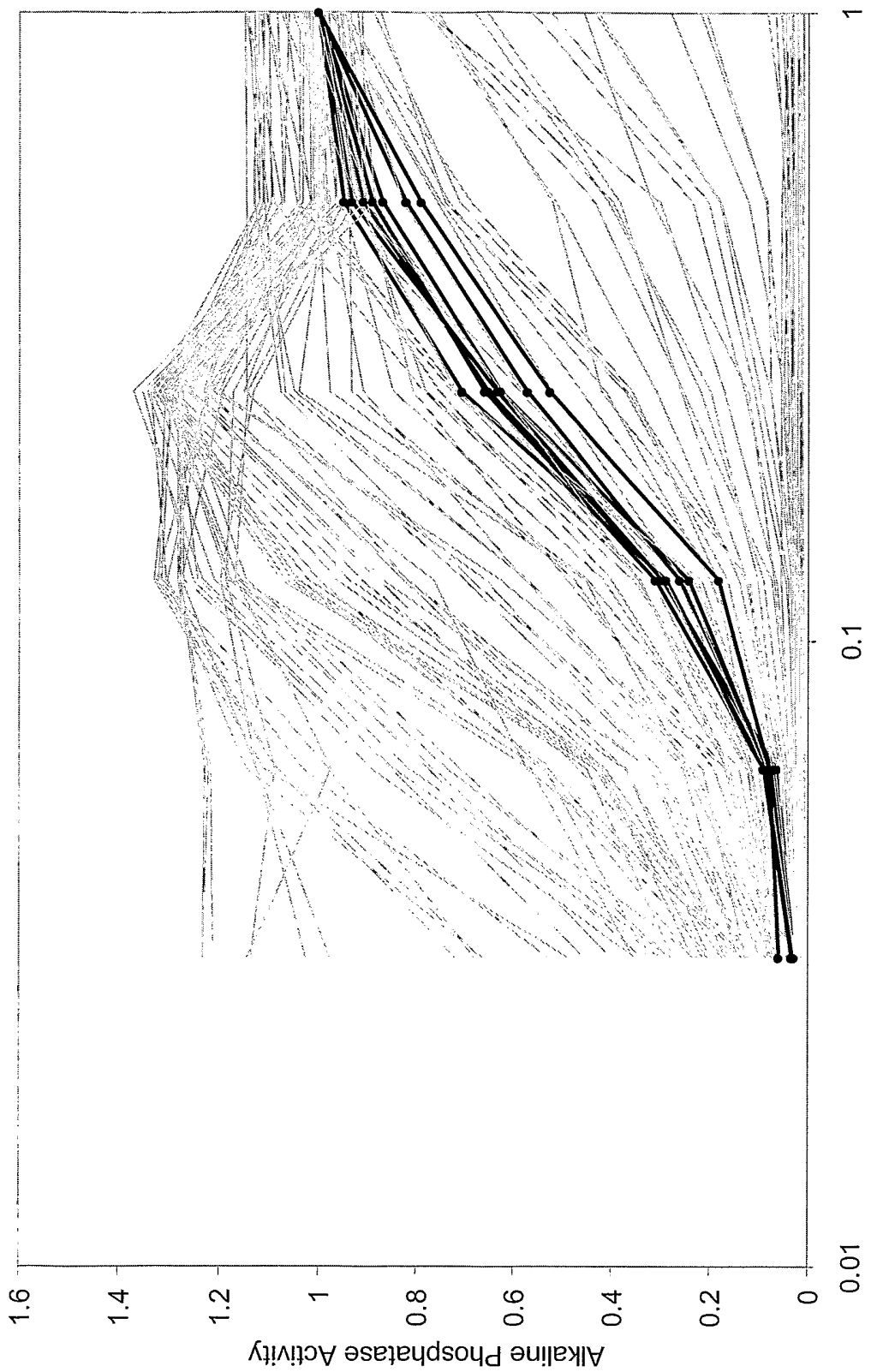
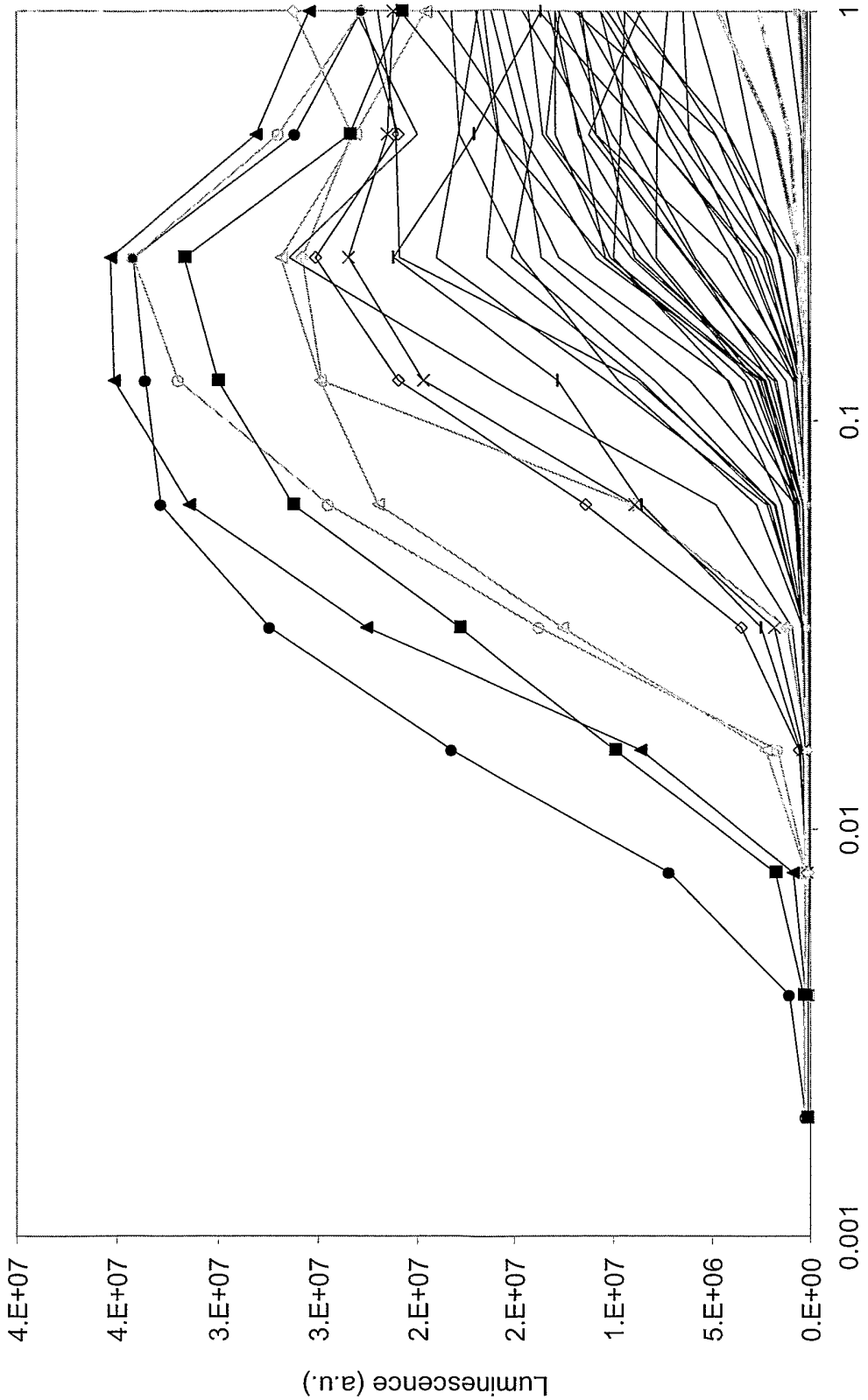


Figure 10



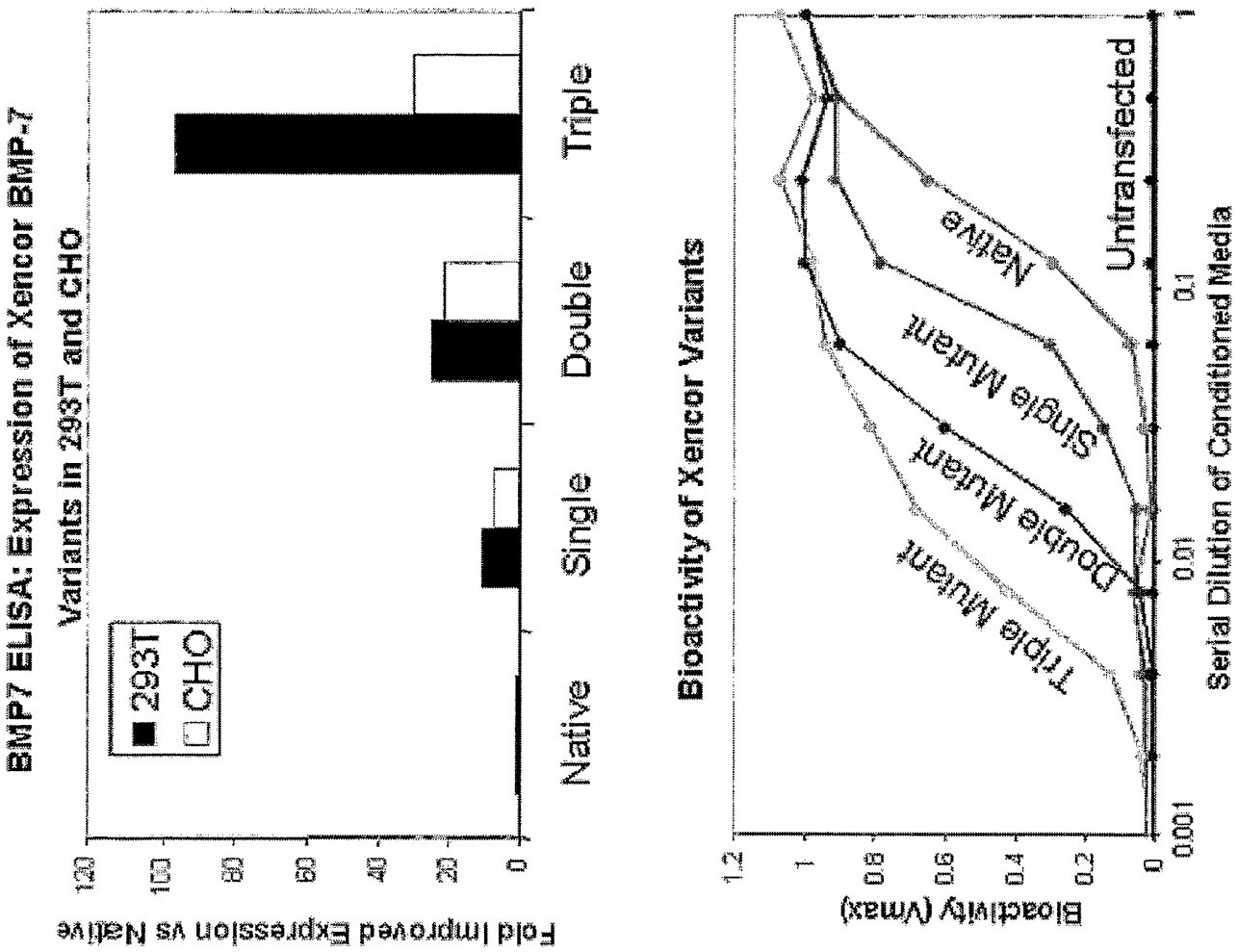
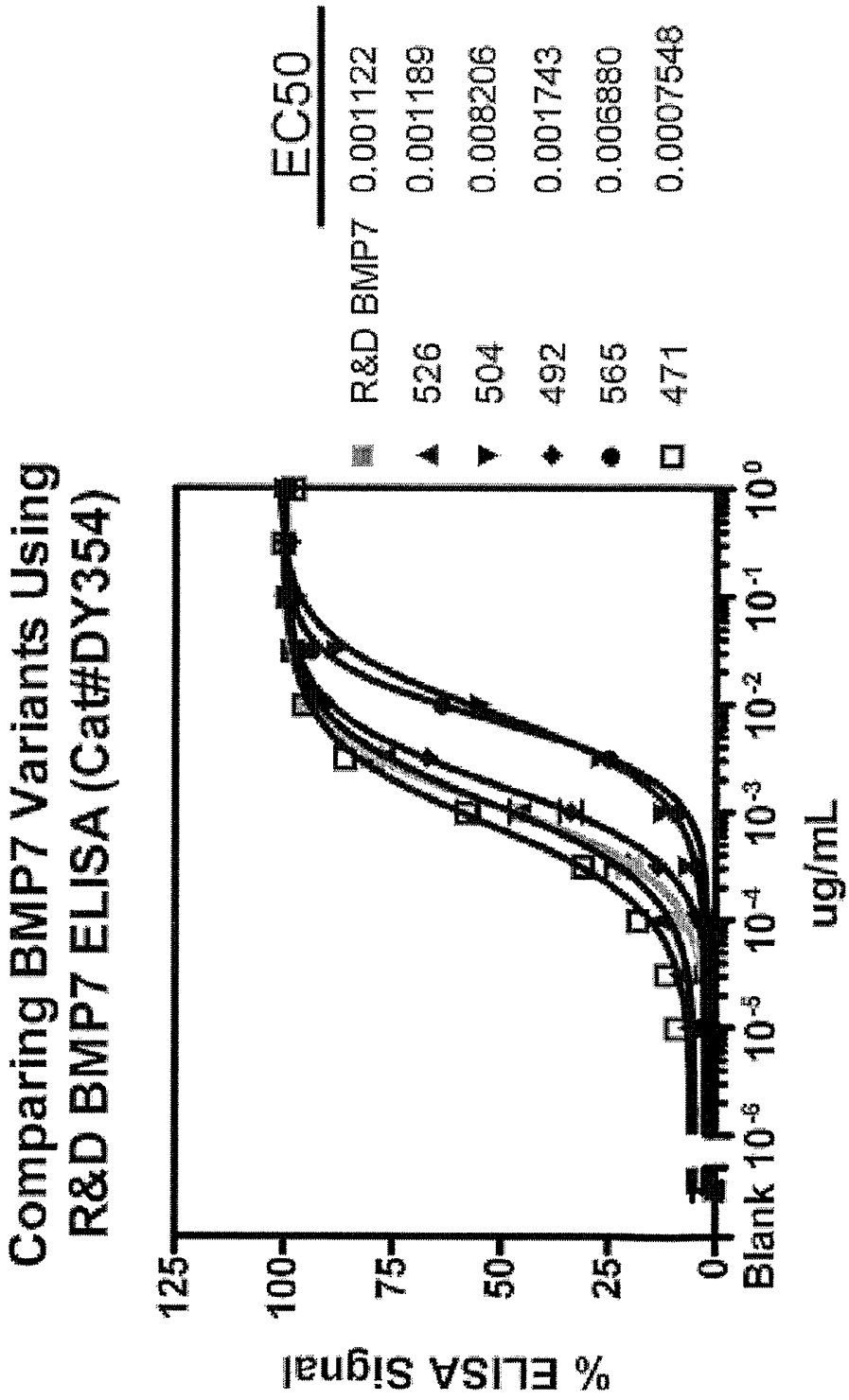


Figure 11
Xencor Confidential

Figure 12



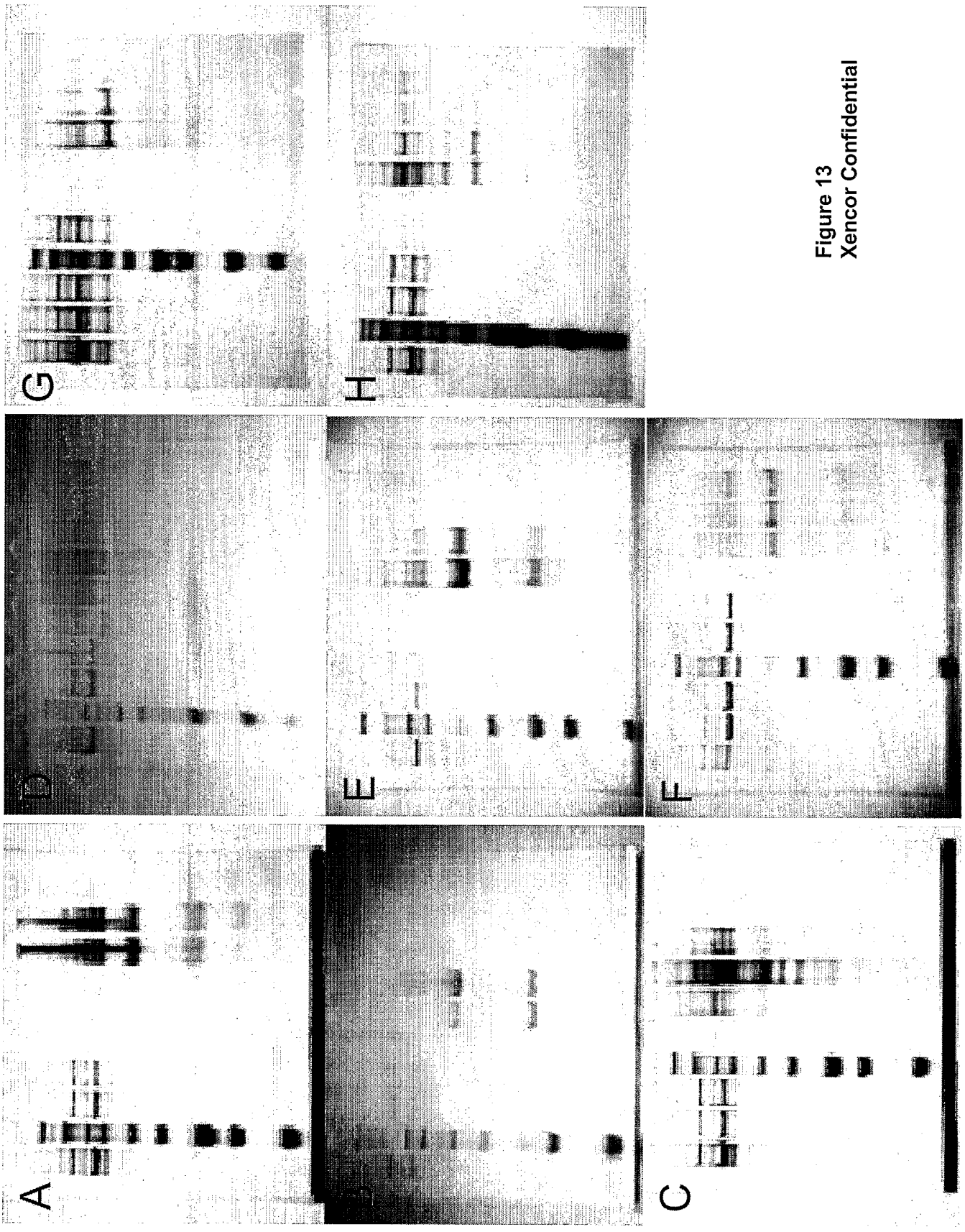


Figure 13
Xencor Confidential

Figure 14
Xencor Confidential

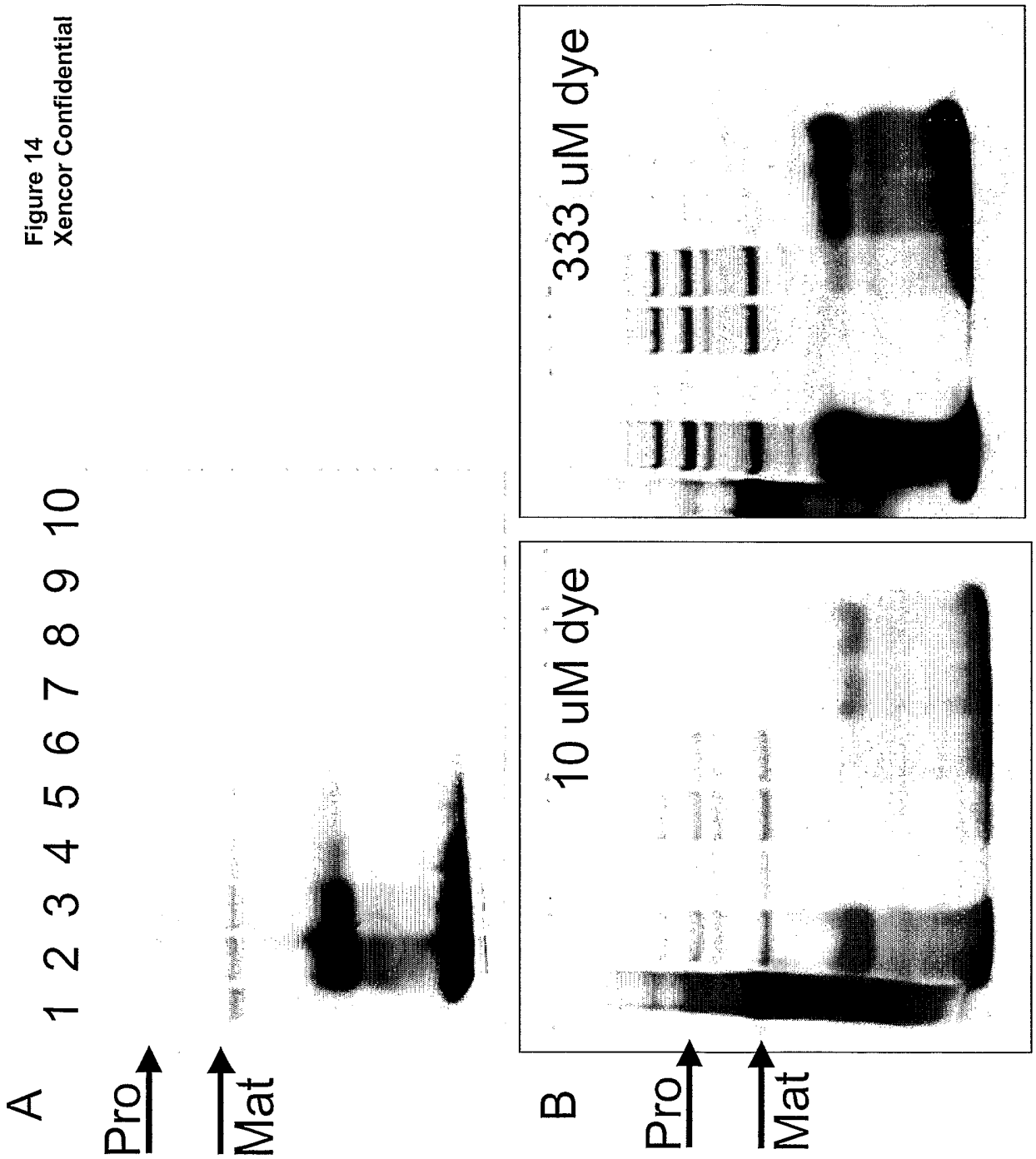


Figure 15

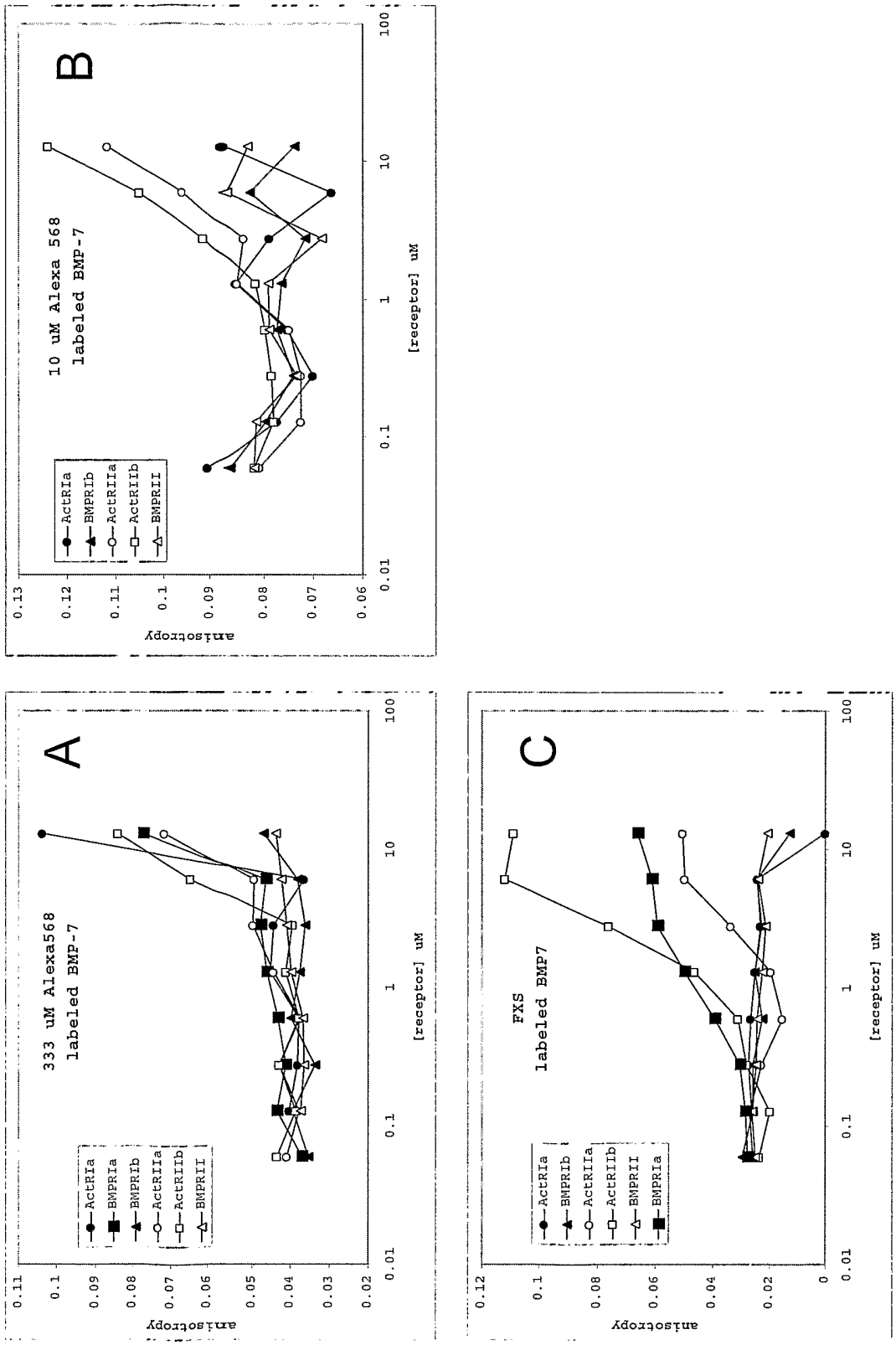
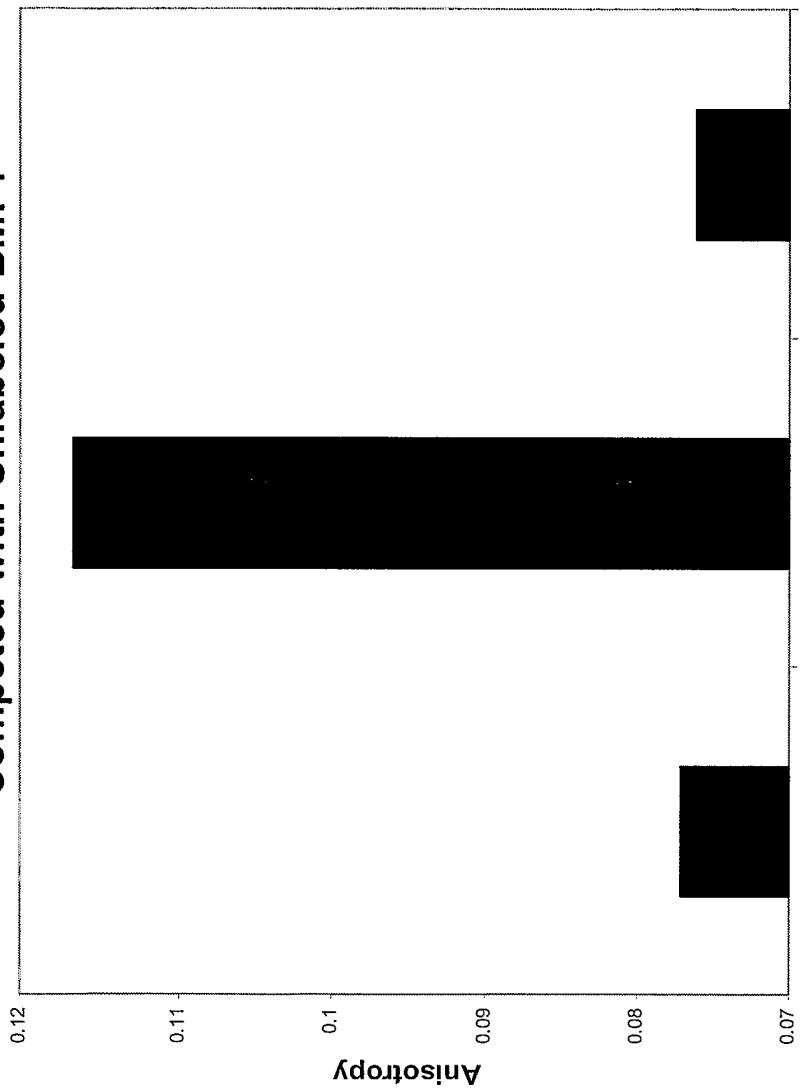


Figure 16



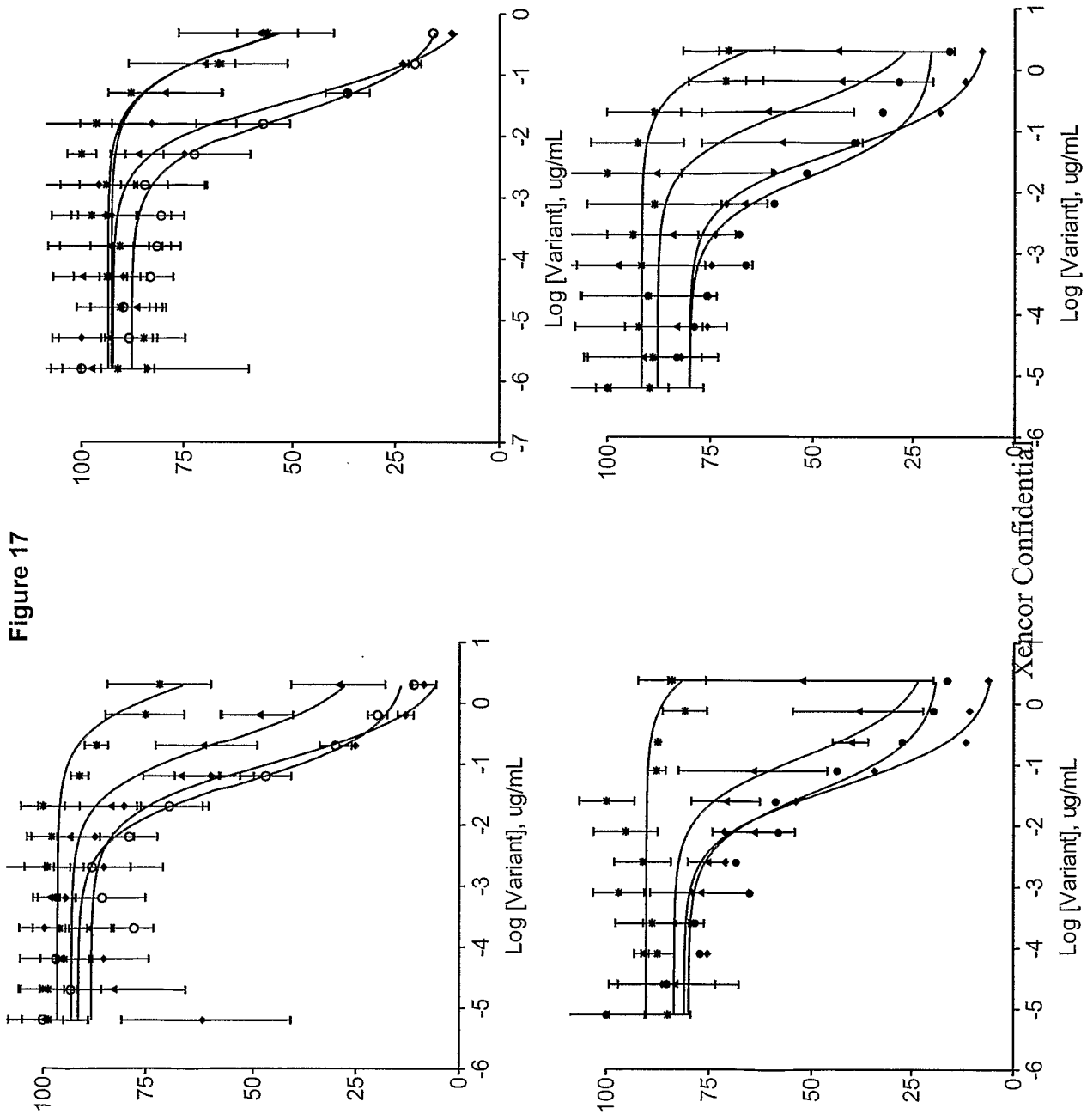
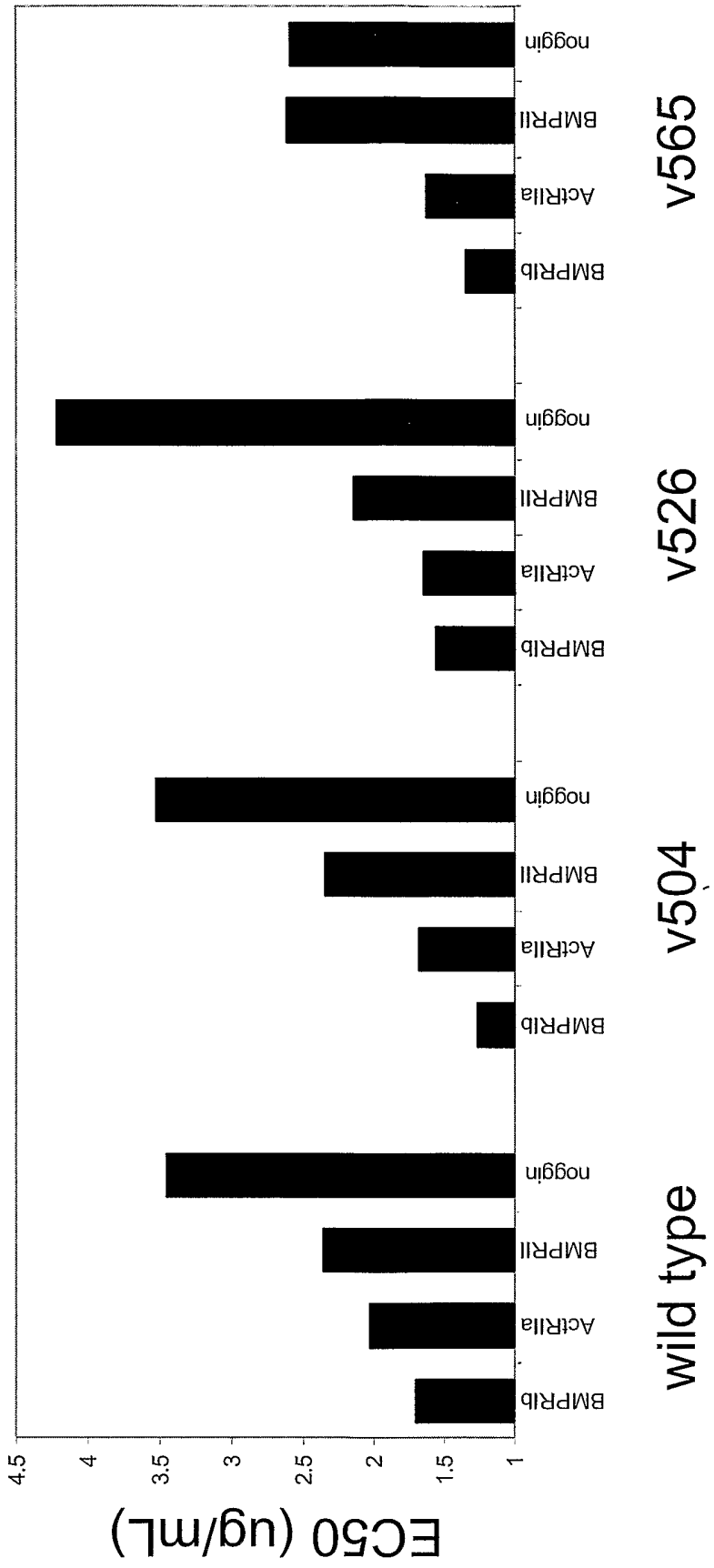


Figure 17

Figure 18



Sequence List

SEQUENCE ID NO: 1 Mature human BMP-2
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GWNDWIVAPPGYHAFYCHGECPFPLA
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PTELSAISMLYLDENEKVVLKNYQDMV
VEGCGCR

SEQUENCE ID NO: 2 Mature human BMP-4
SPKHHSQRARKKNKNCRRHSLYVDFS
DVGWNDWIVAPPGYQAFYCHGDCPFP
LADHLNSTNHAIVQTLVNSVNSSIPKAC
CVPTELSAISMLYLDEYDKVVLKNYQE
MVVEGCGCR

SEQUENCE ID NO: 3 Mature human BMP-5
AANKRKNQNRNKSSSHQDSSRMSSV
GDYNTSEQKQACKKHELYVSFRDLGW
QDWIIAPEGYAAFYCDGECSFPLNAHM
NATNHAIVQTLVHLMFPDHVPKPCCAP
TKLNAISVLYFDDSSNVILKKYRNMVVR
SCGCH

SEQUENCE ID NO: 4 Mature human BMP-6
SASSRRRQQSRNRSTQSQDVARVSSA
SDYNSSELKTACRKHELYVSFQDLGW
QDWIIAPKGYAANYCDGECSFPLNAHM
NATNHAIVQTLVHLMNPEYVPKPCCAP
TKLNAISVLYFDDNSNVILKKYRNMVVR
ACGCH

SEQUENCE ID NO: 5 Mature human BMP-7
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AENSSSDQRQACKKHELYVSFRDLGW
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NATNHAIVQTLVHFINPETVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 6 Mature human BMP-8
AVRPLRRRQPKKSNELPQANRLPGIFD
DVHGSHGRQVCRRHELYVSFQDLGW
LDWVIAPQGYSAYYCEGECSFPLDSC
MNATNHAILQSLVHLMKPNAVPKACCA
PTKLSATSVLYYDSSNNVILRKHRNMV
VKACGCH

SEQUENCE ID NO: 7 Human ActRIa / ALK-2 ligand binding domain
LYMCVCEGLSCGNEDHCEGQQCFSSL
SINDGFHVYQKGCFQVYEQGKMTCKT
PPSPGQAVECCQGDWCNRNITAQL

SEQUENCE ID NO: 8 Human BMPRIa / ALK-3 ligand binding domain
FLKCYCSGHCPDDAINNTCITNGHCFAI
IEEDDQGETTLASGCMKYEGSDFQCK
DSPKAQLRRTIECCRTNLCNQYLQPTL

SEQUENCE ID NO: 9 Human BMPRIb / ALK-6 ligand binding domain
VLRCKCHHHCPEDSVNNICSTDGYCFT
MIEEDDSGLPVVTSGCLGLEGSDFQCR
DTPIPHQRRSIECCTERNECNKDLHPT
L

SEQUENCE ID NO: 10 Human ActRIIa ligand binding domain
ETQECLFFNANWEKDRTNQTGVEPCY
GDKDKRRHCFATWKNISGSIEIVKQGC
WLDDINCYDRTDCVEKKDSPEVYFCCC
EGNMCNEKFSYFP

SEQUENCE ID NO: 11 Human ActRIIb ligand binding domain
ETRECIYYNANWELERTNQSGIERCEG
EQDKRIHCYASWANSSGTIEIVKKGCW
LDDFNCYDRQECVATEENPQVYFCCC
EGNFCNERFTHIP

SEQUENCE ID NO: 12 Human BMPRII ligand binding domain
FKDPYQQDLGIGESRISHENGTILCSKG
STCYGLWEKSKGDINLVKQGCWSHIG

DPQECHYEECVVTTTPPSIQNGTYRFC
CCSTDLCNVNFTENF
SEQUENCE ID NO: 13 Human gremlin
MSRTAYTVGALLLLLGTLLPAAEGKKK
GSQGAIPPPDKAQHNDSEQTQSPQQP
GSRNRGRGQGRGTAMPGEEVLESSQ
EALHVTERKYLKRDWCKTQPLKQTIHE
EGCNSRTIINRFCYGQCNSFYIPRHIRK
EEGSFQSCSFCKPKKFTTMMVTLNCP
ELQPPTKKKRVTRVKQCRCISIDLD
SEQUENCE ID NO: 14 Human noggin
MERCPSLGVTLYALVVVLGLRATPAGG
QHYLHIRPAPSDNLPLVDLIEHPDPIFD
PKEKDLNETLLRSLLGGHYDPGFMATS
PPEDRPGGGGGAAGGAEDLAELDQLL
RQRPSGAMPSEIKGLEFSEGLAQGKK
QRLSKKLRRKLQMWLWSQTFCPVLYA
WNDLGSRFWPRYVKVGSCFSKRSCS
VPEGMVCKPSKSVHLTVLRWRCQRRG
GQRCGWIPIQYPIISECKCSC
SEQUENCE ID NO: 15 BMP-7 variants
at position 21 (X = D,E,G,K,N,R,S)
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AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH
SEQUENCE ID NO: 16 BMP-7 variants
at position 23 (X = D,G,K,N,R,S)
STGGKQRSQNRSKTPKNQEALRXASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH
SEQUENCE ID NO: 17 BMP-7 variants
at position 26 (X = D,E,G,K,N,S)
STGGKQRSQNRSKTPKNQEALRMASX
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH
SEQUENCE ID NO: 18 BMP-7 variants
at position 36 (X = E,N,R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRXACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH
SEQUENCE ID NO: 19 BMP-7 variants
at position 37 (X = D,E,H,K,R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQXCKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH
SEQUENCE ID NO: 20 BMP-7 variants
at position 39 (X = A,D,E,G,N,R,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACXKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH
SEQUENCE ID NO: 21 BMP-7 variants
at position 42 (X = D,Q,R,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHXLYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH
SEQUENCE ID NO: 22 BMP-7 variants
at position 44 (X =
A,D,E,G,H,K,N,P,Q,R,S,T)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELXVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 23 BMP-7 variants at position 48 (X = D,E,H,K,N,Q)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFXDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 24 BMP-7 variants at position 49 (X = E,S)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRXLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 25 BMP-7 variants at position 52 (X = A,E,K,P,Q,T)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGX
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 26 BMP-7 variants at position 53 (X = A,D,E,G,H,K,R,S,T)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
XDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 27 BMP-7 variants at position 54 (X = K,N,R,S)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QXWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 28 BMP-7 variants at position 55 (X = A,E,H,K,N,P,Q,R,T)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDXIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 29 BMP-7 variants at position 57 (X = A,D,E,H,K,L,P,Q,T,V)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIXAPEGYAAYYCEGECAFPLNSY
MNATNHAIVQTLVHFINPDTVPKPCCA
PTQLNAISVLYFDDSSNVILKKYRNMVV
RACGCH

SEQUENCE ID NO: 30 BMP-7 variants at position 60 (X = H,K,N,P,Q,R,S,T)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPXGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 31 BMP-7 variants at position 63 (X = E,Q,R,S)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYXAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 32 BMP-7 variants at position 65 (X = D,E,N)

STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAXYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 33 BMP-7 variants at position 70 (X = A,Q)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGXCAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 34 BMP-7 variants at position 72 (X = D,E,H,K,N,R,S)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 35 BMP-7 variants at position 73 (X = A,D,E,G,H,K,N,Q,R,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAXPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 36 BMP-7 variants at position 76 (X = A,D,N,S,T,Y)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLXSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 37 BMP-7 variants at position 77 (X = A,D,E,H,K,N,P,Q,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNXYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 38 BMP-7 variants at position 78 (X = D,G,H,N,P,R,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSXM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 39 BMP-7 variants at position 80 (X = D,Q,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
XATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 40 BMP-7 variants at position 82 (X = V)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NAXNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 41 BMP-7 variants at position 83 (X = P)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATXHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 42 BMP-7 variants at position 86 (X = A,D,E,K,P,Q,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAXVQTLVHFINPDTVPKPCCAP
TQLNAISVLYFDDSSNVILKKYRNMVVR
ACGCH

SEQUENCE ID NO: 43 BMP-7 variants at position 88 (X = E)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVXTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 44 BMP-7 variants at position 90 (X = E,G,H,K,N,P,Q,R,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTXVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 45 BMP-7 variants at position 93 (X = A,D,E,G,H,P,Q,R,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHXINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 46 BMP-7 variants at position 94 (X = A,E,H,K,P,Q,R,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFXNPDTVPKPCCAP
TQLNAISVLYFDDSSNVILKKYRNMVVR
ACGCH

SEQUENCE ID NO: 47 BMP-7 variants at position 95 (X = D,K,Q,R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFIXPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 48 BMP-7 variants at position 97 (X = D,K,R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPXTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 49 BMP-7 variants at position 98 (X = A,E,K,R or deletion)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDXVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 50 BMP-7 variants at position 105 (X = V)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCXPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 51 BMP-7 variants at position 108 (X = D,K,S)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
XLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 52 BMP-7 variants at position 110 (X = D,E,H)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLXAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 53 BMP-7 variants at position 111 (X = D,S)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNXISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 54 BMP-7 variants at position 115 (X = A,E,K,Q,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVXYFDDSSNVILKKYRNMVVR
ACGCH

SEQUENCE ID NO: 55 BMP-7 variants at position 116 (X = A,D,E,H,K,Q,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLXFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 56 BMP-7 variants at position 117 (X = A,D,E,H,K,Q,R,S,Y)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYXDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 57 BMP-7 variants at position 119 (X = E,N,S,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDXSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 58 BMP-7 variants at position 120 (X = D,E,N,R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDXSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 59 BMP-7 variants at position 121 (X = D,E,K,N,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSXNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 60 BMP-7 variants at position 122 (X = E,Q,R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSXVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 61 BMP-7 variants at position 123 (X = A,D,G,N,R,T)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNXILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 62 BMP-7 variants at position 125 (X = A,E,K,P,Q,T,Y)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVIXKKYRNMVVR
ACGCH

SEQUENCE ID NO: 63 BMP-7 variants at position 126 (X = D,E,G,Q,R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILXKYRNMVVRA
CGCH

SEQUENCE ID NO: 64 BMP-7 variants at position 127 (X = A,D,E,H,N,P,Q,S,T,Y)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKXYRNMVVRA
CGCH

SEQUENCE ID NO: 65 BMP-7 variants at position 128 (X = D,E,H,K,Q)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKXRNMVVRA
CGCH

SEQUENCE ID NO: 66 BMP-7 variants at position 129 (X = D,E,K,N,S)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYXNMVVRA
CGCH

SEQUENCE ID NO: 67 BMP-7 variants at position 130 (X = D)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCH

SEQUENCE ID NO: 68 BMP-7 variants at position 134 (X = D,E,K,L,P,Q,R,S)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVXA
CGCH

SEQUENCE ID NO: 69 BMP-7 variants at position 135 (X = D,E,S)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRX
CGCH

SEQUENCE ID NO: 70 BMP-7 variants at position 139 (X = R)
STGGKQRSQNRSKTPKNQEALRMASV
AENSSSDQRQACKKHELYVSFRDLGW
QDWIIAPEGYAAYYCEGECAFPLNSYM
NATNHAIVQTLVHFINPDTVPKPCCAPT
QLNAISVLYFDDSSNVILKKYRNMVVRA
CGCX