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(54) Title: DESIGNER LIGANDS OF TGF-BETA SUPERFAMILY

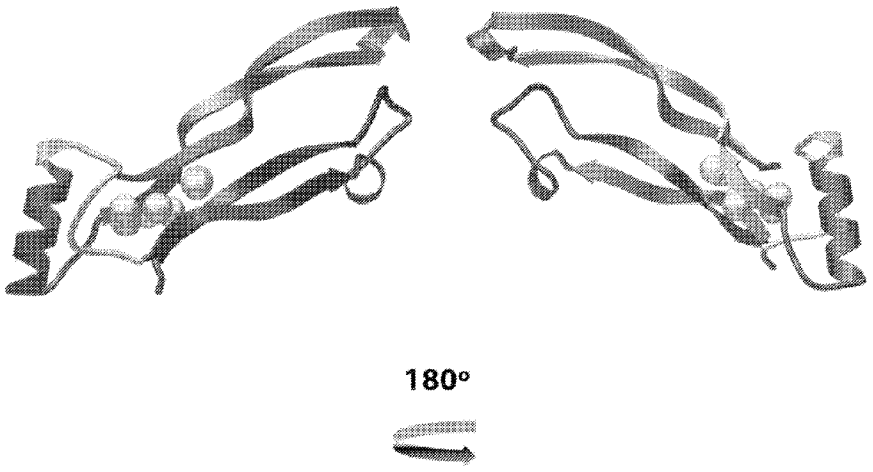


FIGURE 19

(57) Abstract: The present disclosure relates to chimeric polypeptide having TGF- beta activity, nucleic acids encoding the polypeptides, and host cells for producing the polypeptides.

WO 2010/099219 A2

DESIGNER LIGANDS OF TGF-BETA SUPERFAMILY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The application claims priority under 35 U.S.C. §119 to U.S. Provisional Application Serial No. 61/155,066, filed, February 24, 2009, the disclosure of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] The invention was funded, in part, by a grant from the National Institutes of Health grant number HD013527. The government has certain right in this invention.

TECHNICAL FIELD

[0003] The present disclosure relates to biomolecular engineering and design, and engineered proteins and nucleic acids.

BACKGROUND

[0004] Activins and Bone Morphogenetic Proteins (BMPs) are members of the much larger Transforming Growth Factor - beta (TGF-β) superfamily. Due to their pervasiveness in numerous developmental and cellular processes, TGF-β ligands have been the focus of great interest. For TGF-β ligands to be successfully used as therapeutic tools, several hurdles need to be overcome. The ability to specifically modify and alter the properties of TGF-β ligands, as well as generate those ligands in significant quantities is required.

SUMMARY

[0005] The disclosure provides non-naturally occurring chimeric polypeptides having an activity provided by a TGF-beta family of proteins. The chimeric polypeptides of the disclosure comprises two or more segments or fragments from parental TGF-beta proteins operably linked such that the resulting polypeptide is capable of modulating a pathway associated with a TGF-beta family of proteins. In one embodiment, the pathway is a SMAD or DAXX pathway.

[0006] In one embodiment, the disclosure provides designer TGF-beta ligands that can be synthesized by selecting and conjoining different sequence segments of TGF-beta superfamily ligands to construct new ligands (designer ligands). These novel ligands possess entirely new protein sequence library that differs from

naturally existing TGF-beta superfamily ligands. This approach originates primarily from the recognition of the structural commonality among natural TGF-beta superfamily ligands. All ~40 TGF-beta superfamily ligands share the same overall architecture with generic characteristics for each region of the protein. The framework of TGF-beta ligands can be divided into (generally) six subdomains (also called sequence segments; marked in six different colors in Figure 19) that all superfamily members share.

[0007] In one embodiment, the disclosure also provides a recombinant polypeptide comprising: at least two peptide segments, a first segment of the polypeptide comprising a sequence having at least 80% identity to a first TGF-beta family protein and a second segment comprising a sequence having at least 80% identity to a second TGF-beta family protein, wherein the segments are operably linked and have activity of at least one of the first or second parental TGF-beta family protein. In one embodiment, the the polypeptide comprises an N-terminal segment from BMP-2. In another embodiment, the at least two peptide segments comprise 6 peptide segments operably linked N-terminus to C-terminus. In yet another embodiment, each of the first and second TGF-beta family proteins have structural similarity and each segment corresponds to a structural motif. In yet a further embodiment, the first TGF-beta family protein is BMP-2 and the second TGF-beta family protein is activin. In one embodiment, the segments of the BMP-2 protein comprise segment 1: amino acid residue from about 1 to about x_1 of SEQ ID NO:2 ("1b"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:2 ("2b"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:2 ("3b"); segment 4 is from about amino acid residue x_3 to about x_4 of SEQ ID NO:2 ("4b"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:2 ("5b"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:2 ("6b"); and wherein: x_1 is residue 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 of SEQ ID NO:2; x_2 is residue 45, 46, 47, or 48 of SEQ ID NO:2; x_3 is residue 65, 66, 67, or 68 of SEQ ID NO:2; x_4 is residue 76, 77, 78, 79, 80, 81 or 82 of SEQ ID NO:2; x_5 is residue 88, 89, 90, 91, 92, 93, or 94 of SEQ ID NO:2; and x_6 is residue 112, 113, or 114 or SEQ ID NO:2, corresponding to the C-

terminus of BMP-2; and the segments of the activin protein comprise segment 1, amino acid residue from about 1 to about x_1 of SEQ ID NO:5 ("1a"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:5 ("2a"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:5 ("3a"); segment 4 is from about amino acid residue x_3 to about x_4 of SEQ ID NO:5 ("4a"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:5 ("5a"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:5 ("6a"); and wherein: x_1 is residue 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 of SEQ ID NO:5; x_2 is residue 42, 43, 44, or 45 of SEQ ID NO:5; x_3 is residue 61, 62, 63, or 64 of SEQ ID NO:5; x_4 is residue 78, 79, 80, 81, 82, 83 or 84 of SEQ ID NO:5; x_5 is residue 90, 91, 92, 93, 94, 95 or 96 of SEQ ID NO:5; and x_6 is residue 114, 115, or 116 or SEQ ID NO:5; and wherein the polypeptide has an order of segment 1-segment 2-segment 3-segment 4-segment 5-segment 6. In a further embodiment, the polypeptide comprises a sequence of segments selected from the group consisting of 1b2b3b4b5b6a; 1b2b3b4b5a6a; 1b2b3b4b5a6b; 1b2b3a4a5a6a; 1b2b3a4a5b6a; 1b2a3a4a5a6a; 1b2a3a4a5a6a L66V/V67I; 1b(1a_II)2a3a4a5a6a; 1b2a3a4a5a6b; 1b2a3a4a5b6b; 1b2a3a4a5b6a; 1b2a3b4b5b6a; 1b2a3b4b5a6a; and 1b2a3b4b5a6b.

[0008] The disclosure also provides a recombinant polypeptide comprising at least 80%, 90%, 95%, 98% or 99% identity to a sequence as set forth in SEQ ID NO:7, 9, 11, 13, 15, 17, 19, 1, 23, 25, 2, 29, 31, 33, 35, 37, 39 or 41 and wherein the polypeptide modulates the SMAD or DAXX pathway.

[0009] The disclosure also provides a chimeric TGF-beta family polypeptide comprising a segment of a first TGF-beta family protein operably linked to a segment of a second different TGF-beta family protein to provide a chimeric polypeptide having SMAD or DAXX modulating activity.

[0010] The disclosure also provides a polynucleotide encoding a polypeptide of the disclosure. In one embodiment, the polynucleotide has at least 80%, 90%, 95%, 98%, 99% or more identity to a sequence selected from the group consisting of SEQ ID NO: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 24, 26, 28, and 40. Vectors comprising such polynucleotides are also provided along with recombinant cells.

[0011] In an alternate embodiment, the disclosure provides novel ligands for members of the TGF-beta superfamily, wherein the ligand is a chimeric protein with at least one of six subdomians from a foreign or different member of the TGF-beta superfamily.

[0012] The disclosure comprises a chimeric polypeptide comprising a first domain from a first TGF-beta family member, a crossover point at J1 (see, *e.g.*, FIG. 18), a second domain from the same or second TGF-beta family member, a crossover point at J2 (see, *e.g.*, FIG. 18), a third domain from the same or third TGF-beta family member, a crossover point at J3 (see, *e.g.*, FIG. 18), a fourth domain from the same or fourth TGF-beta family member, a crossover point at J4 (see *e.g.*, FIG. 18), a fifth domain from the same or fifth TGF-beta family member, a crossover point at J5 (see, *e.g.*, FIG. 18), and a second domain from the same or sixth TGF-beta family member. In one embodiment, the chimera is derived from 2, 3, 4, 5, or 6 different TGF-beta family members. In yet another embodiment a crossover at J3 is optional.

BRIEF DESCRIPTION OF THE FIGURES

[0013] Figure 1A-B shows BMP2/BMP6 sample characterization. Panel A: BMP2/BMP6 sample on SDS-PAGE. The BMP2/BMP6 sample non-reduced in lane one, molecular weight marker in lane two, and the BMP2/BMP6 sample reduced in lane three. Panel B: SELDI-TOF-MS overlaid results from separate samples of BMP2, BMP6, and BMP2/BMP6 ligands.

[0014] Figure 2 shows C_2C1_2 whole cell Smad1-dependent reporter assay with wild type ligands. The solid black bars represent error.

[0015] Figure 3 shows a visualization of chick limb bud mesenchyme cell micromass culture chrondogenesis assays after 5 days. The top panel shows the culture with no factor and the extracellular antagonist Noggin. The bars in the micrographs represent 1mm. The second through fourth panels show the culture with ligands BMP2, BMP6, and BMP2/BMP6 respectively.

[0016] Figure 4 shows quantification of chick limb bud mesenchyme cell micromass culture chrondogenesis assays after three days. The addition of the specified growth factor at different concentrations is indicated.

[0017] Figure 5 shows Mutant BMP2/BMP6 heterodimers displaying activation of Smad1 reporter gene. Sample a contains no factor (background) and Sample b contains a BMP2 homodimer with no active receptor sites. All quantities are normalized to Sample c which is the fully active BMP2/BMP6 heterodimer (100% activation of reporter gene).

[0018] Figure 6 shows a native-PAGE displaying the quantity of type II receptor ECD remaining after being saturated into a ligand-receptor complex with the specified ligands.

[0019] Figure 7 depicts a structure/sequence aligment for chimera design strategy.

[0020] Figure 8 provides a graph showing cell viability in the presence of xerogel material (lowest concentration, green, 0.3 ug/ul; medium concentration, red, 3 ug/ul; high concentration, blue, 30 ug/ul).

[0021] Figure 9 shows H9 hES cells cultured in mCIVA using different concentrations of AB2-008 in the absence or presence of human FGF2.

[0022] Figure 10 shows mineralization shown by Von Kossa staining with (A) no ligand added, (B) recombinant BMP2 (30 ng/ml), (B) AB2-004 (30 ng/ml), (C) AB2-011 (30 ng/ml) and (D) AB2-015 (30 ng/ml).

[0023] Figure 11 shows the signaling activities of AB2-008, AB2-009, and AB2-010. (A) AB2-008 versus activin-βA (B) AB2-009 versus activin-βA (C) AB2-010 versus activin-βA (D) Relative signaling strength in comparison to activin-βA.

[0024] Figure 12 shows phosphorylation by AB2-008, AB2-009, and AB2-010 in comparison to Activin-βA and BMP2. Activin-βA, AB2-008, and AB2-009 show comparable levels of phosphorylation of SMAD2, whereas BMP2 shows phosphorylation of SMAD1 specifically.

[0025] Figure 13 depicts FSH release by Activin-βA, AB2-008, AB2-009, and AB2-010. (A) Dose dependent FSH stimulation without Inhibin, and (B) decreased release with Inhibin.

[0026] Figure 14 shows co-receptor binding by Activin-βA and AB2-008. Smad-2-Luciferase activity in HEK cells in the presence of and absence Cripto with (A) activin-βA, and with (B) AB2-008.

[0027] Figure 15 shows signaling activity of AB2-011, AB2-012, and AB2-015 by Smad-1 pathway. (A) AB2-004 v.s. BMP2, (B) AB2-011 v.s. BMP2, (C) AB2-012 v.s. BMP2, and (D) AB2-015 v.s. BMP2, all in the concentration range of 3-30 ng/ml in culture media using Smad-1 Luciferase assay with C2C12 cells.

[0028] Figure 16 shows Noggin sensitivity of BMP2, AB2-004, AB2-011, AB2-012, and AB2-015. Smad-1 luciferase signaling acitivity is measured with (light gray) and without (dark gray) Noggin.

[0029] Figure 17 is a schematic description of the RASCH method.

[0030] Figure 18 provides an alignment of the sequences of several members of the TGF-beta superfamily, with the relative segments defined for each member.

[0031] Figure 19 shows the six subdomains (fragments) on a single subunit of the TGF-beta superfamily ligand's scaffold.

DETAILED DESCRIPTION

[0032] As used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a domain" includes a plurality of such domains and reference to "the protein" includes reference to one or more proteins, and so forth.

[0033] Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0034] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0035] Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods and materials are described herein.

[0036] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs.

Thus, as used throughout the instant application, the following terms shall have the following meanings.

[0037] "Amino acid" is a molecule having the structure wherein a central carbon atom (the alpha-carbon atom) is linked to a hydrogen atom, a carboxylic acid group (the carbon atom of which is referred to herein as a "carboxyl carbon atom"), an amino group (the nitrogen atom of which is referred to herein as an "amino nitrogen atom"), and a side chain group, R. When incorporated into a peptide, polypeptide, or protein, an amino acid loses one or more atoms of its amino acid carboxylic groups in the dehydration reaction that links one amino acid to another. As a result, when incorporated into a protein, an amino acid is referred to as an "amino acid residue."

[0038] "Protein" or "polypeptide" refers to any polymer of two or more individual amino acids (whether or not naturally occurring) linked via a peptide bond, and occurs when the carboxyl carbon atom of the carboxylic acid group bonded to the alpha-carbon of one amino acid (or amino acid residue) becomes covalently bound to the amino nitrogen atom of amino group bonded to the -carbon of an adjacent amino acid. The term "protein" is understood to include the terms "polypeptide" and "peptide" (which, at times may be used interchangeably herein) within its meaning. In addition, proteins comprising multiple polypeptide subunits (*e.g.*, DNA polymerase III, RNA polymerase II) or other components (for example, an RNA molecule, as occurs in telomerase) will also be understood to be included within the meaning of "protein" as used herein. Similarly, fragments of proteins and polypeptides are also within the scope of the disclosure and may be referred to herein as "proteins." In one aspect of the disclosure, a polypeptide comprises a chimera of two or more parental peptide segments.

[0039] As used herein, TGF-beta superfamily member refers to a TGF-beta superfamily (including bone morphogenic factors) gene or protein of any species, particularly a mammalian species, including but not limited to bovine, ovine, porcine, murine, equine, and human. "TGF-beta superfamily polypeptide" refers to the amino acid sequences of purified TGF-beta superfamily protein obtained from any species, particularly a mammalian species, including bovine, ovine,

porcine, murine, equine, and human and from any source, whether natural, synthetic, semi-synthetic, or recombinant.

[0040] "Peptide segment" refers to a portion or fragment of a larger polypeptide or protein. A peptide segment need not on its own have functional activity, although in some instances, a peptide segment may correspond to a domain of a polypeptide wherein the domain has its own biological activity. A stability-associated peptide segment is a peptide segment found in a polypeptide that promotes stability, function, or folding compared to a related polypeptide lacking the peptide segment.

[0041] A particular amino acid sequence of a given protein (*i.e.*, the polypeptide's "primary structure," when written from the amino-terminus to carboxy-terminus) is determined by the nucleotide sequence of the coding portion of a mRNA, which is in turn specified by genetic information, typically genomic DNA (including organelle DNA, *e.g.*, mitochondrial or chloroplast DNA). Thus, determining the sequence of a gene assists in predicting the primary sequence of a corresponding polypeptide and more particular the role or activity of the polypeptide or proteins encoded by that gene or polynucleotide sequence.

[0042] "Fused," "operably linked," and "operably associated" are used interchangeably herein to broadly refer to a chemical or physical coupling of two otherwise distinct domains, wherein each domain has independent biological function. As such, the present disclosure provides TGF-beta (*e.g.*, BMP or activins) domains that are fused to one another such that they function as a polypeptide having a TGF-beta family activity or an improvement or change in ligand specificity of a TGF-beta family of polypeptides. In one embodiment, a chimeric polypeptide comprising a plurality of domains from two parental TGF-beta family polypeptides are linked such that they are part of the same coding sequence, each domain encoded by a polynucleotide from a parental TGF-beta family polypeptide, wherein the polynucleotides are in frame such that the polynucleotide when transcribed encodes a single mRNA that when translated comprises a plurality of domains as a single polypeptide. Typically, the coding domains will be linked "in-frame" either directly of separated by a peptide linker and encoded by a single polynucleotide. Various

coding sequences for peptide linkers and peptide are known in the art.

[0043] "Polynucleotide" or "nucleic acid" refers to a polymeric form of nucleotides. In some instances a polynucleotide comprises a sequence that is not immediately contiguous with either of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The nucleotides of the disclosure can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. A polynucleotides as used herein refers to, among others, single-and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. The term polynucleotide encompasses genomic DNA or RNA (depending upon the organism, *i.e.*, RNA genome of viruses), as well as mRNA encoded by the genomic DNA, and cDNA.

[0044] "Nucleic acid segment," "oligonucleotide segment" or "polynucleotide segment" refers to a portion of a larger polynucleotide molecule. The polynucleotide segment need not correspond to an encoded functional domain of a protein; however, in some instances the segment will encode a functional domain of a protein. A polynucleotide segment can be about 6 nucleotides or more in length (*e.g.*, 6-20, 20-50, 50-100, 100-200, 200-300, 300-400 or more nucleotides in length).

[0045] "Chimera" or "chimeric protein" or "chimeric polypeptide" refers to a combination of at least two segments of at least two different parent proteins. As appreciated by one of skill in the art, the segments need not actually come from each of the parents, as it is the particular sequence that is relevant, and not the physical nucleic acids themselves. For example, a chimeric BMP will

have at least two segments from two different parent BMPs; or BMP and other member of the TGF-beta superfamily, or alternatively, an unrelated protein. A chimeric protein may also be an "interspecies," "intergenic," *etc.* fusion of protein structures (the same or different member protein) expressed by different kinds of organisms. In a one embodiment, two segments are connected so as to result in a new chimeric protein. In other words, a protein will not be a chimera if it has the identical sequence of either one of the full-length parents. A chimeric protein can comprise more than two segments from two different parent proteins. For example, there may be 2, 3, 4, 5, 6 or 10-20, or more parents from which the domains may be derived in generating a final chimera or library of chimeras. The segment of each parent protein can be very short or very long, the segments can range in length of contiguous amino acids from 1 to about the full length of the protein. In one embodiment, the minimum length is 5 amino acids. Generally, the segment or subdomain, is one of six subdomains, alternatively five subdomains (see FIGs. 18 and 19). The six segments of a TGF-beta superfamily member are identified based on the structural architecture of the member protein and/or the primary amino acid sequence as aligned against other homologous member proteins. As identified, the member protein is generally divided into 6 distinct sections (although, alternatively, 5 distinct sections) based on segments derived to minimize alterations, or alternatively viewed, maximize alterations, to the aligned native TGF-beta member sequence during chimera engineering. Generally, Fig. 18 shows the relative positions of the distinct segments overlapping the aligned sequences of each of several TGF-beta superfamily members. The vertical line denotes a general position for cross-over between domains in generating the chimera. The amino acids that can overlap the two domains can be defined as being plus or minus about 5 amino acids (or alternatively, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids) in either direction of the vertical line. Also in Figure 18 is shown a boxed set of amino acids that identify additionl junctions that can be used to generate chimera. The J1-J5 junctions are positions general conservation across the TGF-beta family proteins that can be used to generate cross-over points.

[0046] Although relatively distinct, the segments may comprise a particular amino acid sequence or an original amino acid sequence that is amenable to substitution(s), insertion(s), additional amino acid(s) at either or both termini of the original sequence, or other modifications. By "amenable", it is meant that the structural integrity of each segment is maintained as compared to the domain of the original sequence. For example, a segment described herein of a TGF-beta superfamily member may shift by 10, 5, 3, 2, or 1, or preferably no more than 1 amino acid on either or both termini of segment as identified.

[0047] In one embodiment, the disclosure provides a chimeric protein comprising a fusion of at least one segment from a TGF-beta member with a second segment from a second TGF-beta member, wherein the first segment is foreign to the second TGF-beta member. Utilizing the five-six subdomains (segments) on a single subunit of the TGF-beta superfamily ligand as a scaffold framework, new (designer) sequences can be recombinantly linked by mixing segments from different TGF-beta ligands in the same order as they appear in nature. This assembly produces new sequences that are partly similar to one of several different target sequences, but distinctly different from any naturally occurring sequences.

[0048] In one embodiment, a single crossover point is defined for two parents. The crossover location defines where one parent's amino acid segment will stop and where the next parent's amino acid segment will start. Thus, a simple chimera would only have one crossover location where the segment before that crossover location would belong to one parent and the segment after that crossover location would belong to the second parent. In one embodiment, the chimera has more than one crossover location. For example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11-30, or more crossover locations. In a particular embodiment, the parental strands are defined as having 4 or 5 crossover locations. How these crossover locations are named and defined are both discussed below. In an embodiment where there are two crossover locations and two parents, there will be a first contiguous segment from a first parent, followed by a second contiguous segment from a second parent, followed by a third contiguous segment from the first parent. Contiguous is meant to

denote that there is nothing of significance interrupting the segments. These contiguous segments are connected to form a contiguous amino acid sequence. For example, a BMP-2/activin chimera derived from a BMP-2 wild-type parental strand and an activin wild-type parental strand with two crossovers would comprise a first segment from either BMP-2 or activin, a second segment from the opposite parental strand compared to the first segment operably linked to the first strand and comprising a structural motif downstream of the first strand and a third segment strand from the opposite parental strand compared to the second segment and from the same parental strand as the first segment operably linked to the second strand and comprising a structural motif downstream of the second strand all connected in one contiguous amino acid chain.

[0049] As appreciated by one of skill in the art, variants of chimeras exist as well as the exact sequences. In otherwords conservative amino acid substitutions may be incorporated into the chimera (*e.g.*, from about 1-10 conservative amino acid substitutions). Thus, not 100% of each segment need be present in the final chimera if it is a variant chimera. The amount that may be altered, either through additional residues or removal or alteration of residues will be defined as the term variant is defined. Of course, as understood by one of skill in the art, the above discussion applies not only to amino acids but also nucleic acids which encode for the amino acids.

[0050] "Conservative amino acid substitution" refers to the interchangeability of residues having similar side chains, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid, *e.g.*, alanine, valine, leucine, isoleucine, and methionine; an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain, *e.g.*, serine and threonine; an amino acids having aromatic side chains is substituted with another amino acid having an aromatic side chain, *e.g.*, phenylalanine, tyrosine, tryptophan, and histidine; an amino acid with a basic side chain is substituted with another amino acid with

a basis side chain, *e.g.*, lysine, arginine, and histidine; an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain, *e.g.*, aspartic acid or glutamic acid; and a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively.

[0051] "Non-conservative substitution" refers to substitution of an amino acid in the polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (*e.g.*, proline for glycine) (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0052] "Reference sequence" refers to a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence can be at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (*i.e.*, a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two polynucleotides or polypeptides over a "comparison window" to identify and compare local regions of sequence similarity.

[0053] "Sequence identity" means that two amino acid sequences are substantially identical (*i.e.*, on an amino acid-by-amino acid basis) over a window of comparison. The term "sequence similarity" refers to similar amino acids that share the same biophysical characteristics. The term "percentage of sequence identity" or

"percentage of sequence similarity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical residues (or similar residues) occur in both polypeptide sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity (or percentage of sequence similarity). With regard to polynucleotide sequences, the terms sequence identity and sequence similarity have comparable meaning as described for protein sequences, with the term "percentage of sequence identity" indicating that two polynucleotide sequences are identical (on a nucleotide-by-nucleotide basis) over a window of comparison. As such, a percentage of polynucleotide sequence identity (or percentage of polynucleotide sequence similarity, *e.g.*, for silent substitutions or other substitutions, based upon the analysis algorithm) also can be calculated. Maximum correspondence can be determined by using one of the sequence algorithms described herein (or other algorithms available to those of ordinary skill in the art) or by visual inspection.

[0054] As applied to polypeptides, the term substantial identity or substantial similarity means that two peptide sequences, when optimally aligned, such as by the programs BLAST, GAP or BESTFIT using default gap weights or by visual inspection, share sequence identity or sequence similarity. Similarly, as applied in the context of two nucleic acids, the term substantial identity or substantial similarity means that the two nucleic acid sequences, when optimally aligned, such as by the programs BLAST, GAP or BESTFIT using default gap weights (described in detail below) or by visual inspection, share sequence identity or sequence similarity.

[0055] One example of an algorithm that is suitable for determining percent sequence identity or sequence similarity is the FASTA algorithm, which is described in Pearson, W. R. & Lipman, D. J., (1988) Proc. Natl. Acad. Sci. USA 85:2444. See also, W. R. Pearson, (1996) Methods Enzymology 266:227-258. Preferred parameters used in a FASTA alignment of DNA sequences to calculate percent identity or percent similarity are optimized, BL50 Matrix

15: -5, k-tuple=2; joining penalty=40, optimization=28; gap penalty -12, gap length penalty=-2; and width=16.

[0056] Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity or percent sequence similarity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, (1987) J. Mol. Evol. 35:351-360. The method used is similar to the method described by Higgins & Sharp, CABIOS 5:151-153, 1989. The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity (or percent sequence similarity) relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, *e.g.*, version 7.0 (Devereaux *et al.*, (1984) Nuc. Acids Res. 12:387-395).

[0057] Another example of an algorithm that is suitable for multiple DNA and amino acid sequence alignments is the CLUSTALW program (Thompson, J. D. *et al.*, (1994) Nuc. Acids Res. 22:4673-4680). CLUSTALW performs multiple pairwise comparisons between groups of sequences and assembles them into a multiple alignment based on sequence identity. Gap open and Gap extension penalties were 10 and 0.05 respectively. For amino acid alignments, the

BLOSUM algorithm can be used as a protein weight matrix (Henikoff and Henikoff, (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919).

[0058] Figure 18, for example, shows an alignment of a number of TGF-beta family members. One of skill in the art can readily determine from the alignment those amino acids that are conserved across the family as well as those that are not conserved.

[0059] "Functional" refers to a polypeptide which possesses either the native biological activity of the naturally-produced proteins of its type, or any specific desired activity, for example as judged by its ability to bind to ligand or cognate molecules or induce a particular biological function (*e.g.*, stimulate muscle growth, bone growth and the like).

[0060] The Transforming Growth Factor – beta (TGF-β) superfamily of proteins is comprised of extracellular cytokines found in the vast majority of human cells. The TGF-β superfamily ligands, of which there are ~40, can be subdivided into smaller families including TGF-β, Bone Morphogenetic Proteins (BMPs), activin and inhibin, Growth and Differentiation Factors (GDFs), Nodal, Müllerian Inhibiting Substance (MIS), and Glial cell line-Derived Neurotrophic Factors (GDNFs). TGF-β superfamily members are found in a diverse range of cell types and play roles in many fundamental cellular events including dorsal/ventral patterning and left/right axis determination to bone formation and tissue repair. More recently, several TGF-β ligands have been shown to be involved in the maintenance or direct the differentiation of stem cells. Due to their pervasiveness, regulation of TGF-β ligand signaling holds promise for the treatment of a wide range of different diseases from skeletal and muscle abnormalities to numerous neoplastic events. Exemplary sequences are provided herein for various members of this family or proteins, however, one of skill in the art can easily identify homologs and variants using publicly available databases by word search or sequence BLAST searches.

[0061] There are generally recognized several subfamilies within the superfamily of TGF-beta (TGF-β1-β5) as well as the differentiation factors (*e.g.*, Vg-1), the hormones activin and inhibin, the Mullerianinhibiting substance (MIS), osteogenic and morphogenic proteins (*e.g.*, OP-1, OP-2, OP-3, other BMPs), the

developmentally regulated protein Vgr-1, the growth/differentiation factors (*e.g.*, GDF-1, GDF-3, GDF-9 and dorsalin-1), *etc.* See, *e.g.*, Spom and Roberts (1990) in Peptide Growth Factors and Their Receptors, Sporn and Roberts, eds., Springer-Verlag: Berlin pp. 419-472; Weeks and Melton (1987) Cell 51: 861-867; Padgett *et al.* (1987) Nature 325: 81-84; Mason *et al.* (1985) Nature 318: 659-663; Mason *et al.* (1987) Growth Factors 1: 77-88; Cate *et al.* (1986) Cell 45: 685-698; PCT/US90/05903; PCT/US91/07654; PCT/WO94/10202; U.S. Pat. Nos. 4,877,864; 5,141,905; 5,013,649; 5,116,738; 5,108,922; 5,106,748; and 5,155,058; Lyons *et al.* (1989) Proc. Natl. Acad. Sci. USA 86: 4554-58; McPherron *et al.* (1993) J. Biol. Chem. 268: 3444-3449; Basler *et al.* (1993) Cell 73: 687-702.

[0062] Morphogenic proteins of the TGF-beta superfamily include the mammalian osteogenic protein-1 (OP-1, also known as BMP-7), osteogenic protein-2 (OP-2, also known as BMP-8), osteogenic protein-3 (OP3), BMP-2 (also known as BMP-2A or CBMP-2A, and the Drosophila homolog DPP), BMP-3, BMP-4 (also known as BMP-2B or CBMP-2B), BMP-5, BMP-6 and its murine homolog Vgr-1, BMP-9, BMP-10, BMP-11, BMP-12, GDF3 (also known as Vgr2), GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, BMP-13, BMP-14, BMP-15, GDF-5 (also known as CDMP-1 or MP52), GDF-6 (also known as CDMP-2 or BMP13), GDF-7 (also known as CDMP-3 or BMP-12), the Xenopus homolog Vgl and NODAL, UNIVIN, SCREW, ADMP, NEURAL, *etc.*

[0063] One major roadblock for research involving many of the TGF-β superfamily ligands has been the inability to generate significant quantities of the proteins. While, BMP-2 is known to refold efficiently *in vitro*, other TGF-β ligands (activin, Nodal, and BMP-7 for instance) have not shown the same refolding properties. Other expression systems are available to obtain functional TGF-β ligands. Activin, for example, is expressed using stably transfected cell lines, such as CHO or transiently transfected cell lines, such as HEK293 cells.

[0064] The largest sub-family of the TGF-β superfamily is the BMP/GDF family, which comprises nearly half of all known ligands. Many of the ligands in BMP/GDF family share both BMP and GDF designations, such as GDF-7 which also referred to as BMP-12. In conjunction with being largest family, the BMP/GDF family is also

the most extensively studied family. For example, x-ray crystal structures have been solved for BMP-2 alone, bound to its type I receptor, or as a ternary complex bound to both its receptor types. Additionally, BMP-2, along with BMP-7, has been utilized as an effective treatment for certain bone injuries. One of the reasons for the large amount of structural and therapeutic work involving the BMP/GDF family has been the ability to chemically refold these ligands. Indeed, BMP-2 is one of the most successful TGF-β ligands at refolding, with optimized conditions reported to yield up to 63% active dimer from starting material. However, if specific amino acids of BMP-2 could be incorporated into other TGF-β ligands, it may allow for these otherwise non-refoldable ligands to be refolded opening up the remainder of the TGF-β superfamily to be better studied.

[0065] TGF-β ligands are synthesized as inactive precursor molecules composed of an N-terminal pro-domain and a C-terminal mature domain linked by a protease cleavage site. To be become active, the mature domain must be cleaved from the pro-domain, commonly by a convertase, such as furin. Members of the TGF-β superfamily are classified together due to the conserved structural architecture found in their mature domains. In general, each mature ligand monomer contains 7 cysteines, 6 of which form three intra-disulfide bonds arranged in a 'cystine knot' motif. Stretching outward from the 'cystine knot' are 4 beta strands, creating 2 curved fingers. The last remaining cysteine forms an inter-disulfide bond with a second ligand monomer, generating a covalently linked dimer. The dimer has the overall appearance of a butterfly with the 'cystine knot' as the body and the fingers spreading out like wings. The functional subunit for the TGF-β superfamily is the dimer and they been shown to exist both as homo- and heterodimers *in vivo*. Some family members, such as GDF-9 and BMP-15, lack the cysteine required to form the inter-disulfide bond yet they are still able to form stable dimers.

[0066] To initiate the signaling process, TGF-β dimers must recruit two sets of receptors, termed type I and type II. These receptors are serine/threonine kinases possessing an extracellular domain (ECD) ordered into a three-finger toxin fold, a single

transmembrane domain, and a large intracellular kinase domain. TGF-β ligands have been shown to display preferences in their affinity for the different receptor types. Activin and Nodal exhibit high affinity for type II receptors, while BMP-2 and GDF-5 possess higher affinity for type I receptors. Following the binding of two high affinity receptors to a TGF-β ligand, two lower affinity receptors are then able to bind and join the complex. Upon binding of all four receptors to the TGF-β ligand, forming a 6-member ternary complex, the downstream signaling cascade is initiated. The constitutively active type II receptors phosphorylate the type I receptors which, in turn, bind and phosphorylate intracellular signaling molecules called Smads. The Smad molecules then are able to translocate to the nucleus and interact directly with transcriptional regulators. Multiple mechanisms are employed to closely regulate TGF-β signaling at different stages of the cascade: Extracellular antagonists, including Noggin, follistatin, and Inhibin; pseudo-receptors lacking the intracellular kinase domain, similar to BAMBI; or through intracellular molecules, such as inhibitory Smads.

[0067] TGF-β superfamily shows a high degree of promiscuity by receptors for the ligands. While there are over 40 TGF-β ligands, there are only 12 receptors (7 type I and 5 type II). Therefore, receptors must be able to interact with a multitude of different ligands. For instance, ActRII is known to bind activin and BMP-7 with high affinity, but binds BMP-2 with much lower affinity. In GDF-5, a single amino acid has been found which determines its type I receptor preference, while in BMP-3 a single point mutation was discovered which alters type II receptor affinity as well as imparting function to the ligand. The disclosure provides methods to create modified TGF-β ligands with novel receptor binding properties thereby diversifying TGF-β ligand function as well as compositions having such activity.

[0068] The disclosure demonstrates a TGF-beta signaling complex by utilizing novel ligand constructs. Using synthesized chimeric homo- or heterodimeric ligands the ligands the disclosure provides compositions for use in dissecting the signaling of TGF-beta family proteins. Furthermore, utilizing such ligands allows a method for

distinguishing contributions of two type I receptor interfaces from each other, and two type II receptor interfaced each other. The methods and compositions of the disclosure demonstrate a correlation between ligand-receptor affinity, signaling activity, and biological activity. The methods and compositions of the disclosure shed light on the mechanism and requirements of the TGF-beta superfamily signaling complex assembly. In addition the chimeric ligands provide novel polypeptide for use in treating diseases and disorders associated with TGF-β family of proteins.

[0069] The disclosure provides methods of making and novel chimeric TGF-β ligands which possess the ability to be expressed and refolded using, for example, an E.coli or mammalian expression system. These chimeras either mimic a specific TGF-β ligand's signaling characteristics or display unique signaling properties not seen in nature. In one embodiment, the disclosure uses activin-βA and BMP-2 as a template to generate an activin/BMP-2 chimera with the refolding efficiency of BMP-2 and the signaling properties of activin-βA; however it should be recognized that any number of TGF-beta protein family members can be used. The chimera design scheme of the disclosure yielded additional TGF-beta member chimera (*e.g.*, activin/BMP-2) ligands with unnatural signaling characteristics and biological activity. Such chimeric TGF-beta family polypeptides expand the library of TGF-β ligands available for structural studies as well as facilitate the development of novel TGF-β ligands as therapeutic agents.

[0070] In one embodiment the disclosure provides a series of activin/BMP-2 chimeric ligands which possess unique signaling properties. For example, an activin/BMP-2 ligand of the disclosure exhibited the refolding characteristics of wild type BMP-2 while retaining activin-like signaling attributes in both *in vitro* and *in vivo* studies. Further, 'super' ligands were generated which are more potent than wild type BMP-2 and were not inhibited by the BMP antagonist Noggin. The disclosure also provides chimeric TGF-beta polypeptides comprising an N-terminus of wild type BMP-2mq operably linked to a different TGF-β ligand polypeptide segment. The disclosure demonstrates that the N-terminal portion of wild type BMP-2mq is enough to switch a previously non-refolding ligand into a

refoldable ligand. These findings highlight a method for obtaining activin and the other TGF-β ligand mimics and indicate how this strategy can be utilized to expand the library of TGF-β ligands by diversifying their functionality and promote the development of unnatural ligands for therapeutic purposes.

[0071] The nucleic acid sequences and polypeptide sequences of BMP-2 and naturally occurring variants are known. A wild-type BMP-2 nucleic acid sequence (SEQ ID NO:1) and polypeptide (SEQ ID NO:2) from *Rattus sp.* are provided. Met at the position N-terminal to the residue 1 (Q) results from translation of the bacterial initiation codon (ATG). Furthermore activins are also known in the art (see, *e.g.*, SEQ ID NO:5). The disclosure provides a number of chimeric TGF-beta family polypeptides having at least one N-terminal domain from a BMP-2 and at lease one second domain form another TGF-beta family members wherein the chimeric polypeptide display activities different than wild-type parental proteins.

[0072] In one embodiment, two factors were considered when looking to design the segments of the chimeras. First was a structural consideration. The overall TGF-β monomer fold is divided into 6 sections naturally: Beta strand 1 and 2, the pre-helix loop, alpha helix 1, and beta strand 3 and 4. The identification and characterization of these subdomains are further described in Example 4. The disclosure utilized a chimeric structures to mimic these natural regions in the design. Thus, each segment can be indicated by 1, 2, 3, 4, 5, and 6. The second consideration was to minimize alterations to the aligned native TGF-beta member sequence during chimera engineering. Therefore, those regions with sequence identity between the 2 protein sequences were identified as putative cross-over points. These regions are suitable for the overlaps in DNA sequence for PCR strategy and will minimize any changes to the natural sequences. Figure 7 illustrates the sequence and structure of these considerations. The regions are boxed and numbered according to their section and are mapped onto the ligand monomer. The areas which can be used for the cross-over points as segmental boundaries are shaded as a sequence range in orange. Residue numbering in one embodiment is based on BMP-2 (SEQ ID NO:2). Thus, cross-over points in generating a chimeric polypeptide of the

disclosure can be identified by identifying similar struactural motifs in combination with at least 60%, 70%, 80%, 90%, 95%, 98%, 99% or 100% identity in a segment of the sequence between changes in the structural motif. Cross-overs at these regions (which may be between 3 to 20 amino acids) minimize disruption of the resulting chimeric polypeptide providing a stabilized chimera.

[0073] For example, a chimeric polypeptide comprising the algorithm 1b2b3b4b5b6a indicates 6 segments, the letter indicating the parental strand of each segment. Thus, in the example "1b2b3b4b5b6a", segment 1 is from parental strand "b" for BMP-2mq, segment 2 is from parental strand "b" for BMP-2mq, segment 3 is from parental strand "b" for BMP-2mq, segment 4 is from parental strand "b" for BMP-2mq, segment 5 is from parental strand "b" for BMP-2mq, and segment 6 is from parental strand "a" for Activin.

[0074] In one embodiment, crossover between segments of BMP-2mq and a second TGF-beta family protein can occur where structural similarity and sequence similary overlap. Figure 7 depicts such an overlap between BMP-2mq and activin, wherein crossovers can be generated between about residue D25-P35, G45-P48; T65-N68; K76-T82; and S88-E94 (residue numbering is based on BMP-2 (SEQ ID NO:2)). Sequence alignment of BMP-2 and activin-βA highlight the boundaries of segments 1 through 6. Activin has the extra disulfide bond formed between two Cys. Red (or first shaded boxes on lower sequences) box notes two amino acids of AB2-009 swapped into AB2-008. Blue (second shaded box L/Y on lower lines) box notes one amino acid changed in Segment 5 of BMP2 for all chimera. For clarity, BMP-2's Segment 5 contains YYD instead of YLD. Green (KKQ-FFVSFKDI) box denotes a segment introduced into AB2-008 to make AB2-010, marked as (1a_II) of AB2-010.

[0075] Figure 18 further depicts such crossover regions with reference to additional members of the TGF-β family or proteins. For example, with reference to Figure 18, one of skill in the art can see that BMP-3 (SEQ ID NO:43) comprises 110 amino acids. The first vertical line demonstrates a general region of cross over and can comprise from 1-5 amino acids on either side of the vertical line. Accordingly, a first domain from BMP-3 can comprise amin acid 1 to about x_1, wherein x_1 is an amino acid corresponding to residue

20-29 (*e.g.*, x1 is 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29). As further shown in Figure 18, "J1" corresponds to residues 20-23 of SEQ ID NO:43). J1 refers to a junction region having conservation across the various species in the TGF-β family of proteins. Accordingy, a first domain of BMP-3 comprises amino acids 1 to about x_1, wherein x_1 will be either V or G and the following chimeric domain from a second TGF-β family member will begin with either G or W. Using Figure 18 as a template one of skill in the art can readily identify the cross-over regions (or junctions points) for the various members of the TGF-β family. It is important to note that not every chimera is required to have 6 distinct domains. For example, a cross over at junction 3 (J3) may not be necessary such that only 5 or fewer domains from distinct family member are present in the final chimera.

[0076] Other methods for identifying crossover locations may be employed in the generation of chimeric TGF-beta family polypeptides. For example, SCHEMA is a computational based method for predicting which fragments of homologous proteins can be recombined without affecting the structural integrity of the protein (see, *e.g.*, Meyer *et al.*, (2003) Protein Sci., 12:1686-1693). Chimeras with higher stability are identifiable by determining the additive contribution of each segment to the overall stability, either by use of linear regression of sequence-stability data, or by reliance on consensus analysis of the MSAs of folded versus unfolded proteins. SCHEMA recombination ensures that the chimeras retain biological function and exhibit high sequence diversity by conserving important functional residues while exchanging tolerant ones.

[0077] As presented in this disclosure, it has been found that when these recombined, functional chimeric TGF-beta family polypeptides are generated their ligand specificity can be improved or biological activity can be altered or improved compared to a unrecombined parental polypeptide. Because of differences in activity/ligand profiles, these engineered chimeric TGF-beta family polypeptides provide a unique basis to screen for activities for ligand specific activation and inhibition, provide novel therapeutic polypeptides and research reagents.

[0078] For example, in the chimeras of the disclosure, domain 1, 2, 3, 4, 5, and 6 can be selected from the following sequences (Table A) wherein the polypeptide comprises a structure (domain 1-domain 2-domain 3-domain 4-domain 5-domain 6):

Table A:

Amino acids (domain #)	SEQ ID NO:	Variable definition
1-x_1 (1)	2	x_1 is 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35
1-x_1 (1)	5	x_1 is 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32
1-x_1 (1)	43	$x_1$is 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29
1-x_1 (1)	45	$x_1$is 27, 28, 29, 30, 31, 32, 33, 34,35, or 36
1-x_1 (1)	47	$x_1$is 43, 44, 44, 46 47, 48, 49, 50, 51, or 52
1-x_1 (1)	49	$x_1$is 43, 44, 44, 46 47, 48, 49, 50, 51, or 52
1-x_1 (1)	51	$x_1$is 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60
1-x_1 (1)	53	$x_1$is 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60
1-x_1 (1)	55	$x_1$is 19, 20, 21, 22, 23, 24, 25, 26 27, or 28
1-x_1 (1)	57	$x_1$is 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27
1-x_1 (1)	59	$x_1$is 36, 37, 38, 29, 40, 41, 42, 43, 44, or 45
1-x_1 (1)	61	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34
1-x_1 (1)	63	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34
1-x_1 (1)	65	$x_1$is 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40
1-x_1 (1)	67	$x_1$is 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40
1-x_1 (1)	69	$x_1$is 40, 41, 42, 43, 44, 45, 46, 47, 48 or 49
1-x_1 (1)	71	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34
1-x_1 (1)	73	$x_1$is 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55
1-x_1 (1)	75	$x_1$is 28, 29, 30, 31, 32, 33, 34, 35, 36, or 37
1-x_1 (1)	77	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34
1-x_1 (1)	79	$x_1$is 24, 25, 26, 27, 28, 29, 30, 31, 32, or 33
1-x_1 (1)	81	$x_1$is 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30

1-x_1 (1)	83	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31
1-x_1 (1)	85	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31
1-x_1 (1)	87	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31
1-x_1 (1)	89	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31
1-x_1 (1)	91	$x_1$is 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51
1-x_1 (1)	93	$x_1$is 28, 29, 30, 31, 32, 33, 34, 35, 36 or 37
1-x_1 (1)	95	$x_1$is 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33
1-x_1 (1)	97	$x_1$is 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33
$x_1 - x_2$ (2)	2	x_1 is 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, or 52
$x_1 - x_2$ (2)	5	x_1 is 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51
$x_1 - x_2$ (2)	43	$x_1$is 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29 x_2 is 38, 39, 40, 41, 42, 43, 44, 45, 46 or 47
$x_1 - x_2$ (2)	45	$x_1$is 27, 28, 29, 30, 31, 32, 33, 34,35 or 36 x_2 is 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55
$x_1 - x_2$ (2)	47	$x_1$is 43, 44, 44, 46 47, 48, 49, 50, 51 or 52 x_2 is 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70
$x_1 - x_2$ (2)	49	$x_1$is 43, 44, 44, 46 47, 48, 49, 50, 51 or 52 x_2 is 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70
$x_1 - x_2$ (2)	51	$x_1$is 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60 x_2 is 68, 69, 70, 71, 72, 73, 74, 75, 76, 77 or 78
$x_1 - x_2$ (2)	53	$x_1$is 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60 x_2 is 68, 69, 70, 71, 72, 73, 74, 75, 76, 77 or 78
$x_1 - x_2$ (2)	55	$x_1$is 19, 20, 21, 22, 23, 24, 25, 26 27, or 28 x_2 is 38, 39, 40, 41, 42, 43, 44, 45, 46 or 47
$x_1 - x_2$ (2)	57	$x_1$is 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 x_2 is 37, 38, 39, 40, 41, 42, 43, 44, 45 or 49
$x_1 - x_2$ (2)	59	$x_1$is 36, 37, 38, 29, 40, 41, 42, 43, 44 or 45 x_2 is 53, 54, 55, 56, 57, 58, 59, 60, 61 or 62
$x_1 - x_2$ (2)	61	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34 x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53
$x_1 - x_2$ (2)	63	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34 x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53
$x_1 - x_2$ (2)	65	$x_1$is 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 x_2 is 48, 49, 50, 51, 52, 53, 54, 55, 56, 57 or 58
$x_1 - x_2$ (2)	67	$x_1$is 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 x_2 is 48, 49, 50, 51, 52, 53, 54, 55, 56, 57 or 58
$x_1 - x_2$ (2)	69	$x_1$is 40, 41, 42, 43, 44, 45, 46, 47, 48 or 49 x_2 is 57, 58, 59, 60, 61, 62, 63, 64, 65 ot 66
$x_1 - x_2$ (2)	71	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34 x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, or 52
$x_1 - x_2$ (2)	73	$x_1$is 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55 x_2 is 63, 64, 65, 66, 67, 68, 69, 70, 71, 72 or 73
$x_1 - x_2$ (2)	75	$x_1$is 28, 29, 30, 31, 32, 33, 34, 35, 36, or 37 x_2 is 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55

$x_1 - x_2$ (2)	77	$x_1$is 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34 x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53
$x_1 - x_2$ (2)	79	$x_1$is 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 x_2 is 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53
$x_1 - x_2$ (2)	81	$x_1$is 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 x_2 is 39, 40, 41, 42, 43, 44, 45,46, 47 or 48
$x_1 - x_2$ (2)	83	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31 x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51
$x_1 - x_2$ (2)	85	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31 x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51
$x_1 - x_2$ (2)	87	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31 x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51
$x_1 - x_2$ (2)	89	$x_1$is 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31 x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51
$x_1 - x_2$ (2)	91	$x_1$is 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51 x_2 is 59, 60, 61, 62, 63, 65, 65, 66, 67 or 68
$x_1 - x_2$ (2)	93	$x_1$is 28, 29, 30, 31, 32, 33, 34, 35, 36 or 37 x_2 is 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55
$x_1 - x_2$ (2)	95	$x_1$is 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 x_2 is 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53
$x_1 - x_2$ (2)	97	$x_1$is 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 x_2 is 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53
$x_2 - x_3$ (3)	2	x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, or 52 x_3 is 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70
$x_2 - x_3$ (3)	5	x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51 x_3 is 55, 56, 57, 58, 59, 60, 61, 62, 63, 6,4 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74
$x_2 - x_3$ (3)	43	x_2 is 38, 39, 40, 41, 42, 43, 44, 45, 46 or 47 x_3 is 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65
$x_2 - x_3$ (3)	45	x_2 is 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 x_3 is 61, 62, 63, 64, 65, 66,67, 68, 69, 70, 71, 72, 73 or 74
$x_2 - x_3$ (3)	47	x_2 is 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 x_3 is 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87 or 88
$x_2 - x_3$ (3)	49	x_2 is 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 x_3 is 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87 or 88
$x_2 - x_3$ (3)	51	x_2 is 68, 69, 70, 71, 72, 73, 74, 75, 76, 77 or 78 x_3 is 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 or 95
$x_2 - x_3$ (3)	53	x_2 is 68, 69, 70, 71, 72, 73, 74, 75, 76, 77 or 78 x_3 is 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 or 95
$x_2 - x_3$ (3)	55	x_2 is 38, 39, 40, 41, 42, 43, 44, 45, 46 or 47 x_3 is 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65
$x_2 - x_3$ (3)	57	x_2 is 37, 38, 39, 40, 41, 42, 43, 44, 45 or 49 x_3 is 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, or 64
$x_2 - x_3$ (3)	59	x_2 is 53, 54, 55, 56, 57, 58, 59, 60, 61 or 62 x_3 is 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80 or 81
$x_2 - x_3$ (3)	61	x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53 x_3 is 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75 or 76
$x_2 - x_3$ (3)	63	x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53 x_3 is 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70
$x_2 - x_3$ (3)	65	x_2 is 48, 49, 50, 51, 52, 53, 54, 55, 56, 57 or 58 x_3 is 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74 or 75
$x_2 - x_3$ (3)	67	x_2 is 48, 49, 50, 51, 52, 53, 54, 55, 56, 57 or 58 x_3 is 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74 or 75

$x_2 - x_3$ (3)	69	x_2 is 57, 58, 59, 60, 61, 62, 63, 64, 65 ot 66 x_3 is 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83 or 84
$x_2 - x_3$ (3)	71	x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, or 52 x_3 is 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65
$x_2 - x_3$ (3)	73	x_2 is 63, 64, 65, 66, 67, 68, 69, 70, 71, 72 or 73 x_3 is 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89 or 90
$x_2 - x_3$ (3)	75	x_2 is 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 x_3 is 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72 or 73
$x_2 - x_3$ (3)	77	x_2 is 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53 x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65
$x_2 - x_3$ (3)	79	x_2 is 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53 x_3 is 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 or 68
$x_2 - x_3$ (3)	81	x_2 is 39, 40, 41, 42, 43, 44, 45,46, 47 or 48 x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65 or 66
$x_2 - x_3$ (3)	83	x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51 x_3 is 55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74
$x_2 - x_3$ (3)	85	x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51 x_3 is 55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74
$x_2 - x_3$ (3)	87	x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51 x_3 is55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74
$x_2 - x_3$ (3)	89	x_2 is 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or 51 x_3 is 55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74
$x_2 - x_3$ (3)	91	x_2 is 59, 60, 61, 62, 63, 65, 65, 66, 67 or 68 x_3 is 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86 or 87
$x_2 - x_3$ (3)	93	x_2 is 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 x_3 is 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70
$x_2 - x_3$ (3)	95	x_2 is 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53 x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65
$x_2 - x_3$ (3)	97	x_2 is 44, 45, 46, 47, 48, 49, 50, 51, 52 or 53 x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65
$x_3 - x_4$ (4)	2	x_3 is 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 x_4 is 78, 79, 80, 81, 82, 83, 84 or 85
$x_3 - x_4$ (4)	5	x_3 is 55, 56, 57, 58, 59, 60, 61, 62, 63, 6,4 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74 x_4 is 79, 80, 81, 82, 83, 84, 85 or 86
$x_3 - x_4$ (4)	43	x_3 is 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 x_4 is 73, 74, 75, 76, 77, 78, 79 or 80
$x_3 - x_4$ (4)	45	x_3 is 61, 62, 63, 64, 65, 66,67, 68, 69, 70, 71, 72, 73 or 74 x_4 is 79, 80, 81, 82, 83, 84, 85 or 86
$x_3 - x_4$ (4)	47	x_3 is 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87 or 88 x_4 is 95, 96, 97, 98, 99, 100, 101, 102 or 103
$x_3 - x_4$ (4)	49	x_3 is 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87 or 88 x_4 is 95, 96, 97, 98, 99, 100, 101, 102 or 103
$x_3 - x_4$ (4)	51	x_3 is 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 or 95 x_4 is 102, 103, 104, 105, 106, 107, 108, 109 or 110
$x_3 - x_4$ (4)	53	x_3 is 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 or 95 x_4 is 102, 103, 104, 105, 106, 107, 108, 109 or 110
$x_3 - x_4$ (4)	55	x_3 is 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 x_4 is 72, 73, 74, 75, 76, 77, 78, 79 or 80
$x_3 - x_4$	57	x_3 is 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, or 64

(4)		x_4 is 71, 72, 73, 74, 75, 76, 77, 78 or 79
$x_3 - x_4$ (4)	59	x_3 is 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80 or 81 x_4 is 78, 79, 80, 81, 82, 83, 84, 85 or 86
$x_3 - x_4$ (4)	61	x_3 is 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75 or 76 x_4 is 82, 83, 84, 85, 86, 87, 88, 89 or 90
$x_3 - x_4$ (4)	63	x_3 is 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 x_4 is 77, 78, 79, 80, 81, 82, 83, 84 or 85
$x_3 - x_4$ (4)	65	x_3 is 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74 or 75 x_4 is 83, 84, 85, 86, 87, 88, 89, 90 or 91
$x_3 - x_4$ (4)	67	x_3 is 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74 or 75 x_4 is 83, 84, 85, 86, 87, 88, 89, 90 or 91
$x_3 - x_4$ (4)	69	x_3 is 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83 or 84 x_4 is 92, 93, 94, 95, 96, 97, 98, 99 or 100
$x_3 - x_4$ (4)	71	x_3 is 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 x_4 is 72, 73, 74, 75, 76, 77, 78, 79 or 80
$x_3 - x_4$ (4)	73	x_3 is 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89 or 90 x_4 is 98, 99, 100, 101, 102, 103, 104, 105 or 106
$x_3 - x_4$ (4)	75	x_3 is 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72 or 73 x_4 is 82, 83, 84, 85, 86, 87, 88, 89, or 90
$x_3 - x_4$ (4)	77	x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 x_4 is 72, 73, 74, 75, 76, 77, 78, 79 or 80
$x_3 - x_4$ (4)	79	x_3 is 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 or 68 x_4 is 76, 77, 78, 79, 80, 81, 82, 83 or 84
$x_3 - x_4$ (4)	81	x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65 or 66 x_4 is 74, 75, 76, 77, 78, 79, 80, 81 or 82
$x_3 - x_4$ (4)	83	x_3 is 55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74 x_4 is 79, 80, 81, 82, 83, 84, 85, 86 or 87
$x_3 - x_4$ (4)	85	x_3 is 55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74 x_4 is 78, 79, 80, 81, 82, 83, 84, 85 or 86
$x_3 - x_4$ (4)	87	x_3 is55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74 x_4 is 79, 80, 81, 82, 83, 84, 85, 86 or 87
$x_3 - x_4$ (4)	89	x_3 is 55, 56, 5, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, or 74 x_4 is 77, 78, 79, 80, 81, 82, 83, 84 or 85
$x_3 - x_4$ (4)	91	x_3 is 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86 or 87 x_4 is 96, 97, 98, 99, 100, 101, 102, 103 or 104
$x_3 - x_4$ (4)	93	x_3 is 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 x_4 is 77, 78, 79, 80, 81, 82, 83, 84, or 85
$x_3 - x_4$ (4)	95	x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 x_4 is 76, 77, 78, 79, 80, 81, 82, 83 or 84
$x_3 - x_4$ (4)	97	x_3 is 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 x_4 is 76, 77, 78, 79, 80, 81, 82, 83 or 84
$x_4 - x_5$ (5)	2	x_4 is 78, 79, 80, 81, 82, 83, 84 or 85 x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98
$x_4 - x_5$ (5)	5	x_4 is 79, 80, 81, 82, 83, 84, 85 or 86 x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100
$x_4 - x_5$ (5)	43	x_4 is 73, 74, 75, 76, 77, 78, 79 or 80 x_5 is 85, 86, 87, 88, 89, 90 91, 92 or 93
$x_4 - x_5$ (5)	45	x_4 is 79, 80, 81, 82, 83, 84, 85 or 86 x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100

$x_4 - x_5$ (5)	47	x_4 is 95, 96, 97, 98, 99, 100, 101, 102 or 103 x_5 is 107, 108, 109, 110, 111, 112, 113, 114 or 115
$x_4 - x_5$ (5)	49	x_4 is 95, 96, 97, 98, 99, 100, 101, 102 or 103 x_5 is 107, 108, 109, 110, 111, 112, 113, 114 or 115
$x_4 - x_5$ (5)	51	x_4 is 102, 103, 104, 105, 106, 107, 108, 109 or 110 x_5 is 114, 115, 116, 117, 118, 119, 120, 121, or 122
$x_4 - x_5$ (5)	53	x_4 is 102, 103, 104, 105, 106, 107, 108, 109 or 110 x_5 is 114, 115, 116, 117, 118, 119, 120, 121, or 122
$x_4 - x_5$ (5)	55	x_4 is 72, 73, 74, 75, 76, 77, 78, 79 or 80 x_5 is 84, 85, 86, 87, 88, 89, 90, 91, 92 or 93
$x_4 - x_5$ (5)	57	x_4 is 71, 72, 73, 74, 75, 76, 77, 78 or 79 x_5 is 83, 84, 85, 86, 87, 88, 89, 90, 91, or 92
$x_4 - x_5$ (5)	59	x_4 is 78, 79, 80, 81, 82, 83, 84, 85 or 86 x_5 is 100, 101, 102, 103, 104, 106, 106, 107 or 108
$x_4 - x_5$ (5)	61	x_4 is 82, 83, 84, 85, 86, 87, 88, 89 or 90 x_5 is 94, 95, 96, 97, 98, 99, 100, 101 or 102
$x_4 - x_5$ (5)	63	x_4 is 77, 78, 79, 80, 81, 82, 83, 84 or 85 x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98
$x_4 - x_5$ (5)	65	x_4 is 83, 84, 85, 86, 87, 88, 89, 90 or 91 x_5 is 95, 96, 97, 98, 99, 100, 101, 102 or 103
$x_4 - x_5$ (5)	67	x_4 is 83, 84, 85, 86, 87, 88, 89, 90 or 91 x_5 is 95, 96, 97, 98, 99, 100, 101, 102 or 103
$x_4 - x_5$ (5)	69	x_4 is 92, 93, 94, 95, 96, 97, 98, 99 or 100 x_5 is 104, 105, 106, 107, 108, 109, 110, 111 or 112
$x_4 - x_5$ (5)	71	x_4 is 72, 73, 74, 75, 76, 77, 78, 79 or 80 x_5 is 84, 85, 86, 87, 88, 89, 90, 91, 92 or 93
$x_4 - x_5$ (5)	73	x_4 is 98, 99, 100, 101, 102, 103, 104, 105 or 106 x_5 is 110, 111, 112, 113, 114, 115, 116, 117 or 118
$x_4 - x_5$ (5)	75	x_4 is 82, 83, 84, 85, 86, 87, 88, 89, or 90 x_5 is 94, 95, 96, 97, 98, 99, 100, 101 or 102
$x_4 - x_5$ (5)	77	x_4 is 72, 73, 74, 75, 76, 77, 78, 79 or 80 x_5 is 84, 85, 86, 87, 88, 89, 90, 91, 92 or 93
$x_4 - x_5$ (5)	79	x_4 is 76, 77, 78, 79, 80, 81, 82, 83 or 84 x_5 is 88, 89, 90, 91, 92, 93, 94, 95 or 96
$x_4 - x_5$ (5)	81	x_4 is 74, 75, 76, 77, 78, 79, 80, 81 or 82 x_5 is 86, 87, 88, 89, 90, 91, 92, 93 or 94
$x_4 - x_5$ (5)	83	x_4 is 79, 80, 81, 82, 83, 84, 85, 86 or 87 x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100
$x_4 - x_5$ (5)	85	x_4 is 78, 79, 80, 81, 82, 83, 84, 85 or 86 x_5 is 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99
$x_4 - x_5$ (5)	87	x_4 is 79, 80, 81, 82, 83, 84, 85, 86 or 87 x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100
$x_4 - x_5$ (5)	89	x_4 is 77, 78, 79, 80, 81, 82, 83, 84 or 85 x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98
$x_4 - x_5$ (5)	91	x_4 is 96, 97, 98, 99, 100, 101, 102, 103 or 104 x_5 is 108, 109, 110, 111, 112, 113, 114, 115 or 116
$x_4 - x_5$ (5)	93	x_4 is 77, 78, 79, 80, 81, 82, 83, 84, or 85 x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98
$x_4 - x_5$ (5)	95	x_4 is 76, 77, 78, 79, 80, 81, 82, 83 or 84 x_5 is 88, 89, 90, 91, 92, 93, 94, 95 or 96
$x_4 - x_5$ (5)	97	x_4 is 76, 77, 78, 79, 80, 81, 82, 83 or 84 x_5 is 88, 89, 90, 91, 92, 93, 94, 95 or 96
$x_5 - 114$ (6)	2	x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98

x_5 – 116 (6)	5	x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100
x_5 – 110 (6)	43	x_5 is 85, 86, 87, 88, 89, 90 91, 92 or 93
x_5 – 116 (6)	45	x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100
x_5 – 132 (6)	47	x_5 is 107, 108, 109, 110, 111, 112, 113, 114 or 115
x_5 – 132 (6)	49	x_5 is 107, 108, 109, 110, 111, 112, 113, 114 or 115
x_5 – 139 (6)	51	x_5 is 114, 115, 116, 117, 118, 119, 120, 121, or 122
x_5 – 139 (6)	53	x_5 is 114, 115, 116, 117, 118, 119, 120, 121, or 122
x_5 – 110 (6)	55	x_5 is 84, 85, 86, 87, 88, 89, 90, 91, 92 or 93
x_5 – 108 (6)	57	x_5 is 83, 84, 85, 86, 87, 88, 89, 90, 91, or 92
x_5 – 125 (6)	59	x_5 is 100, 101, 102, 103, 104, 106, 106, 107 or 108
x_5 – 119 (6)	61	x_5 is 94, 95, 96, 97, 98, 99, 100, 101 or 102
x_5 – 114 (6)	63	x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98
x_5 – 120 (6)	65	x_5 is 95, 96, 97, 98, 99, 100, 101, 102 or 103
x_5 – 120 (6)	67	x_5 is 95, 96, 97, 98, 99, 100, 101, 102 or 103
x_5 – 129 (6)	69	x_5 is 104, 105, 106, 107, 108, 109, 110, 111 or 112
x_5 – 109 (6)	71	x_5 is 84, 85, 86, 87, 88, 89, 90, 91, 92 or 93
x_5 – 135 (6)	73	x_5 is 110, 111, 112, 113, 114, 115, 116, 117 or 118
x_5 – 119 (6)	75	x_5 is 94, 95, 96, 97, 98, 99, 100, 101 or 102
x_5 – 109 (6)	77	x_5 is 84, 85, 86, 87, 88, 89, 90, 91, 92 or 93
x_5 – 113 (6)	79	x_5 is 88, 89, 90, 91, 92, 93, 94, 95 or 96
x_5 – 110 (6)	81	x_5 is 86, 87, 88, 89, 90, 91, 92, 93 or 94
x_5 – 116 (6)	83	x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100
x_5 – 115 (6)	85	x_5 is 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99
x_5 – 116 (6)	87	x_5 is 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100
x_5 – 114 (6)	89	x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98
x_5 – 134 (6)	91	x_5 is 108, 109, 110, 111, 112, 113, 114, 115 or 116
x_5 – 113 (6)	93	x_5 is 89, 90, 91, 92, 93, 94, 95, 96, 97 o r 98

$x_5 - 112$ (6)	95	x_5 is 88, 89, 90, 91, 92, 93, 94, 95 or 96
$x_5 - 112$ (6)	97	x_5 is 88, 89, 90, 91, 92, 93, 94, 95 or 96

[0079] In some embodiments, domain 3 may be derived from the same parent as either domain 2, domain 4 or both domain 3 and 4.

[0080] In some embodiment, J1 (Junction 1) between domain 1 and domain 2 comprises the consensus sequence Z_1Z_2W, wherein Z_1 is selected from the group L, V, F, and M, and Z_2 is G or K, wherein 2 of the 3 amino acids are found at the C-terminus of the first domain or the N-terminus of the second domain. In some embodiment, J2 (Junction 2) between domain 2 and domain 3 comprises the consensus sequence CZ_1G, wherein Z_1 is selected from the group H, S, A, L, I, E, K, Q and D, wherein 2 of the 3 amino acids are found at the C-terminus of the second domain or the N-terminus of the third domain. In some embodiment, J3 (Junction 3) between domain 3 and domain 4 comprises the consensus sequence $Z_1Z_2Z_3$, wherein Z_1 is selected from the group T, S, P, G and I, Z_2 is selected from the group consisting of N, K, V, M, H and Y, and Z_3 is selected from the group consisting of H, Y, S, T and P, wherein 2 of the 3 amino acids are found at the C-terminus of the third domain or the N-terminus of the fouth domain. In some embodiment, J4 (Junction 4) between domain 4 and domain 5 comprises the consensus sequence Z_1CZ_2, wherein Z_1 is selected from the group C, S and V, and Z_2 is selected from the group consisting of V, A, I and T, wherein 2 of the 3 amino acids are found at the C-terminus of the fourth domain or the N-terminus of the Fifth domain. In some embodiment, J5 (Junction 1) between the domain 5 and domain 6 comprises the consensus sequence $Z_1Z_2Z_3$, wherein Z_1 is selected from the group L, R and V, Z_2 is selected from the group consisting of T, Q, Y, F and M, and Z_3 is selected from the group consisting of L, F, Y, K, I, Q, V and T, wherein 2 of the 3 amino acids are found at the C-terminus of the fifth domain or the N-terminus of the sixth domain.

[0081] In one embodiment, the disclosure provides the following domains (Table B) for reach of the TGF-beta family members that may be recombined to form a chimera of the disclosure having increased

or improved biological activity (*e.g.*, resistance to inactivation and the like).

[0082] Table B

	Domain 1	Domain 2	Domain 3	Domain 4	Domain 5	Domain 6
BMP-2	1-30	31-48	49-68	69-81	82-93	94-114
BMP-3	1-24	25-42	43-62	63-77	78-89	90-110
BMP-4	1-32	33-50	51-70	71-83	84-95	96-116
BMP-5	1-47	48-65	66-85	86-99	100-111	112-132
BMP-6	1-47	48-65	66-85	86-99	100-111	112-132
BMP-7	1-54	55-72	73-92	93-106	107-118	119-139
BMP-8	1-54	55-72	73-92	93-106	107-118	119-139
BMP-9	1-24	25-42	43-62	63-76	77-88	89-110
BMP-10	1-23	24-41	42-61	62-75	76-87	88-108
BMP-15	1-40	41-58	59-78	79-92	93-104	105-125
GDF-1	1-30	31-48	49-72	73-86	87-98	99-119
GDF-3	1-30	31-48	49-68	69-81	82-93	94-114
GDF-5	1-35	36-53	54-73	74-87	88-99	100-120
GDF-6	1-35	36-53	54-73	74-87	88-99	100-120
GDF-7	1-44	45-62	63-82	83-96	97-108	109-129
GDF-8	1-30	31-48	49-63	64-76	77-88	89-109
GDF-9	1-50	51-68	69-88	89-102	103-114	115-135
GDF-10	1-33	34-51	52-71	72-86	87-98	99-119
GDF-11	1-30	31-48	49-63	64-76	77-88	89-109
GDF-15	1-31	32-49	50-66	67-80	81-92	93-112
NODAL	1-26	27-44	45-64	65-78	79-90	91-110
ACTIVIN-A	1-27	28-45	46-68	69-83	84-95	96-116
Activin-B	1-27	28-45	46-68	69-82	83-94	95-115
Activin-C	1-27	28-45	46-68	69-83	84-95	96-116
Activin-E	1-27	28-45	46-68	69-81	82-93	94-114
INHIBIN-A	1-46	47-64	65-84	85-100	101-112	113-134
TGF-beta1	1-32	33-50	51-68	69-81	82-93	94-113
TGF-beta2	1-31	32-49	50-67	68-80	81-92	93-112
TGF-beta3	1-31	32-49	50-67	68-80	81-92	93-112

[0083] Thus, as illustrated by various embodiments herein, the disclosure provides chimeric TGF-beta family polypeptides, wherein a first TGF-beta family protein (*i.e.*, a first parental protein) is recombined with a second different TGF-beta family protein to provide a chimeric polypeptide. Table 2, below, provides exemplary chimeric polypeptides of the disclosure. In some embodiments, the polypeptide comprises one or more domains of a BMP-2 protein, wherein the segments of the BMP-2 protein comprise segment 1: amino acid residue from about 1 to about x_1 of SEQ ID NO:2 ("1b"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:2 ("2b"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:2 ("3b"); segment 4 is from about amino acid residue x_3 to

about x_4 of SEQ ID NO:2 ("4b"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:2 ("5b"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:2 ("6b"); and wherein: x_1 is residue 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 of SEQ ID NO:2; x_2 is residue 45, 46, 47, or 48 of SEQ ID NO:2; x_3 is residue 65, 66, 67, or 68 of SEQ ID NO:2; x_4 is residue 76, 77, 78, 79, 80, 81 or 82 of SEQ ID NO:2; x_5 is residue 88, 89, 90, 91, 92, 93, or 94 of SEQ ID NO:2; and x_6 is residue 112, 113, or 114 or SEQ ID NO:2, corresponding to the C-terminus of BMP-2, such that a continguous polypeptide comprising segments 1b2b3b4b5b6b comprises a wild-type BMP-2 following the translation initiation codon (ATG). Homologs and proteins having at least about 80%, 90%, 95%, 98%, and 99% identity to the foregoing sequences are also included by the disclosure.

[0084] In other embodiments, the polypeptide comprises one or more domains of an activin protein, wherein the segments of the activin protein comprise segment 1: amino acid residue from about 1 to about x_1 of SEQ ID NO:5 ("1a"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:5 ("2a"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:5 ("3a"); segment 4 is from about amino acid residue x_3 to about x_4 of SEQ ID NO:5 ("4a"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:5 ("5a"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:5 ("6a"); and wherein: x_1 is residue 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 of SEQ ID NO:5; x_2 is residue 42, 43, 44, or 45 of SEQ ID NO:5; x_3 is residue 61, 62, 63, or 64 of SEQ ID NO:5; x_4 is residue 78, 79, 80, 81, 82, 83 or 84 of SEQ ID NO:5; x_5 is residue 90, 91, 92, 93, 94, 95 or 96 of SEQ ID NO:5; and x_6 is residue 114, 115, or 116 or SEQ ID NO:5, corresponding to the C-terminus of activin, such that a continguous polypeptide comprising segments 1a2a3a4a5a6a comprises a wild-type mature activin protein. Homologs and proteins having at least about 80%, 90%, 95%, 98%, and 99% identity to the foregoing sequences are also included by the disclosure.

[0085] In some embodiments, chimeric TGF-beta family polypeptide has a chimeric segmental structure selected from the group consisting of: 1b2b3b4b5b6b; 1b2b3b4b5b6a; 1b2b3b4b5a6a;

1b2b3b4b5a6b; 1b2b3a4a5a6a; 1b2b3a4a5b6a; 1b2a3a4a5a6a; 1b2a3a4a5a6a L66V/V67I; 1b(1a_II)2a3a4a5a6a; 1b2a3a4a5a6b; 1b2a3a4a5b6b; 1b2a3a4a5b6a; 1b2a3b4b5b6a; 1b2a3b4b5a6a; and 1b2a3b4b5a6b.

[0086] In other embodiment, the chimeric polypeptide may be fused to an additional heterologous polypeptide to generate a chimeric fusion polypeptide. The heterologous polypeptide may be, for example, a peptide useful for purification or that permits oligomerization of multiple chimeric polypeptides of the disclosure. The heterologous may be chemically conjugated to the chimeric polypeptide or may be operably linked in-frame with a coding sequence for the chimeric polypeptide.

[0087] In more particular embodiments, the polypeptide comprises a sequence that is (a) at least 80%, 90%, 95%, 98%, or 99% identical to sequence selected from the group consisting of SEQ ID NO: 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33 and has BMP-2 activity; (b) comprises a sequence as set forth in SEQ ID NO: 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33; (c) is encoded by a polynucleotide comprising a sequence selected from the group consisting of SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32; or (d) comprises a sequence described by an algorithm selected from the group consisting of 1b2b3b4b5b6b; 1b2b3b4b5b6a; 1b2b3b4b56a; 1b2b3b4b5a6b; 1b2b3b4a5a6a; 1b2b3b4a5b6b; 1b2b3b4a5a6b; 1b2b3b4a5b6a; 1b2b3a4a5a6a; 1b2b3a4a5a6b; 1b2b3a4a5b6b; 1b2b3a4a5b6a; 1b2b3a4b5b6b; 1b2b3a4b5b6a; 1b2b3a4b5a6a; 1b2b3a4b5a6b; 1b2a3a4a5a6a; 1b2a3a4a5a6b; 1b2a3a4a5b6b; 1b2a3a4a5b6a; 1b2a3a4b5b6b; 1b2a3a4b5b6a; 1b2a3a4b5a6b; 1b2a3a4b5a6a; 1b2a3b4b5b6b; 1b2a3b4b5b6a; 1b2a3b4b5a6a; 1b2a3b4b5a6b; 1b2a3b4a5a6a; 1b2a3b4a5b6a; 1b2a3b4a5b6b; 1b2a3b4a5a6b; 1b2a3a4a5a6a L66V/V67I; and 1b(1a_II)2a3a4a5a6a. In yet another embodiment, the disclosure provides a chimeric TGF-beta polypeptide comprising a segment from BMP-2 and segments from BMP-7 (*e.g.*, a 1b-BMP7 polypetpide; see, *e.g.*, SEQ ID NO:35). In yet another embodiment, the disclosure provides a chimeric TGF-beta polypeptide comprising a segment from BMP-2 and segments from BMP-9 (*e.g.*, a 1b-BMP9; see, *e.g.*, SEQ ID NO:37). In yet another embodiment, the disclosure provides a chimeric TGF-beta polypeptide comprising a segment from BMP-2 and

segments from GDF-7 (*e.g.*, a 1b-GDF7; see, *e.g.*, SEQ ID NO:39). In yet another embodiment, the disclosure provides a chimeric TGF-beta polypeptide comprising a segment from BMP-2 and segments from GDF-8 (*e.g.*, a 1b-GDF8; see, *e.g.*, SEQ ID NO:41). The chimeric polypeptides of the disclosure retain a TGF-beta protein family member activity. Such activity can be measured in any number of ways as described below. In some embodiments, the chimeric polypeptide has BMP-2 activity, but is not inhibited by Noggin.

[0088] In some embodiments, segment of a chimeric polypeptide is 100% identical to the parental strand from which the segment was derived. In other embodiments the segment can comprise an amino acid sequence that has at least 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% or more identity to a corresponding segment in a parental strand. For example, the segment may have one or more conservative amino acid substitutions (*e.g.*, 1-5 conservative amino acid substitutions).

[0089] In some embodiments, the chimeric TGF-beta family polypeptide may have improved activity compared to one or more of the parental strands from which the chimeric polypeptide is generated. Biological activity of a chimeric polypeptide of the disclosure can be measured using any number of recognized assays in the art for TGF-beta activity. Such assays include, but are not limited to, BIAcore (Surface Plasmon Resonance); C_2C1_2 luciferase assay: Smad 1/5 reporter system; HEK293 luciferase assay: Smad 2/3 reporter system; FSH (Follicle Stimulating Hormone) release assay: in rat pituitary cells; BRE (BMP Response Element) luciferase assay: Smad 1/5 reporter HEK 293 cells; Cripto binding assay: Luciferase response measured in presence/absence of Crptio; Human Stem Cell assay: Maintenance or Differentiation of H9 cells; NMR binding Studies; Micro mass culture: Bone formation measured in Chick embryos; X-ray Crystallography: Determine Structure of ligand:receptor complexes; Native Gel: Visualization of ligand:receptor complexes; Size Exclusion Chromatography (SEC): Visualization of ligand:receptor complexes; Velocity Scan Ultracentrifugation: Visualize ligand:receptor complex formation; and Seldi mass Spectrometry: Accurately determine size of ligands.

[0090] The chimeric TGF-beta family polypeptides described herein may be prepared in various forms, such as lysates, crude extracts, or isolated preparations.

[0091] In some embodiments, the isolated chimeric polypeptide is a substantially pure polypeptide composition. A "substantially pure polypeptide" refers to a composition in which the polypeptide species is the predominant species present (*i.e.*, on a molar or weight basis it is more abundant than any other individual macromolecular species in the composition), and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. Generally, a substantially pure polypeptide composition will comprise about 60 % or more, about 70% or more, about 80% or more, about 90% or more, about 95% or more, and about 98% or more of all macromolecular species by mole or % weight present in the composition. In some embodiments, the object species is purified to essential homogeneity (*i.e.*, contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species.

[0092] In certain embodiments, the disclosure contemplates making functional variants by modifying the structure of chimeras. Such modifications may be made, for example, for such purposes as enhancing therapeutic efficacy, or stability (*e.g.*, ex vivo shelf life and resistance to proteolytic degradation *in vivo*, improve stability, solubility, bioavailability, or biodistribution of the chimeric protein, *etc.*). For example, but not by way of limitation, the derivatives include chimeras that have been modified, *e.g.*, by acetylation, carboxylation, acylation glycosylation, pegylation, phosphorylation, farnesylation, biotinylation, lipidation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein such as an organic deriatizing agent, *etc.* Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation,

metabolic synthesis, *etc.* Additionally, the derivative may contain one or more non-natural amino acids, such as those with ketone-containing side chain, polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-natural amino acid elements on the functionality of a chimeric TGF-beta superfamily protein may be tested as described herein for other TGF-beta superfamily protein variants. When a chimeric TGF-beta superfamily protein is produced in cells by cleaving a nascent form of the precursor protein, post-translational processing may also be important for correct folding and/or function of the protein. Different cells (such as CHO, HeLa, MDCK, 293, W138, NIH-3T3 or HEK293) have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct post-translational modification and processing of the precursor protein into a chimeric TGF-beta superfamily protein. *In vitro* cell-free expression system in combination with its associated engineered tRNA synthase and tRNA can be utilized to ensure the correct modification in a specific amino acid position genetically tagged to introduce non-natural amino acids.

[0093] Modified chimeras can also be produced, for instance, by amino acid substitution, deletion, or addition. For instance, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (*e.g.*, conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains.

[0094] In certain embodiments, the disclosure contemplates making mutations in a proteolytic cleavage site of the chimera sequence to make the site less susceptible to proteolytic cleavage. Computer analysis (using a commercially available software, *e.g.*, MacVector, Omega, PCGene, Molecular Simulation, Inc.) can be used to identify proteolytic cleavage sites. As will be recognized by one of skill in the art, most of the described mutations, variants or modifications may be made at the nucleic acid level or, in some

cases, by post translational modification or chemical synthesis. Such techniques are well known in the art.

[0095] In certain embodiments, the disclosure contemplates specific mutations of a chimera sequences so as to alter the glycosylation of the chimera. Such mutations may be selected so as to introduce or eliminate one or more glycosylation sites, such as O-linked or N-linked glycosylation sites. Asparagine-linked glycosylation recognition sites generally comprise a tripeptide sequence, asparagine-X-threonine (where "X" is any amino acid) which are specifically recognized by appropriate cellular glycosylation enzymes. The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the wild-type polypeptide (for O-linked glycosylation sites). A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Another means of increasing the number of carbohydrate moieties is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine; (b) free carboxyl groups; (c) free sulfhydryl groups such as those of cysteine; (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston (1981) CRC Crit. Rev. Biochem., pp. 259-306, incorporated by reference herein. Removal of one or more carbohydrate moieties present on a chimera may be accomplished chemically and/or enzymatically. Chemical deglycosylation may involve, for example, exposure to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the amino acid sequence intact. Chemical deglycosylation is further described by Hakimuddin *et al.* (1987) Arch. Biochem. Biophys. 259:52 and by Edge *et al.* (1981) Anal. Biochem. 118:131. 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.* (1987) Meth. Enzymol. 138:350. The nucleic acid and/or amino acid sequence of a propeptide may be adjusted, as appropriate, depending on the type of expression system used, as mammalian, yeast, insect and plant cells may all introduce differing glycosylation patterns that can be affected by the amino acid sequence of the peptide.

[0096] In some embodiments, the chimeric polypeptide can be in the form of arrays. The polypeptide may be in a soluble form, for example as solutions in the wells of mircotitre plates, or immobilized onto a substrate. The substrate can be a solid substrate or a porous substrate (e.g, membrane), which can be composed of organic polymers such as polystyrene, polyethylene, polypropylene, polyfluoroethylene, polyethyleneoxy, and polyacrylamide, as well as co-polymers and grafts thereof. A solid support can also be inorganic, such as glass, silica, controlled pore glass (CPG), reverse phase silica or metal, such as gold or platinum. The configuration of a substrate can be in the form of beads, spheres, particles, granules, a gel, a membrane or a surface. Surfaces can be planar, substantially planar, or non-planar. Solid supports can be porous or non-porous, and can have swelling or non-swelling characteristics. A solid support can be configured in the form of a well, depression, or other container, vessel, feature, or location. A plurality of supports can be configured on an array at various locations, addressable for robotic delivery of reagents, or by detection methods and/or instruments.

[0097] The disclosure also provides polynucleotides encoding the chimeric TGF-beta family polypeptides disclosed herein. The polynucleotides may be operably linked to one or more heterologous regulatory or control sequences that control gene expression to create a recombinant polynucleotide capable of expressing the polypeptide. Expression constructs containing a polynucleotide encoding the chimeric polypeptide can be introduced into appropriate host cells to express the polypeptide. Polynucleotide sequences encoding various domains or full chimera of the disclosure can be determined without undue efforts based upon the various codons that are associated with an amino acid of in a polypeptide. Furthermore,

the disclosure provides exemplary sequences of the TGF-β family member. Deriving the sequences of a domain or chimera from the sequences provided herein is readily performed by one of skill in the art. Given the knowledge of specific sequences of the TGF-beta family of proteins, and the specific descriptions of the chimeric polypeptides herein (*e.g.*, the segment structure of the chimeric domains), the nucleic acid sequence of the engineered chimera will be apparent to the skilled artisan. The knowledge of the codons corresponding to various amino acids coupled with the knowledge of the amino acid sequence of the polypeptides allows those skilled in the art to make different polynucleotides encoding the polypeptides of the disclosure. Thus, the present disclosure contemplates each and every possible variation of the polynucleotides that could be made by selecting combinations based on possible codon choices, and all such variations are to be considered specifically disclosed for any of the polypeptides described herein.

[0098] In some embodiments, the polynucleotides comprise polynucleotides that encode the polypeptides described herein but have about 80% or more sequence identity, about 85% or more sequence identity, about 90% or more sequence identity, about 91% or more sequence identity, about 92% or more sequence identity, about 93% or more sequence identity, about 94% or more sequence identity, about 95% or more sequence identity, about 96% or more sequence identity, about 97% or more sequence identity, about 98% or more sequence identity, or about 99% or more sequence identity at the nucleotide level to a reference polynucleotide encoding a chimera or parental TGF-beta family polypeptide.

[0099] In some embodiments, the isolated polynucleotides encoding the polypeptides may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides and nucleic acid sequences utilizing recombinant DNA methods are well known in the art. Guidance is provided in Sambrook *et al.*, 2001, Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press; and

Current Protocols in Molecular Biology, Ausubel. F. ed., Greene Pub. Associates, 1998, updates to 2007.

[00100] In some embodiments, the polynucleotides are operatively linked to control sequences for the expression of the polynucleotides and/or polypeptides. In some embodiments, the control sequence may be an appropriate promoter sequence, which can be obtained from genes encoding extracellular or intracellular polypeptides, either homologous or heterologous to the host cell.

[00101] In some embodiments, the control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used.

[00102] In some embodiments, the control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used.

[00103] In some embodiments, the control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region that is foreign to the coding sequence. The foreign signal peptide-coding region may be required where the coding sequence does not naturally contain a signal peptide coding region.

[00104] The disclosure is further directed to a recombinant expression vector comprising a polynucleotide encoding the chimeric TGF-beta polypeptides described herein, and one or more expression regulating regions such as a promoter and a terminator, a replication origin, *etc.*, depending on the type of hosts into which

they are to be introduced. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[00105] The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus), which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the polynucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell or *in vitro* cell-free reaction mixture into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

[00106] The expression vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

[00107] In some embodiments, the expression vector of the disclosure contains one or more selectable markers, which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

[00108] In another embodiments, the disclosure provides a host cell comprising a polynucleotide encoding the chimeric TGF-beta polypeptide, the polynucleotide being operatively linked to one or more control sequences for expression of the fusion polypeptide in the host cell. Host cells for use in expressing the fusion polypeptides encoded by the expression vectors of the present disclosure are well known in the art. Appropriate culture mediums and growth conditions for the above-described host cells are well known in the art.

[00109] Expression vectors can be designed for expression of chimeras in prokaryotic or eukaryotic cells. For example, chimeras of the disclosure can be expressed in bacterial or prokaryote cells such as E. Coli, insect cells (*e.g.*, the baculovirus expression system), yeast cells, microalgae, plant cells or mammalian cells as well as *in vitro* cell-free expression system. Some suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990).

[00110] While one example of an expression system discussed is an *E. coli* expression system, to those skilled in the art, these proteins can be easily be cloned into and expressed from a large number of other expression systems. The advantages include, but are not limited to, achieving post-translational modifications as would be seen in the organism the protein was derived from (in this case *H. sapiens*), expression of the ligands without the start methionine required for bacterial expression, and easy incorporation of non-natural amino acids or additional chemical modifications. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as E. coli. Various E. coli strains are publicly available, such as E. coli K12 strain MM294 (ATCC 31,446); E. coli X1776 (ATCC 31,537); E. coli strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635). In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for VEGF-E-encoding vectors. Saccharomyces cerevisiae is a commonly used lower eukaryotic host microorganism.

[00111] Suitable host cells for the expression of chimeras are derived from unicellular and multicellular organisms. Examples of invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sf9, as well as plant cells. Plant expression systems have also been used successfully to express modified proteins. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol., 36:59 (1977)); Chinese hamster ovary cells/-DHFR(CHO, Urlaub and Chasin, Proc.

Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

[00112] Alternate protein expression systems include human embryonic kidney (HEK) 293 cells, insect cell line (*S. frugiperda*) utilizing the baculovirus expression system, yeast expression systems not limited to *P. pastoris* and *S. cerevisiae*, and numerous Microalgae strains. Transgenic animals can be used to express correctly modified protein. In essence, the animals become living 'bioreactors' capable of expressing large amounts of the desired protein in an easily harvested fluid or tissue, such as the milk from a cow. Cell-free *in vitro* expression systems using either the bacterial or wheat germ cell lysate can be employed. Cell-free expression system will permit inserting a wide range of non-natural amino acids or epitope tags with higher efficiency and greater specificity.

[00113] Examples of bacterial vectors include pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, and pRIT5 (Pharmacia). Examples of vectors for expression in the yeast S. cerevisiae include pYepSec1 (Baldari *et al.*, EMBO J. 6:229 (1987)), pMFa (Kurjan and Herskowitz, Cell 30:933 (1982)), pJRY88 (Schultz *et al.*, Gene 54:113 (1987)), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of nucleic acids to produce proteins in cultured insect cells (*e.g.*, Sf9 cells) include the pAc series (Smith *et al.*, Mol. Cell. Biol. 3:2156 (1983)) and the pVL series (Lucklow and Summers Virology 170:31 (1989)).

[00114] Examples of mammalian expression vectors include pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, PBPV, pMSG, PSVL (Pharmacia), pCDM8 (Seed, Nature 329:840 (1987)) and pMT2PC (Kaufman *et al.*, EMBO J. 6:187 (1987)). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are

derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40.

[00115] Viral vectors have been used in a wide variety of gene delivery applications in cells, as well as living animal subjects. Viral vectors that can be used include, but are not limited to, retrovirus, lentivirus, adeno-associated virus, poxvirus, alphavirus, baculovirus, vaccinia virus, herpes virus, Epstein-Barr virus, adenovirus, geminivirus, and caulimovirus vectors. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), nucleic acid-protein complexes, and biopolymers. In addition to a nucleic acid of interest, a vector may also comprise one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (delivery to specific tissues, duration of expression, *etc.*).

[00116] The chimera of the disclosure can be made by using methods well known in the art. Polynucleotides can be synthesized by recombinant techniques, such as that provided in Sambrook *et al.*, 2001, Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press; and Current Protocols in Molecular Biology, Ausubel. F. ed., Greene Pub. Associates, 1998, updates to 2007. Polynucleotides encoding the enzymes, or the primers for amplification can also be prepared by standard solid-phase methods, according to known synthetic methods, for example using phosphoramidite method described by Beaucage *et al.*, (1981) Tet Lett 22:1859-69, or the method described by Matthes *et al.*, (1984) EMBO J. 3:801-05, *e.g.*, as it is typically practiced in automated synthetic methods. In addition, automated peptide synthesizers are commercially available (*e.g.*, Advanced ChemTech Model 396; Milligen/Biosearch 9600).

[00117] In a one embodiment, the disclosure is directed to a method to accelerate construction of large chimera libraries. Accordingly, the disclosure provides a recombinant strategy termed RASCH (RAndom Segmental CHimera) See FIG. 17. It uses a template sequence (first strand from one TGF-beta superfamily member) and a few target sequences (second (third, fourth, fifth, sixth) strand from one or more alternate TGF-beta superfamily members), whose subdomains are to be linked. The template DNA sequence is used to

promote efficient coupling of the target sequences and is degraded once subdomains are linked. Following the gene construction to create the chimeric sequences, the new ligands are chemically refolded into functional dimer. This dimerization process permits additional diversification of the final sequence by mixing and dimerizing two different sequences of both natural and designer origins. Therefore, the RASCH method can be used to diversify the approximate 40 natural protein sequences of TGF-beta superfamily ligands into ten of thousands or more variant sequences, each distinct from any naturally-ocurring TGF-beta superfamily ligand sequences.

[00118] Engineered polypeptide expressed in a host cell can be recovered from the cells and or the culture medium using any one or more of the well known techniques for protein purification, including, among others, lysozyme treatment, sonication, filtration, salting-out, ultra-centrifugation, chromatography, and affinity separation (*e.g.*, substrate bound antibodies).

[00119] Chromatographic techniques for isolation of the polypeptides include, among others, reversed phase chromatography high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, and affinity chromatography. Conditions for purifying a particular enzyme will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, molecular weight, molecular shape, *etc.*, and will be apparent to those having skill in the art.

[00120] Assays to determine activity are well known in the art. The present disclosure relates to assays to test for biological activity of chimeric proteins, more preferably, to assays to test for clinical activity. Such activity can include enhanced agonistic or antagonistic TGF-beta activity, combined or novel biological activity, and the like.

[00121] In certain embodiments, a chimeric protein of the disclosure comprising an agonist of a TGF-beta superfamily protein comprises an antagonist of a different TGF-beta superfamily protein.

[00122] Irrespective of which protein expression, harvesting, and, folding methodologies are used, certain of the subject chimeric proteins can bind, preferentially to a pre-selected receptor and can

now be identified using standard methodologies, *e.g.*, ligand/receptor binding assays, well known, and thoroughly documented in the art. See, *e.g.*, Legerski gl al. (1992) Bio h˜_Biophys. Res. Comm. 183: 672679; Frakar *et al.* (1978) Biochem. Bio12-hys. Res. Comm 80:849-857; Chio et el. (1990) Nature 343: 266-269; Dahlman *et al.* (1988) Biochem 27: 1813-1817; Strader et el. (1989) J. Biol. Chem. 264: 13572-13578; and DDowd *et al.* (1988) J. Biol. Chem. 263: 15985-15992.

[00123] Typically, in a ligand/receptor binding assay, the native or parent TGF-beta superfamily member of interest having a known, quantifiable affinity for a pre-selected receptor is labeled with a detectable moiety, for example, a radiolabel, a chromogenic label, or a fluorogenic label. Aliquots of purified receptor, receptor binding domain fragments, or cells expressing the receptor of interest on their surface are incubated with the labeled TGF-beta superfamily member in the presence of various concentrations of the unlabeled chimeric protein. The relative binding affinity of a candidate chimeric protein may be measured by quantitating the ability of the chimeric protein to inhibit the binding of the labeled TGF-beta superfamily member with the receptor. In performing the assay, fixed concentrations of the receptor and the TGF-beta superfamily member are incubated in the presence and absence of unlabeled chimeric protein. Sensitivity may be increased by preincubating the receptor with the chimeric protein before adding the labeled template TGF-beta superfamily member. After the labeled competitor has been added, sufficient time is allowed for adequate competitor binding, and then free and bound labeled TGF-beta superfamily members are separated from one another, and one or the other measured. Labels useful in the practice of the screening procedures include radioactive labels, chromogenic labels, spectroscopic labels such as those disclosed in Haughland (1994) "Handbook of Fluorescent and Research Chemicals," 5 ed. by Molecular Probes, Inc., Eugene, Oreg., or conjugated enzymes having high turnover rates, *i.e.*, horseradish peroxidase, alkaline phosphatase, or agalactosidase, used in combination with chemiluminescent or fluorogenic substrates. The biological activity, namely the agonist or antagonist properties of the resulting chimeric protein

constructs can subsequently be characterized using conventional *in vivo* and *in vitro* assays that have been developed to measure the biological activity of any TGF-beta superfamily member. It is appreciated, however, the type of assay used preferably depends on the TGF-a superfamily member upon which the chimeric protein is based. For example, chimeric constructs based upon naturally occurring BMP-2 protein may be assayed using any of the biological assays that have been developed to date for measuring BMP-2 activity, described in more detail below.

[00124] The presence of multimers among the subject chimeric proteins can be detected visually either by standard SDS-PAGE in the absence of a reducing agent such as DTT or by HPLC (*e.g.*, C18 reverse phase HPLC). Multimeric proteins of the present disclosure can have an apparent molecular weight proportionally greater than the monomeric subunit, *e.g.*, in the range about 28-36 kDa for a dimer, as compared to monomeric subunits, which may have an apparent molecular weight of about 14-18 kDa. The multimeric protein can readily be visualized on an electrophoresis gel by comparison to commercially available molecular weight standards. The dimeric protein also elutes from a C18 RP HPLC (45-50% acetonitrile: 0.1% TFA) at a time point different from that for its monomeric counterpart.

[00125] A second assay evaluates the presence of dimer (*e.g.*, OP-1 dimers) by its ability to bind to hydroxyapatite. Optimally-folded dimer binds a hydroxyapatite column well in pH7, 10 mM phosphate, 6M urea, and 0.142M NaCl (dimer elutes at 0.25 M NaCl) as compared to monomer, which does not bind substantially at those concentrations (monomer elutes at 0.1M NaCl). A third assay evaluates the presence of dimer by the protein's resistant to trypsin or pepsin digestion. The folded dimeric species is substantially resistant to both enzymes, particularly trypsin, which cleaves only a small portion of the N-terminus of the mature protein, leaving a biologically active dimeric species only slightly smaller in size than the untreated dimer (each monomer in amino acids smaller after trypsin cleavage). By contrast, the monomers and misfolded dimers are substantially degraded. In the assay, the protein is subjected to an enzyme digest using standard conditions, *e.g.*, digestion in a standard buffer such

as 50 mM Tris buffer, pH 8, containing 4 M urea, 100 mM NaCl, 0.3% Tween-80 and 20 mM methylamine. Digestion is allowed to occur at 37° C. for on the order of 16 hours, and the product visualized by any suitable means, preferably SDS PAGE.

[00126] The biological activity of the subject chimeric proteins, for example, the chimeric proteins having one or more segments from BMPs, can be assessed by any of a number of means as described in WO00/20607. For example, the protein's ability to induce endochondral bone formation can be evaluated using the well characterized rat subcutaneous bone assay. In the assay bone formation is measured by histology, as well as by alkaline phosphatase and/or osteoclacin production. In addition, osteogenic proteins having high specific bone forming activity, such as OP-1, BMP-2, BMR4, BMP-5 and BMP-6, also induce alkaline phosphatase activity in an *in vitro* rat osteoblast or osteosarcoma cell-based assay. Such assays are well described in the art. See, for example, Sabokdar of al. (1994) Bone and Mineral 27:57-67.; Knutsen *et al.* (1993) Biochem Biophvs Res. Commun 194:1352-1358; and Maliakal *et al.* (1994) Growth Factors 1:227-234).

[00127] By contrast, osteogenic proteins having low specific bone forming activity, such as CDMP-1 and CDMP-2, for example, do not induce alkaline phosphatase activity in the cell based osteoblast assay. The assay thus provides a ready method for evaluating biological activity of B1b9 mutants. For example, CDMP-1, CDMP-2 and CMDP-3 all are competent to induce bone formation, although with a lower specific activity than BMP-2, BW-4, BV-5, BMP-6 or OP-1. Conversely, BMP-2, BMP-4, BMP-5, BPylP-6 and OP-1 all can induce articular cartilage formation, albeit with a lower specific activity than CDMP-1, CDMP-2 or CDMP-3. Accordingly, a chimeric protein having one or more segment from CDMP, designed and described herein to be a chimeric protein competent to induce alkaline phosphatase activity in the cell-based assay, is expected to demonstrate a higher specific bone forming activity in the rat animal bioassay.

[00128] The chimeric protein's biological activity can also be readily evaluated by the protein's ability to inhibit epithelial cell growth. A useful, well characterized *in vitro* assay utilizes mink lung cells or melanoma cells. See WO00/20607. Other assays for

other members of the TGF-beta superfamily are well described in the literature and can be performed without undue experimentation.

[00129] In certain embodiment, the disclosure provides methods and agents for control and maintain skeletal muscle mass in a host, preferably a human. Therefore, any chimeric protein of the disclosure that is expected to affect muscle-related function of a TGF-beta superfamily protein such as for example GDF-8 can be tested in whole cells or tissues, *in vitro* or *in vivo*, to confirm their ability to modulate skeletal muscle mass. GDF-8 (also known as myostatin) is a negative regulator of skeletal muscle growth. GDF-8 knockout mice have approximately twice the skeletal muscle mass of normal mice. The effects of increased muscle mass on bone modeling may be investigated, *e.g.*, by examining bone mineral content (BMC) and bone mineral density (BMD) in the femora of female GDF-8 knockout mice. Dual-energy X-ray absorptiometry (DEXA) densitometry can be used to measure whole-femur BMC and BMD, and PQCT densitometry can be used to calculate BMC and BMD from cross-sections of tissues. Hamrick, Anat Rec. 2003 May; 272A(1):388-91. As is known in the art, a chimeric protein of the disclosure may be introduced into the GDF-8 knockout mice, and similar assays can be used to determine the effect of the chimeric protein on skeletal muscle mass and bone density.

[00130] The dystrophic phenotype in the mdx mouse model of Duchenne muscular dystrophy (DMD) may also be employed to test the biological activity of a chimeric protein of the disclosure. It was reported that blockade of endogenous myostatin by using intraperitoneal injections of blocking antibodies for three months resulted in an increase in body weight, muscle mass, muscle size and absolute muscle strength in mdx mouse muscle along with a significant decrease in muscle degeneration and concentrations of serum creatine kinase. Bogdanovich *et al.*, Nature. 2002 Nov. 28; 420(6914):418-21. Similar study may be employed to determine whether a chimeric protein of the disclosure potentiates or inhibits the endogenous GDF-8 activity.

[00131] In certain embodiments, the disclosure provides methods and agents for modulating neurogenesis. For example, GDF-11 is known to inhibit olfactory epithelium neurogenesis *in vitro* by inducing

p27(Kip1) and reversible cell cycle arrest in progenitors. Wu *et al.* Neuron. 2003 Jan. 23; 37(2):197-207. The effect of a chimeric protein of the disclosure on neurogenesis can be similarly tested. Further, the effect of a chimeric protein of the disclosure on GDF-11's effect on neurogenesis can also be tested using similar assays as described in Wu *et al.* Id.

[00132] In certain embodiment, the disclosure provides methods and agents for stimulating bone formation and increasing bone mass. Therefore, any chimeric protein of the disclosure that is expected to affect bone-related function of a TGF-beta superfamily protein such as for example BMP-2, BMP-3, GDF-10, BMP-4, BMP-7, or BMP-8, can be tested in whole cells or tissues, *in vitro* or *in vivo*, to confirm their ability to modulate bone or cartilage growth. Various methods known in the art can be utilized for this purpose. For example, BMP-3 inhibits BMP2-mediated induction of Msx2 and blocks BMP2-mediated differentiation of osteoprogenitor cells into osteoblasts. Thus, the effect of a subject chimer protein, preferably one comprising a segment from a BMP-2 or BMP-3, on bone or cartilage growth can be determined by their effect on the osteogenic activity of BMP-2, for example, by measuring induction of Msx2 or differentiation of osteoprogenitor cells into osteoblasts in cell based assays (see, *e.g.*, Daluiski *et al.*, Nat. Genet. 2001, 27(1):84-8; Hino *et al.*, Front Biosci. 2004, 9:1520-9). Similarly, a subject chimeric protein, preferably one comprising a segment from a BMP-2 or BMP-3, may be tested for its osteogenic or anti-osteogenic activity or its agonistic or antagonistic effect on BMP-2-mediated osteogenesis.

[00133] Another example of cell-based assays includes analyzing the osteogenic or anti-osteogenic activity of a subject chimeric and test compounds in mesenchymal progenitor and osteoblastic cells. To illustrate, recombinant adenoviruses expressing a subject chimeric protein were constructed to infect pluripotent mesenchyimal progenitor C3H10T1/2 cells, preosteoblastic C2C12 cells, and osteoblastic TE-85 cells. Osteogenic activity is then determined by measuring the induction of alkaline phosphatase, osteocalcin, and matrix mineralization (see, *e.g.*, Cheng *et al.*, J bone Joint Surg Am. 2003, 85-A(8):1544-52).

[00134] Further, the disclosure contemplates *in vivo* assays to measure bone or cartilage growth. For example, Namkung-Matthai *et al.*, Bone, 28:80-86 (2001) discloses a rat osteoporotic model in which bone repair during the early period after fracture is studied. Kubo *et al.*, Steroid Biochemistry & Molecular Biology, 68:197-202 (1999) also discloses a rat osteoporotic model in which bone repair during the late period after fracture is studied. These references are incorporated by reference herein in their entirety for their disclosure of rat model for study on osteoporotic bone fracture. In certain aspects, the present disclosure makes use of fracture healing assays that are known in the art. These assays include fracture technique, histological analysis, and biomechanical analysis, which are described in, for example, U.S. Pat. No. 6,521,750, which is incorporated by reference in its entirety for its disclosure of experimental protocols for causing as well as measuring the extent of fractures, and the repair process.

[00135] It is understood that the screening assays of the disclosure apply to not only the subject chimeric proteins and variants thereof, but also any test compounds including agonists and antagonist of the chimeric proteins or their variants themselves. Further, these screening assays are useful for drug target verification and quality control purposes.

[00136] In other embodiment, the disclosure relates to the use of the subject chimeric TGF-beta superfamily proteins to identify compounds which can modulate activities of the chimeric proteins. Compounds identified through this screening can be tested in tissues (*e.g.*, bone and/or cartilage) or cells (*e.g.*, muscle cells) to assess their ability to modulate the test tissues or cells (*e.g.*, bone/cartilage growth or muscle cell growth) *in vitro*. Optionally, these compounds can further be tested in animal models to assess their ability to modulate, *e.g.*, bone/cartilage growth or muscle control and maintenance *in vivo*.

[00137] A variety of assay formats will suffice and, in light of the disclosure, those not expressly described herein will nevertheless be comprehended by one of ordinary skill in the art. As described herein, the test compounds (agents) of the disclosure may be created by any combinatorial chemical method. Alternatively, the

subject compounds may be naturally occurring biomolecules synthesized *in vivo* or *in vitro*. Compounds (agents) to be tested for their ability to act as modulators of bone or cartilage growth can be produced, for example, by bacteria, yeast, plants or other organisms (*e.g.*, natural products), produced chemically (*e.g.*, small molecules, including peptidomimetics), or produced recombinantly. Test compounds contemplated by the present disclosure include non-peptidyl organic molecules, peptides, polypeptides, peptidomimetics, sugars, hormones, and nucleic acid molecules. In a specific embodiment, the test agent is a small organic molecule having a molecular weight of less than about 2,000 daltons.

[00138] The test compounds of the disclosure can be provided as single, discrete entities, or provided in libraries of greater complexity, such as made by combinatorial chemistry. These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, esters, aldehydes, ethers and other classes of organic compounds. Presentation of test compounds to the test system can be in either an isolated form or as mixtures of compounds, especially in initial screening steps. Optionally, the compounds may be optionally derivatized with other compounds and have derivatizing groups that facilitate isolation of the compounds. Non-limiting examples of derivatizing groups include biotin, fluorescein, digoxygenin, green fluorescent protein, isotopes, polyhistidine, magnetic beads, glutathione S transferase, photoactivatible crosslinkers or any combinations thereof.

[00139] In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an

alteration of binding affinity between a chimeric TGF-beta superfamily protein and its binding protein (*e.g.*, the chimeric protein itself or a TGF-beta receptor protein or fragments thereof).

[00140] Merely to illustrate, in an exemplary screening assay of the present disclosure, the compound of interest is contacted with an isolated and purified chimeric protein which is ordinarily capable of binding to a TGF-beta receptor protein or fragments thereof, as appropriate for the intention of the assay. To the mixture comprising a subject chimeric protein and a TGF-beta receptor protein is then added a composition containing a test compound. Detection and quantification of the chimeric protein receptor complexes provides a means for determining the compound's efficacy at inhibiting (or potentiating) complex formation between the chimeric TGF-beta superfamily protein and its binding protein, *e.g.*, the TGF-beta receptor or fragments thereof. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. For example, in a control assay, an isolated and purified chimeric TGF-beta superfamily protein is added to a composition (cell-free or cell-based) containing a TGF-beta receptor protein or fragment thereof, and the formation of the chimeric protein-receptor complex is quantitated in the absence of the test compound. It will be understood that, in general, the order in which the reactants may be admixed can be varied, and can be admixed simultaneously. Moreover, in place of purified proteins, cellular extracts and lysates may be used to render a suitable cell-free assay system. Alternatively, cells expressing a TGF-beta receptor protein or fragments thereof on their surfaces can be used in certain assays.

[00141] Complex formation between a subject chimeric TGF-beta superfamily protein and its binding protein may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabelled (*e.g.*, 32 P, 35 S, 14 C or 3 H), fluorescently labeled (*e.g.*, FITC), or enzymatically labeled

chimeric protein or its binding protein, by immunoassay, or by chromatographic detection.

[00142] In certain embodiments, the present disclosure contemplates the use of fluorescence polarization assays and fluorescence resonance energy transfer (FRET) assays in measuring, either directly or indirectly, the degree of interaction between a chimeric TGF-beta superfamily protein and its binding protein (*e.g.*, a TGF-beta receptor protein or fragments thereof). Further, other modes of detection such as those based on optical waveguides (PCT Publication WO 96/26432 and U.S. Pat. No. 5,677,196), surface plasmon resonance (SPR), surface charge sensors, and surface force sensors are compatible with many embodiments of the disclosure.

[00143] Moreover, the present disclosure contemplates the use of an interaction trap assay, also known as the "two hybrid assay," for identifying agents that disrupt or potentiate interaction between a chimeric TGF-beta superfamily protein and its binding protein (*e.g.*, a TGF-beta receptor protein or fragments thereof). See for example, U.S. Pat. No. 5,283,317; Zervos *et al.* (1993) Cell 72:223-232; Madura *et al.* (1993) J Biol Chem 268:12046-12054; Bartel *et al.* (1993) Biotechniques 14:920-924; and Iwabuchi *et al.* (1993) Oncogene 8:1693-1696).

[00144] Chimera polynucleotides, polypeptides, antibodies, cells and other reagents of the disclosure have a wide variety of uses, both *in vitro* and *in vivo*. For example, in representative embodiments, these reagents may be used *in vitro* or *in vivo* (*e.g.*, in an animal model) to study the processes of mineralization, bone formation, and bone loss. Further, "knock in" and "knock out" animals can be used as animal models of disease or as screening tools (discussed more below) for compounds that interact with the chimera polynucleotides or polypeptides. It will be apparent to those skilled in the art that any suitable vector can be used to deliver the polynucleotide to a cell or subject. The choice of delivery vector can be made based on a number of factors known in the art, including age and species of the target host, *in vitro* versus *in vivo* delivery, level and persistence of expression desired, intended purpose (*e.g.*, for therapy or screening), the

target cell or organ, route of delivery, size of the isolated polynucleotide, safety concerns, and the like.

[00145] Chimeric polypeptide of the disclosure may be formulated for use in various biological systems including *in vivo*. Any of a variety of art-known methods can be used to administer a chimera either alone or in combination with other active agents. For example, administration can be parenterally by injection or by gradual infusion over time. The agent(s) can be administered by such means as oral, rectal, buccal (*e.g.*, sub-lingual), vaginal, parenteral (*e.g.*, subcutaneous, intramuscular including skeletal muscle, cardiac muscle, diaphragm muscle and smooth muscle, intradermal, intravenous, intraperitoneal), topical (*i.e.*, both skin and mucosal surfaces, including airway surfaces), intranasal, transdermal, intraarticular, intrathecal, intracavity, and inhalation administration, administration to the liver by intraportal delivery, as well as direct organ injection (*e.g.*, into the liver, into the brain for delivery to the central nervous system, into the pancreas). The most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular compound which is being used.

[00146] The disclosure also provides a pharmaceutical preparation comprising a subject chimeric protein and a pharmaceutically acceptable carrier. A pharmaceutical preparation may be employed to promote growth of a tissue or diminishing or prevent loss of a tissue in a subject, preferably a human. The targeted tissue can be, for example, bone, cartilage, skeletal muscle, cardiac muscle and/or neuronal tissue.

[00147] In another aspect, a chimeric TGF-beta polypeptide can be formulated either alone or in combination with other agents for administration (*e.g.*, as a lotion, cream, spray, gel, or ointment). It may be formulated into liposomes to reduce toxicity or increase bioavailability. Other methods for delivery include oral methods that entail encapsulation of the in microspheres or proteinoids, aerosol delivery (*e.g.*, to the lungs), or transdermal delivery (*e.g.*, by iontophoresis or transdermal electroporation). Other methods of administration will be known to those skilled in the art.

[00148] Preparations for parenteral administration of a composition comprising a chimeric TGF-beta polypeptide include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils (*e.g.*, olive oil), and injectable organic esters such as ethyl oleate. Examples of aqueous carriers include water, saline, and buffered media, alcoholic/aqueous solutions, and emulsions or suspensions. Examples of parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives such as, other antimicrobial, anti-oxidants, cheating agents, inert gases and the like also can be included.

[00149] The disclosure provides various disease and disorders that may be modulated by a TGF-beta protein family member comprising contacting or administering a therapeutically effective amount of a chimeric TGF-beta polypeptide either alone or in combination with other agents to a subject who has, or is at risk of having, such a disorder.

[00150] A therapeutically effective amount can be measured as the amount sufficient to decrease a subject's symptoms associated with the diseases or disorder. Typically, the subject is treated with an amount of a therapeutic composition sufficient to reduce a symptom of a disease or disorder by at least 50%, 90% or 100%. Generally, the optimal dosage will depend upon the disorder and factors such as the weight of the subject, the age, the weight, sex, and degree of symptoms. For example, with respect to bone morphogenesis, optionally, the dosage may vary with the type of matrix used in the reconstitution and the types of compounds in the composition. The addition of other known growth factors to the final composition, may also affect the dosage. Progress can be monitored by periodic assessment of bone growth and/or repair, for example, X-rays, histomorphometric determinations, and tetracycline labeling. Nonetheless, suitable dosages can readily be determined by one

skilled in the art. Typically, a suitable dosage is 0.5 to 40 mg/kg body weight, *e.g.*, 1 to 8 mg/kg body weight.

[00151] As mentioned previously, the compositions and methods of the disclosure can include the use of additional (*e.g.*, in addition to a chimeric TGF-beta polypeptide) therapeutic agents (*e.g.*, an inhibitor of TNF, an antibiotic, and the like). The chimeric TGF-beta polypeptide, other therapeutic agent(s), and/or antibiotic(s) can be administered, simultaneously, but may also be administered sequentially.

[00152] A pharmaceutical composition comprising a chimera according to the disclosure can be in a form suitable for administration to a subject using carriers, excipients, and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol, and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents, and inert gases. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described, for instance, in Remington's Pharmaceutical Sciences, 15th ed., Easton: Mack Publishing Co., 1405-1412, 1461-1487 (1975), and The National Formulary XIV., 14th ed., Washington: American Pharmaceutical Association (1975), the contents of which are hereby incorporated by reference. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art. See Goodman and Gilman's, The Pharmacological Basis for Therapeutics (7th ed.).

[00153] The pharmaceutical compositions according to the disclosure may be administered locally or systemically. A "therapeutically effective dose" is the quantity of an agent according to the disclosure necessary to prevent, to cure, or at least partially arrest a symptoms associated with a disease or disorder or to promote cell growth, proliferation or

differentiation. Amounts effective for this use will, of course, depend on the severity of the disease, disorder, or desired effect and will depend on weight and general state of the subject. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of infections. Various considerations are described, *e.g.*, in Langer, Science, 249: 1527, (1990); Gilman *et al.* (eds.) (1990), each of which is herein incorporated by reference. Dosages of pharmaceutically active compounds can be determined by methods known in the art, see, *e.g.*, Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.); Remington, The Science & Practice of Pharmacy, (Lippincott Williams & Wilkins; Twenty first Edition). The therapeutically effective dosage of any specific compound will vary somewhat from compound to compound, and patient to patient, and will depend upon the condition of the patient and the route of delivery. As a general proposition, a dosage from about 0.1 to about 100 mg/kg will have therapeutic efficacy, with all weights being calculated based upon the weight of the compound, including the cases where a salt is employed. Toxicity concerns at the higher level can restrict intravenous dosages to a lower level such as up to about 10 to about 20 mg/kg, with all weights being calculated based upon the weight of the compound, including the cases where a salt is employed. A dosage from about 10 mg/kg to about 50 mg/kg can be employed for oral administration. Typically, a dosage from about 0.5 mg/kg to 15 mg/kg can be employed for intramuscular injection. Particular dosages are about 1 µmol/kg to 50 µmol/kg, and more particularly to about 22 µmol/kg and to 33 µmol/kg of the compound for intravenous or oral administration, respectively.

[00154] In particular embodiments of the disclosure, more than one administration (*e.g.*, two, three, four, or more administrations) can be employed over a variety of time intervals (*e.g.*, hourly, daily, weekly, monthly, *etc.*) to achieve therapeutic effects.

[00155] The compositions and chimera of the disclosure find use in veterinary and medical applications. Suitable subjects include both avians and mammals, with mammals being preferred. The term

"avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys, and pheasants. The term "mammal" as used herein includes, but is not limited to, humans, bovines, ovines, caprines, equines, felines, canines, lagomorphs, *etc.* Human subjects include neonates, infants, juveniles, and adults. In other embodiments, the subject is an animal model of bone disease.

[00156] As used herein, "administering a therapeutically effective amount" is intended to include methods of giving or applying a pharmaceutical composition of the disclosure to a subject that allow the composition to perform its intended therapeutic function.

[00157] The pharmaceutical composition can be administered in a convenient manner, such as by injection (subcutaneous, intravenous, *etc.*), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the pharmaceutical composition can be coated with a material to protect the pharmaceutical composition from the action of enzymes, acids, and other natural conditions that may inactivate the pharmaceutical composition. The pharmaceutical composition can also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

[00158] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the composition should be sterile and should be fluid to the extent that easy syringability exists. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size, in the case of dispersion, and by the use of surfactants. Prevention of the action of microorganisms can be

achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be typical to include isotonic agents, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

[00159] Sterile injectable solutions can be prepared by incorporating the pharmaceutical composition in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the pharmaceutical composition into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above.

[00160] The pharmaceutical composition can be orally administered, for example, with an inert diluent or an assimilable edible carrier. The pharmaceutical composition and other ingredients can also be enclosed in a hard or soft-shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. For oral therapeutic administration, the pharmaceutical composition can be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations can, of course, be varied and can conveniently be between about 5% to about 80% of the weight of the unit.

[00161] The tablets, troches, pills, capsules, and the like can also contain the following: a binder, such as gum gragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid, and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin, or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a

capsule, it can contain, in addition to materials of the above type, a liquid carrier. Various other materials can be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules can be coated with shellac, sugar, or both. A syrup or elixir can contain the agent, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye, and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic/biocompatible in the amounts employed. In addition, the pharmaceutical composition can be incorporated into sustained-release preparations and formulations.

[00162] Thus, a "pharmaceutically acceptable carrier" is intended to include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the pharmaceutical composition, use thereof in the therapeutic compositions and methods of treatment is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[00163] In certain embodiments, the therapeutic method of the disclosure includes administering the composition topically, systemically, or locally as an implant or device. When administered, the therapeutic composition described by the disclosure are generally in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than the chimeras of the disclosure may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the chimeras in the methods of the described herein. For example, preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering BMP chimeras or other therapeutic compounds to the site of bone and/or cartilage damage,

providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. For example, the matrix may provide slow release of the BMP chimeras. Such matrices may be formed of materials presently in use for other implanted medical applications.

[00164] The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the subject compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are non-biodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the aforementioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

[00165] Certain compositions disclosed herein may be administered topically, either to skin or to mucosal membranes. The topical formulations may further include one or more of the wide variety of agents known to be effective as skin or stratum corneum penetration enhancers. Examples of these are 2-pyrrolidone, N-methyl-2-pyrrolidone, dimethylacetamide, dimethylformamide, propylene glycol, methyl or isopropyl alcohol, dimethyl sulfoxide, and azone. Additional agents may further be included to make the formulation cosmetically acceptable. Examples of these are fats, waxes, oils, dyes, fragrances, preservatives, stabilizers, and surface active agents. Keratolytic agents such as those known in the art may also be included. Examples are salicylic acid and sulfur.

[00166] It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and

uniformity of dosage. "Dosage unit form" as used herein, refers to physically discrete units suited as unitary dosages for the individual to be treated; each unit containing a predetermined quantity of pharmaceutical composition is calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the disclosure are related to the characteristics of the pharmaceutical composition and the particular therapeutic effect to be achieve.

[00167] The principal pharmaceutical composition is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in an acceptable dosage unit. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

[00168] One of the challenges to using chimeras as therapeutics is the ability to deliver the proteins effectively. The chimeras of the disclosure can be delivered by several different methods. In the blood stream, the half-life of most TGF-β ligands is on the order of minutes. To compensate for the ligands being degraded so quickly, current therapies involving TGF-β ligands use very high doses of the proteins. Alternatively, several means to directly modify the ligands or delivery systems are available to help improve the stability or sustained release properties of the ligands.

[00169] (1) Direct modification of the protein includes PEGylation as one common form of modification. In this method, polyethylene glycol (PEG) is covalently attached to the protein in hopes of improving stability by increasing solubility, resistance to proteolysis, and decreased immunogenicity.

[00170] (2) Rational modification of residues on the protein surface. By improving any electrostatic instability, without changing overall protein function, the overall stability of molecule can be improved. Using continuum electrostatic models, residues contributing to instability can be located and then analyzed to see if it can be mutated to a more favorable residue.

[00171] (3) Fusing the ligand to another protein or portion of a protein is another technique to increase protein stability and

solubility. The antibody constant fragment (Fc) is common fusion partner used to improve the stability and solubility.

[00172] (4) The use of liposomes can be used as a protein delivery vehicle. Liposomes are composed of any number of different phospholipids, which self assemble to form spheres. The protein of interest is encapsulated inside the bilayer, protecting it from the outside environment. The phospholipid composition influences the exact properties of the liposome and can be tailored to release the protein under any number of desired conditions. Polymer/liposome composite systems are also available to be used as delivery systems. Ideally, this type of system combines the advantages of each system to improve protein delivery.

[00173] (5) Similar to liposomes, polymers can be used as protein drug delivery systems. The polymers are used to make a matrix, commonly what is termed a hydrogel due to the high water content of the material. The advantage of using the gel is it allows for long term, sustained release as well as protecting the protein from proteolysis. As with the liposomes, the polymers used to make the gel influence its properties. There are two general classifications for the materials used to make the hydorgels: natural and unnatural polymers. Common materials used to create hydrogels using natural polymers include collagen, gelatin, fibrin, Hyaluronic acid, alginate, chitosan, and dextran. Synthetic polymers used to make hydrogels include Poly(ethylene oxide), Poly(acrylic acid), Poly(N-isopropylacrylamide), Poly(vinyl alcohol), and Polyphosphazene.

[00174] (6) A different kind of hydrogel can be created without the use of polymers, either natural or unnatural. Considered to be a bioactive glass, or Xerogel, this material is created from silica and calcium phosphate layer capable of absorbing the protein of interest. See, *e.g.*, Figure 8. The Xerogel increases the sustained release time of the protein up to weeks. Figure 1 shows results from cell viability assay using osteoblast cell line MC3T3 by MTT assay, which shows that the xerogel material is nontoxic up to the highest concentration of 30 mg/ml in the culture media we tested.

[00175] Chimera of the disclosure alone or in combination with a pharmaceutically acceptable carrier can be used to treat any number of disease and disorder or modulate cellular or tissue activity.

[00176] The chimeric polypeptides of the disclosure can be used to treat any number of disease or disorders where modulating of TGF-beta activity provides a therapeutic benefit. For example, the chimera of the disclosure can be used in subjects suffering from osteoporosis, cartilage disease or periodontal diseases. The chimera can be used to promote bone and/or cartilage formation, inhibiting bone loss/density or demineralization, promoting bone deposition and the like. Alternatively, the chimera can be used to inhibit excessive bone density and growth. In other embodiment, the chimera can be used in the treatment of endocrine diseases and disorders, hyperparathyroidism, Cushing's disease, malabsorption, renal tubular acidosis, or thyrotoxicosis.

[00177] The chimera of the disclosure can also be used in the treatment or modulating of sexual development, pituitary hormone production, and creation of bone and cartilage. The chimera can also be used for the treatment of cell proliferative diseases and disorders, cell growth and differentiation associated with inflammation, allergy, autoimmune diseases, infectious diseases, and tumors.

[00178] In a further aspect, the chimera of the disclosure can be used in the treatment of neuromuscular disorders, such as muscular dystrophy and muscle atrophy, congestive obstructive pulmonary disease, muscle wasting syndrome, obesity or other metabolic diseases including, for example,, type 2 diabetes.

[00179] The chimera of the disclosure can be used in degenerative muscle diseases characterized by abnormal amount, development or metabolic activity of muscle tissue, including gradual weakening and deterioration of skeletal muscles. Examples of muscle disease and disorders include, but are not limited to, a muscle wasting disorder, cachexia, anorexia, AIDS wasting syndrome, muscular dystrophies, Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Myotonic Dystrophy (MMD) (also known as Steinert's Disease), Oculopharyngeal Muscular Dystrophy (OPMD), Emery-Dreifuss Muscular Dystrophy (EDMD), Limb-Girdle Muscular Dystrophy (LGMD), Facioscapulohumeral Muscular Dystrophy (FSH or FSHD) (also known as Landouzy-Dejerine), Congenital Muscular Dystrophy (CMD), and Distal Muscular Dystrophy (DD).

[00180] The chimera of the disclosure can be used in methods and compositions to prevent, treat, or alleviate symptoms of a neurodegenerative disease or disorder including, but not limited to, Alzheimer's Disease (AD), Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Huntington's disease (HD), and other neuromuscular diseases, motor neuron diseases, diseases of the neuromuscular junction, and/or inflammatory myopathies.

[00181] A subject may have a disorder associated with abnormal cell growth and differentiation which may cause inflammation, allergy, autoimmune diseases, infectious diseases, and/or tumors. A subject may have a heart disorder, such as a disorder associated with excessive cardiomyocyte proliferation or growth, or a disorder in which it would be desirable to stimulate cardiomyocyte growth or proliferation. Subject chimeric TGF-beta superfamily proteins may be designed for the treatment of essentially any disorder that is amenable to treatment by agonists or antagonists of a member of the TGF-beta superfamily.

[00182] The following examples are meant to further explain, but not limited the foregoing disclosure or the appended claims.

EXAMPLES

Example 1

[00183] **Generation of TGF-β Chimeras.** To generate these novel TGF-β ligands, a modified directed evolution approach was utilized. Typically, this technique involves making a large number of random protein sequences, greater than 10^3, either by mixing the sequences of homologous genes or inserting random mutations and then screening for the desired ligand properties. In one set of experiments, sequences that were known to refold efficiently, termed the backbone ligand, were combined with a second ligand sequence containing signaling properties desire to mimic the target ligand. Using a structure guided approach, several TGF-β ligand crystal structures were analyzed and divided into 6 distinct sections. These sections roughly encompass the following regions of the ligand: section 1, N-terminus and beta strand 1; section 2, beta strand 2; section 3, pre-helix loop; section 4, alpha helix; section 5, beta strand 3; and section 6, beta stand 4 and C-terminus. Using this protocol, 64 different ligand combinations are possible for each set of TGF-β

ligands chosen to be recombined. When two or more parental chains are from different subfamilies (*e.g.* BMP/GDF v.s. TGFbeta), the difference between their signaling mechanisms may not be captured if sections 3 and 4 are separated. To be broadly applicable as the design principle, it is also part of the design to keep two structural segments, sections 3 and 4, can be treated as one section of either of the parental gene (referred to as section 3*4).

[00184] The strategy was implemented by making activin/BMP-2 chimeras using activin-βA as a target ligand and BMP-2 as the backbone ligand. Activin-βA was picked as the target ligand as it is biologically very interesting. BMP-2 was chosen as the backbone ligand because it has been shown to refold with excellent efficiency, >10% dimer yield from starting denatured inclusion bodies, and these dimers have been shown to be active in both *in vitro* and *in vivo* experiments. To design the various sections, a sequence alignment of BMP-2 and activin-βA was performed to locate regions of sequence identity between the ligands (Figure 7). These regions were used as the boundaries for the different sections. By using these parts of the sequence as the overlap regions for the oligonucleotides during PCR changes will not be introduced into either the BMP-2 or activin-βA sequences. The sequence alignment was then used in conjunction with data from previously solved BMP-2 and activin-βA structures to ultimately determine the 6 sections (Figure 7a-c). Due to limitations with regions of identity between the sequences, the sections had to be shifted slightly from ideal. Particularly, the pre-helix loop and the majority of the α-helix were combined into one section, while the remainder of the α-helix to the beginning of beta strand 3 was placed into a different section (Figure 7b and c). Additionally, 3-point mutations were inserted to allow for the cloning strategy to be successful. At the end of section 3, the BMP-2 sequence is TLVN, while the activin-βA sequence is TVIN (Figure 7a). Since these residue differences are conservative, the leucine and valine from the BMP-2 were introduced into the corresponding activin-βA sequence. The third mutation is found at the end of section 5. Here, the BMP-2 sequence is LYLD, while the equivalent activin-βA sequence is LYYD (Figure 7a). Since this residue difference is less conserved than the previous two, the

tyrosine from activin-βA was inserted into the corresponding BMP-2 sequence.

[00185] The N-terminus of activin-βA contains 2 additional cysteines (Figure 7a) which form a 4th intra-disulfide bond. To eliminate the potential of this extra disulfide bond complicating the refolding process, the section which contained these residues was eliminated from section 1 of activin-βA chimera design.

[00186] For the activin/BMP-2 chimeras, the mature domains of human BMP-2 and human activin-βA were initially divided into 6 sections each and primers were designed for each section. For BMP-2, the primers coded for the following protein sequences: Section 1, QAKHKQRKRLKSSCKRHPLYVDFSDVGWND; Section 2, WIVAPPGYHAFYCHGECP; Section 3, FPLADHLNSTNHAIVQTLVN; Section 4, SVNSKIPKACCVP; Section 5, TELSAISMLYYD; Section 6, ENEKVVLKNYQDMVVEGCGCR. For activin-βA, the primers coded for the following protein sequences: Section 1, RGLECDGKVNICCKKQFFVSFKDIGWNDW; Section 2, WIIAPSGYHANYCEGECP; Section 3, SHIAGTSGSSLSFHSTLVN; Section 4, HYRMRGHSPFANLKSCCVP; Section 5, TKLRPMSMLYYD; Section 6, DGQNIIKKDIQNMIVEECGCS. An overlapping PCR strategy was used to mix the various sections together to generate full length chimeras. To generate the 1b chimeras, two oligos were used to insert the BMP-2 sequence QAKHKQRKRLKSSCKRHPLYVDFSDVGWNDII into the target gene. Outer primers for all constructs were constructed to incorporate a 5' *Nde*I site and a 3' *Xho*I site for cloning into pET21a expression vector. The desired protein sequences were confirmed by DNA sequencing.

[00187] The chimeras were labeled according to the sections they contained. For example, 1b2b3b4a5a6b, in which the b's represent that the section was taken from BMP-2 and the a's represent that the section was derived from activin-βA. The chimeras were also given shorthand numeric designations, such as A/B2-020, so that any functional assays could be undertaken in a blind manner. Table 1 sets forth some of the various chimeras:

Table 1:

BMP-2/activin constructs	Sample Designation	DNA Sequence	Protein Sequence	Exemplary Characteristics
1b2b3a4a5a6a	AB2-001	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACCAAGCTGAGACCCATGTCCATGTTGTACT ATGATGATGGTCAAAACATCATCAAAAAGGACATTC AGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	Potential universal antagonist because it competes receptor binding but not signaling. Acts as neither BMP-2 nor activin-βA.
1b2b3a4a5b6a	AB2-002	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTACACTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACAGAGCTCAGTGCTATCTCGATGTTGTACT ATGATGATGGTCAAAACATCATCAAAAAGGACATTC AGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S L H S T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	Activity in stem cell differentiation assays unlike BMP-2.
1b2a3a4a5b6a	AB2-003	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACAGAACTCAGTGCTATCTCGATGTTGTACT ATGATGATGGTCAAAACATCATCAAAAAGGACATTC AGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	
1b2a3b4b5a6a	AB2-004	ATGCAAGCCAAACACAAACAGCGGAAGCGTCTTAAG TCCAGCTGCAAAAGGCACCCTTTGTATGTGGACTTCA GTGATGTGGGGTGGAATGACTGGATCATTGCTCCCT CTGGCTATCATGCCAACTACTGCGACGGAGAATGCC CTTTTCCTCTGGCTGATCATCTGAACTCCACTAATCA TGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTCT AAGATTCCTAAGGCATGCTGTGTCCCGACCAAGCTG AGACCCATGTCCATGTTGTACTATGATGATGGTCAA AACATCATCAAAAAGGACATTCAGAACATGATCGTG GAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C D G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	'Super" BMP-2 activity, unable to be inhibited by Noggin

1b2b3b4b5b6b (BMP-2_{mq})	AB2-005	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTC TAAGATTCCTAAGGCATGCTGTGTCCCGACAGAACT CAGTGCTATCTCGATGCTGTACCTTGACGAGAATGA AAAGGTTGTATTAAAGAACTATCAGGACATGGTTGT GGAGGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	AB2-005 (BMP-2_{mq}) contains one amino acid (Met) added at the N-terminus of mature BMP-2 in nature. Met originates from the translation initiation codon (ATG). Unless it is truncated during the folding process, it can remain as the N-terminus of AB2-005.
1b2a3a4a5b6b	AB2-006	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACAGAACTCAGTGCTATCTCGATGTTGTACC TTGACGAGAATGAAAAGGTTGTATTAAAGAACTATC AGGACATGGTTGTGGAGGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	Activity in stem cell differentiation assays unlike BMP-2.
1b2a3a4a5a6b	AB2-007	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACCAAGCTGAGACCCATGTCCATGTTGTAC CTTGACGAGAATGAAAAGGTTGTATTAAAGAACTAT CAGGACATGGTTGTGGAGGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	Activity in stem cell differentiation assays unlike BMP-2.
1b2a3a4a5a6a	AB2-008	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCCCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCATTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACCAAGCTGAGACCCATGTCCATGTTGTACT ATGATGATGGTCAAAACATCATCAAAAAGGACATTC AGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	Functions like activin-βA in cell signaling and *in vivo* experiments, ~4-fold lower potency; replaces TGF-beta 1 in chemically-defined stem cell media containing FGF2.

1b2a3a4a5a6a L66V/V67I	AB2-009	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCCCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCATTCAACGGTGATCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACCAAGCTGAGACCCATGTCCATGTTGTACT ATGATGATGGTCAAAACATCATCAAAAAGGACATTC AGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T V I N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	Functions like activin-βA in cell signaling and *in vivo* experiments, ~10-fold lower potency in activin-βA signaling activity.
1b(1a_II)2a3a4a5a6a	AB2-010	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAAACAGTTCTTTGTCAGTTTCAAG GACATCGGGTGGAATGACTGGATCATTGCTCCCTCT GGCTATCATGCCAACTACTGCGAGGGAGAATGCCCT TCCCATATAGCAGGCACGTCCGGGTCCTCACTGTCCT TCCATTCAACGTTGGTCAACCACTACCGCATGCGGG GCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGTGT CCCGACCAAGCTGAGACCCATGTCCATGTTGTACTAT GATGATGGTCAAAACATCATCAAAAAGGACATTCAG AACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K K Q F F V S F K D I G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	Functions like activin-βA in cell signaling and *in vivo* experiments, ~20-fold lower potency in activin-βA signaling.
1b2b3b4b5a6a	AB2-011	ATGCAAGCCAAACACCAACAGCGGAAACGCCTTAAG TCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTCA GTGACGTGGGGTGGAATGACTGGATTGTGGCTCCCC CGGGGTATCACGCCTTTTACTGCCACGGAGAATGCC CTTTTCCTCTGGCTGATCATCTGAACTCCACTAATCA TGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTCT AAGATTCCTAAGGCATGCTGTGTCCCGACCAAGCTG AGACCCTCCATGTTGTACTATGATGATGGTCAAAAC ATCATCAAAAAGGACATTCAGAACATGATCGTGGAG GAGTGTGGGTGCTCA	M Q A K H Q Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T K L R P S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	'Super" BMP-2 activity, unable to be inhibited by Noggin
1b2b3b4b5b6a	AB2-012	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTC TAAGATTCCTAAGGCATGCTGTGTCCCGACAGAACT CAGTGCTATCTCGATGTTGTACTATGATGATGGTCGA AACATCATCAAAAAGGACATTCAGAACATGATCGTG GAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T E L S A I S M L Y Y D D G R N I I K K D I Q N M I V E E C G C S	'Super" BMP-2 activity, partially inhibited by Noggin

1b2b3b4b5a6b	AB2-013	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTC TAAGATTCCTAAGGCATGCTGTGTCCCGACCAAGCT GAGACCCATGTCCATGTTGTACTATGATGAGAATGA AAAGGTTGTATTAAAGAACTATCAGGACATGGTTGT GGAGGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T K L R P M S M L Y Y D E N E K V V L K N Y Q D M V V E G C G C R	Activity comparable to BMP-2, inhibited by Noggin
1b2a3b4b5a6b	AB2-014	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTC TAAGATTCCTAAGGCATGCTGTGTCCCGACCAAGCT GAGACCCATGTCCATGTTGTACCTTGACGAGAATGA AAAGGTTGTATTAAAGAACTATCAGGACATGGTTGT GGAGGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C D G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T K L R P M S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	Activity comparable to BMP-2, partially blocked by Noggin
1b2a3b4b5b6a	AB2-015	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTC TAAGATTCCTAAGGCATGCTGTGTCCCGACAGAACT CAGTGCTATCTCGATGTTGTACTATGATGATGGTCAA AACATCATCAAAAAGGACATTCAGAACATGATCGTG GAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C D G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T E L S A I S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	'Super" BMP-2 activity, unable to be inhibited by Noggin
1b,2a3b4b5b6b	AB2-016	ATGCAAGCCAAACACAAACAGCGGAAGCGTCTTAAG TCCAGCTGCAAAAGGCACCCTTTGTATGTGGACTTCA GTGATGTGGGGTGGAATGACTGGATCATTGCTCCCT CTGGCTATCATGCCAACTACTGCGAGGGAGAATGCC CTTTTCCTCTGGCTGATCATCTGAACTCCACTAATCA CGCCATTGTTCAGACGTTGGTCAACTCTGTTAACTCT AAGATTCCTAAGGCATGCTGTGTCCCGACAGAACTC AGTGCTATCTCGATGCTGTACCTTGACGAGAATGAA AAGGTTGTATTAAAGAACTATCAGGACATGGTTGTG GAGGGTTGCGGGTGTCGT	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	Activity comparable to BMP-2, inhibited by Noggin

1b2b3b4a5a6a	AB2-017	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACCACTACCGCAT GCGGGGCCATAGCCCCTTTGCCAACCTCAAATCGTG CTGTGTCCCGACCAAGCTGAGACCCATGTCCATGTTG TACTATGATGATGGTCAAAACATCATCAAAAAGGAC ATTCAGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	
1b2b3b4a5b6b	AB2-018	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACCACTACCGCAT GCGGGGCCATAGCCCCTTTGCCAACCTCAAATCGTG CTGTGTCCCGACAGAACTCAGTGCTATCTCGATACTG TACCTTGACGAGAATGAAAAGGTTGTATTAAAGAAC TATCAGGACATGGTTGTGGAGGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S I L Y L D E N E K V V L K N Y Q D M V V E G C G C R	
1b2b3b4a5a6b	AB2-019	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACCACTACCGCAT GCGGGGCCATAGCCCCTTTGCCAACCTCAAATCGTG CTGTGTCCCGACCAAGCTGAGACCCATGTCCATGTTG TACTATGATGAGAATGAAAAGGTTGTATTAAAGAAC TATCAGGACATGGTTGTGGAGGGTTGCGGGTGTCGT	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D E N E K V V L K N Y Q D M V V E G C G C R	
1b2b3b4a5b6a	AB2-020	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACCACTACCGCAT GCGGGGCCATAGCCCCTTTGCCAACCTCAAATCGTG CTGTGTCCCGACAGAACTCAGTGCTATCTCGATGTTG TACTATGATGATGGTCAAAACATCATCAAAAAGGAC ATTCAGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	

1b2b3a4a5a6b	AB2-021	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTATGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACCAAGCTGAGACCCATGTCCATGTTGTAC CTTGACGAGAATGAAAAGGTTGTATTAAAGAACTAT CAGGACATGGTTGTGGAGGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	
1b2b3a4a5b6b	AB2-022	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACCACTACCGCATGCG GGGCCATAGCCCCTTTGCCAACCTCAAATCGTGCTGT GTCCCGACAGAACTCAGTGCTATCTCGATGTTGTACT ATGATGAGAATGAAAAGGTTGTATTAAAGAACTATC AGGACATGGTTGTGGAGGGTTGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S F H S T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	
1b2b3a4b5b6b	AB2-023	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACTCTGTTAACTCTAA GATTCCTAAGGCATGCTGTGTCCCGACAGAACTCAG TGCTATCTCGATGCTGTACCTTGACGAGAATGAAAA GGTTGTATTAAAGAACTATCAGGACATGGTTGTGGA GGGTTGCGGGTGTCGT	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S F H S T L V N S V N S K I P K A C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	
1b2b3a4b5b6a	AB2-024	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACTCTGTTAACTCTAA GATTCCTAAGGCATGCTGTGTCCCGACAGAACTCAG TGCTATCTCGATGTTGTACTATGATGATGGTCAAAAC ATCATCAAAAAGGACATTCAGAACATGATCGTGGAG GAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S F H S T L V N S V N S K I P K A C C V P T E L S A I S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	

1b2b3a4b5a6a	AB2-025	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACTCTGTTAACTCTAA GATTCCTAAGGCATGCTGTGTCCCGACCAAGCTGAG ACCCATGTCCATGTTGTACTATGATGATGGTCAAAAC ATCATCAAAAAGGACATTCAGAACATGATCGTGGAG GAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S F H S T L V N S V N S K I P K A C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	
1b2b3a4b5a6b	AB2-026	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTGTGGCTCCC CCGGGGTATCACGCCTTTTACTGCCACGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACTCTGTTAACTCTAA GATTCCTAAGGCATGCTGTGTCCCGACCAAGCTGAG ACCCATGTCCATGTTGTACTATGATGAGAATGAAAA GGTTGTATTAAAGAACTATCAGGACATGGTTGTGGA GGGTTGCGGGTGTCGT	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P S H I A G T S G S S L S F H S T L V N S V N S K I P K A C C V P T K L R P M S M L Y Y D E N E K V V L K N Y Q D M V V E G C G C R	
1b2a3a4b5b6b	AB2-027	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACTCTGTTAACTCTAA GATTCCTAAGGCATGCTGTGTCCCGACAGAACTCAG TGCTATCTCGATGTTGTACCTTGACGAGAATGAAAA GGTTGTATTAAAGAACTATCAGGACATGGTTGTGGA GGGTTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N S V N S K I P K A C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	
1b2a3a4b5b6a	AB2-028	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACTCTGTTAACTCTAA GATTCCTAAGGCATGCTGTGTCCCGACAGAACTCAA TGCTATCTCGATGTTGTACTATGATGATGGTCAAAAC ATCATTAAAAAGGACATTCAGAACATGATCGTGGAG GAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N S V N S K I P K A C C V P T E L N A I S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	

1b2a3a4b5a6a	AB2-030	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTCTCATATAGCAGGCACGTCCGGGTCCTCACTGT CCTTCCACTCAACGTTGGTCAACTCTGTTAACTCTAA GATTCCTAAGGCATGCTGTGTCCCGACCAAGCTGAG ACCCATGTCCATGTTGTACTATGATGATGGTCAAAAC ATCATCAAAAAGGACATTCAGAACATGATCGTGGAG GAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T L V N S V N S K I P K A C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	
1b2a3b4a5a6a	AB2-031	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACCACTACCGCAT GCGGGGCCATAGCCCCTTTGCCAACCTCAAATCGTG CTGTGTCCCGACCAAGCTGAGACCCATGTCCATGTTG TACTATGATGATGGTCAAAACATCATCAAAAAGGAC ATTCAGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	
1b2a3b4a5b6a	AB2-032	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCCACTAATC ATGCCATTGTTCAGACGTTGGTCAACCACTACCGCAT GCGGGGCCATAGCCCCTTTGCCAACCTCAAATCGTG CTGTGTCCCGACAGAACTCAGTGCTATCTCGATGCTG TACCTTGACGATGGTCAAAACATCATCAAAAAGGAC ATTCAGAACATGATCGTGGAGGAGTGTGGGTGCTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S M L Y Y D D G Q N I I K K D I Q N M I V E E C G C S	
1b2a3b4a5b6b	AB2-033	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATCATTGCTCCC TCTGGCTATCATGCCAACTACTGCGAGGGAGAATGC CCTTTTCCTCTGGCTGATCATCTGAACTCTACTAATC ATGCCATTGTTCAGACGTTGGTCAACCACTACCGCAT GCGGGGCCATAGCCCCTTTGCCAACCTCAAATCGTG CTGTGTCCCGACAGAACTCAGTGCTATCTCGATGCTG TACCTTGACGAGAATGAAAAGGTTGTATTAAAGAAC TATCAGGACATGGTTGTGGAGGGTTGCGGGTGTCGT	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C E G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	

1b2a3b4a5a6b	AB2-034	ATGCAAGCCAAACACAAACAGCGGAAGCGTCTTAAG TCCAGCTGCAAAAGGCACCCTTTGTATGTGGACTTCA GTGATGTGGGGTGGAATGACTGGATCATTGCTCCCT CTGGCTATCATGCCAACTACTGCGACGGAGAATGCC CTTTTCCTCTGGCTGATCATCTGAACTCCACTAATCA TGCCATTGTTCAGACGTTGGTCAACCACTACCGCATG CGGGGCCATAGCCCCTTTGCCAACCTCAAATCATGCT GTGTCCCGACCAAGCTGAGACCCATGTCCATGTTGT ACTATGATGAGAATGAAAAGGTTGTATTAAAGAACT ATCAGGACATGGTTGTGGAGGGTTGCGGGTGTCGT	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P S G Y H A N Y C D G E C P F P L A D H L N S T N H A I V Q T L V N H Y R M R G H S P F A N L K S C C V P T K L R P M S M L Y Y D E N E K V V L K N Y Q D M V V E G C G C R	
BMP-2_{ma}	BMP-2_{ma}	ATGGCTCAAGCCAAACACAAACAGCGGAAACGCCTT AAGTCCAGCTGTAAGAGACACCCTTTGTACGTGGAC TTCAGTGACGTGGGGTGGAATGACTGGATTGTGGCT CCCCCGGGGTATCACGCCTTTTACTGCCACGGAGAA TGCCCTTTTCCTCTGGCTGATCATCTGAACTCCACTA ATCATGCCATTGTTCAGACGTTGGTCAACTCTGTTAA CTCTAAGATTCCTAAGGCATGCTGTGTCCCGACAGA ACTCAGTGCTATCTCGATGCTGTACCTTGACGAGAAT GAAAAGGTTGTATTAAAGAACTATCAGGACATGGTT GTGGAGGGTTGTGGGTGTCGC	M A Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I V A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V N S V N S K I P K A C C V P T E L S A I S M L Y L D E N E K V V L K N Y Q D M V V E G C G C R	BMP-2_{ma} contains two additional amino acids (Met-Ala) at the N-terminal side of mature BMP-2 in nature (QAKH...). Met is often truncated during the folding process, but Ala remains as the N-terminus of the final product. Either form is regarded as BMP-2_{ma} .
1b_BMP7	NB2-BMP7	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTATCGCGCCT GAAGGCTACGCCGCCTACTACTGTGAGGGGGAGTGT GCCTTCCCTCTGAACTCCTACATGAACGCCACCAACC ACGCCATCGTGCAGACGCTGGTCCACTTCATCAACC CGGAAACGGTGCCCAAGCCCTGCTGTGCGCCCACGC AGCTCAATGCCATCTCCGTCCTCTACTTCGATGACAG CTCCAACGTCATCCTGAAGAAATACAGAAACATGGT GGTCCGGGCCTGTGGCTGCCAC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P E G Y A A Y Y C E G E C A F P L N S Y M N A T N H A I V Q T L V H F I N P E T V P K P C C A P T Q L N A I S V L Y F D D S S N V I L K K Y R N M V V R A C G C H	
1b_BMP9	NB2-BMP9	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTATTGCCCCA AAAGAGTACGAGGCATACGAGTGTAAGGGCGGCTGT TTCTTTCCGCTGGCCGACGATGTCACCCCGACCAAGC ACGCAATTGTCCAAACCTTAGTGCACCTGAAGTTCCC AACGAAAGTGGGTAAGGCATGTTGTGTGCCAACCAA GTTATCTCCAATTAGCGTGCTGTATAAGGATGATATG GGCGTGCCGACGTTAAAGTATCATTACGAGGGCATG AGCGTCGCAGAGTGTGGCTGCCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P K E Y E A Y E C K G G C F F P L A D D V T P T K H A I V Q T L V H L K F P T K V G K A C C V P T K L S P I S V L Y K D D M G V P T L K Y H Y E G M S V A E C G C R	

1b_GDF7	NB2-GDF7	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTATCGCGCCG CTGGACTACGAGGCGTACCACTGCGAGGGCCTATGC GATTTTCCTCTGCGTTCGCACCTCGAACCCACCAACC ATGCCATCATTCAGACGTTGGTCAACTCCATGGCACC AGACGCGGCGCCGGCCTCCTGCTGTGTCCCGGCGCG CCTCAGCCCCATCAGCATCTTGTACTATGATGCCGCC AACAACGTTGTCTACAAGCAATACGAGGACATGGTG GTGGAGGCCTGTGGGTGTCGC	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P L D Y E A Y H C E G L C D F P L R S H L E P T N H A I I Q T L V N S M A P D A A P A S C C V P A R L S P I S I L Y Y D A A N N V V Y K Q Y E D M V V E A C G C R	
1b_GDF8	NB2-GDF8	ATGCAAGCCAAACACAAACAGCGGAAACGCCTTAA GTCCAGCTGTAAGAGACACCCTTTGTACGTGGACTTC AGTGACGTGGGGTGGAATGACTGGATTATTGCACCC AAAAGATATAAGGCCAATTACTGCTCTGGAGAGTGT GAATTTGTATTTTTACAAAAATACCCTCACACTCATC TTGTGCACCAAGCAAACCCCAGAGGTTCAGCAGGCC CCTGCTGTACTCCCACAAAGATGTCTCCAATCAATAT GCTATATTTTAATGGCAAAGAACAAATAATATATGG GAAAATTCCAGCCATGGTAGTAGATCGCTGTGGGTG CTCA	M Q A K H K Q R K R L K S S C K R H P L Y V D F S D V G W N D W I I A P K R Y K A N Y C S G E C E F V F L Q K Y P H T H L V H Q A N P R G S A G P C C T P T K M S P I N M L Y F N G K E Q I I Y G K I P A M V V D R C G C S	
BMP2/BMP6	B2/B6	BMP-2_{wt} and BMP-6 are added together during the refolding to generate the BMP2/BMP6 heterodimer. BMP-2_{wt} sequence herein is also reported as AB2-005. BMP-6 DNA sequence is: ATGCAACAGAGTCGTAATCGCTCTACCCAGTCCCAG GACGTGGCGCGGGTCTCCAGTGCTTCAGATTACAAC AGCAGTGAATTGAAAACAGCCTGCAGGAAGCATGA GCTGTATGTGAGTTTCCAAGACCTGGGATGGCAGGA CTGGATCATTGCACCCAAGGGCTATGCTGCCAATTA CTGTGATGGAGAATGCTCCTTCCCACTCAACGCACA CATGAATGCAACCAACCACGCGATTGTGCAGACCTT GGTTCACCTTATGAACCCCGAGTATGTCCCCAAACC GTGCTGTGCGCCAACTAAGCTAAATGCCATCTCGGTT CTTTACTTTGATGACAACTCCAATGTCATTCTGAAAA AATACAGGAATATGGTTGTAAGAGCTTGTGGATGCC AC	BMP-2_{wt} sequence is reported above as AB2-005. BMP-6 amino acid sequence. Two amino acids, MA, are present in BMP-2_{wt} in contrast to mature form of BMP-2 existent in nature. M Q Q S R N R S T Q S Q D V A R V S S A S D Y N S S E L K T A C R K H E L Y V S F Q D L G W Q D W I I A P K G Y A A N Y C D G E C S F P L N A H M N A T N H A I V Q T L V H L M N P E Y V P K P C C A P T K L N A I S V L Y F D D N S N V I L K K Y R N M V V R A C G C H	Has increased SMAD-mediated signaling activity as compared to either BMP-2 or BMP-6.

[00188] **Protein Expression and Purification.** The activin/BMP-2, 1b chimeras, and BMP-2_{ma} chimeras were expressed using a typical *E. coli* expression system, and all 32 chimeras were found in the inclusion body fractions. The expressed inclusion bodies were isolated, purified, and refolded. The refolded ligands were purified using a Hi-trap heparin column (GE Healthcare) and reversed phase chromatography (GraceVydac). The ligands were lyophilized and re-suspended in 4mM HCl, pH 1 for use in all cell based assays or 10mM Na acetate, pH 4 for all biophysical assays. Activin-βA was expressed in a stably transfected CHO cell line and purified using techniques known in the art. Noggin was expressed and purified based on previously described protocols.

[00189] The activin/BMP-2 chimera inclusion bodies were seen as single bands on a reduced, SDS-PAGE gel and found at the expected size of ~13 kDa (Figure 1a). To standardize the refoldings, all activin/BMP-2 chimeras were refolded in 100mL volumes at a concentration of 50mg/L. The concentration was chosen based on previously successful BMP-2, BMP-3, and GDF-5 refoldings. The volume was picked so that any dimer yield of 2% or greater would generate enough protein for biophysical activity assays, yet small enough to be manageable with the large number of samples. Following refolding, the activin/BMP-2 samples were analyzed for the formation of pure dimer, the desired product, after elution from Heparin column (Figure 1b and c). Surprisingly, all 32 activin/BMP-2 samples showed the presence of some dimer and the chimeras were ranked based on their refolding efficiency (dimer yield) and grouped into 4 categories, from poor (<1%, -) to wild type (>10%, +++) (Table 2). To be classified as a 'successful' chimera, the ligand needed to have a refolding efficiency equal to or greater than 5%. This efficiency would yield 2.5mg/L of dimeric protein from a standard 1L refolding at 50mg/L concentration, and would be considered suitable for experiments where large quantities are required, such as x-ray crystallography. When refolding efficiency was calculated, 24 out 32 (75%) of the activin/BMP-2 chimeras met this criteria (Table 2, supplemental, ++ or +++).

Table 2

Construct	Name	Dimer Yield	Rating	Rating System	
1b2b3a4a5a6a	AB2001	5%	++	+++(wt)	>10%
1b2b3a4a5b6a	AB2002	7%	++	++	5-9%
1b2a3a4a5b6a	AB2003	1%	-	+	2-4%
1b2a3b4b5a6a	AB2004	9%	++	-	<1%
1b2b3b4b5b6b	AB2005	>10%	+++		
1b2a3a4a5b6b	AB2006	9%	++		
1b2a3a4a5a6b	AB2007	>10%	+++		
1b2a3a4a5a6a	AB2008	>10%	+++		
1b2a3a4a5a6a L66V/V67I	AB2009	~4%	+		
1b(1a_II)2a3a4a5a6a	AB2010	3%	+		
1b2b3b4b5a6a	AB2011	>10%	+++		
1b2b3b4b5b6a	AB2012	>10%	+++		
1b2b3b4b5a6b	AB2013	>10%	+++		
1b2a3b4b5a6b	AB2014	>10%	+++		
1b2a3b4b5b6a	AB2015	>10%	+++		
1b2a3b4b5b6b	AB2016	>10%	+++		
1b2b3b4a5a6a	AB2017	5%	++		
1b2b3b4a5b6b	AB2018	2%	+		
1b2b3b4a5a6b	AB2019	3%	+		
1b2b3b4a5b6a	AB2020	3%	+		
1b2b3a4a5a6b	AB2021	6%	++		
1b2b3a4a5b6b	AB2022	5%	++		
1b2b3a4b5b6b	AB2023	3%	+		
1b2b3a4b5b6a	AB2024	6%	++		
1b2b3a4b5a6a	AB2025	4%	+		
1b2b3a4b5a6b	AB2026	5%	++		
1b2a3a4b5b6b	AB2027	>10%	+++		
1b2a3a4b5b6a	AB2028	4%	+		
1b2a3a4b5a6b	AB2029	1%	-		
1b2a3a4b5a6a	AB2030	2%	+		
1b2a3b4a5a6a	AB2031	4%	+		
1b2a3b4a5b6a	AB2032	4%	+		
1b2a3b4a5b6b	AB2033	4%	+		
1b2a3b4a5a6b	AB2034	1%	-		

[00190] To be considered a successful ligand, the activin/BMP-2 chimeras not only have to be refoldable but they also need to display signaling characteristics. To test for these properties, all activin/BMP-2 chimeras, regardless of refolding efficiency, were initially subjected to activin activity assays. Activin-like signaling characteristics were tested using a whole cell luciferase

reporter assay sensitive to Smad-2/3 activation (as described below). Activin-βA is known to signal through and activate the Smad-2/3 pathway, so if any of the activin/BMP-2 chimeras mimic activin-βA functionality, they should signal in a similar manner. Out of all 32 chimeras, only 1, 1b2a3a4a5a6a (AB2-008), signaled in an activin-like manner. AB2-008 activates the luciferase reporter in a dose dependent manner similar to activin-βA. When the potency of the AB2-008 chimera was determined, the EC_{50} was calculated to be 64.5 pM. This value is ~2 fold lower than activin-βA with an EC_{50} of 28.8 pM. To confirm that the luciferase results were a direct response to Smad-2 activation, phospho-Smad-2 was tested in the presence of AB2-008. Similar to activin-βA, the addition of AB2-008 promotes an increase in phospho-Smad-2 levels. As expected, AB2-008, along with activin-βA, does not stimulate phospho-Smad-1 production, indicating only activation of a specific signaling pathway. Interestingly, AB2-008 exhibits BMP-2_{ma} refolding efficiency with >10% dimer yield (Table 2).

[00191] To fully test if AB2-008 possesses complete activin-βA functionality, additional biophysical assays were performed. Cripto is a known co-receptor for many of the TGF-β ligands and elicits a wide range of responses. For instance, the presence of Cripto is required for proper Nodal signaling, while it antagonizes TGF-β1 and activin signaling. Therefore, using the Smad-2/3 luciferase assay activin-βA and AB2-008 signaling were monitored in the presence or absence of Cripto. In the presence of Cripto, activin-βA signaling is decreased by ~43% compared activin-βA alone. The AB2-008 chimera exhibits a similar decrease in signaling of ~38% when Cripto is added to the assay. This result confirms that the AB2-008 chimera is a fully functioning activin mimic by being able to activate the activin signaling pathway as well having the ability to interact with other known activin binding partners.

[00192] Based on the results of AB2-008, 2 additional activin/BMP-2 chimeras were generated to see if the potency of the chimeras could be increased to wild type activin-βA levels. The first chimera, named 1b2a3a4a5a6a L66V/V67I (AB2-009), introduced a valine and iso-leucine into the chimera. These residues are found in the wild type sequence of activin-βA and were originally mutated

to the corresponding BMP-2 residues due to experimental design constraints (Figure 7a). The second chimera, named 1b(1a_II)2a3a4a5a6a (AB2-010), replaces the second half of the 1b section with the corresponding sequence from activin (Figure 7a). This leaves only the 13 N-terminal residues preceding the first structurally conserved cysteine, Leu-66, and Val-67 as components from BMP-2 in this chimera construct. It is predicted that the introduction of additional activin residues into AB2-008 will improve its functional characteristics (*i.e.* potency). AB2-009 and AB2-010 were expressed and refolded as previously described for the other activin/BMP-2 chimeras. Unexpectedly, both of these new chimeras exhibited decreased refolding efficiency compared to AB2-008. AB2-009 and AB2-010 saw a decrease from >10% dimer yield to ~4% and ~3% for AB2-009 and AB2-010, respectively (Table 2). While this result may not be surprising for AB2-010 since an 11 residue section was mutated, the drastic decrease for AB2-009 was unexpected. Both the L66V and V67I mutations are very conservative changes with only a 1 carbon difference between the side chains of the mutated residues.

[00193] Following refolding, the new chimeras were subjected to the same Smad-2/3 luciferase assay as AB2-008 previously. AB2-009 activated the reporter in a dose dependent manner and displayed activity comparable to AB2-008 with an EC_{50} of 79.4 pM. However, while AB2-010 also activated the reporter, it showed a significant decrease in activity with an EC_{50} of 198.6 pM, or ~3-fold weaker than AB2-008 and ~7-fold weaker than activin-βA. As with AB2-008, both AB2-009 and AB2-0010 showed Smad-2 phosphorylation. Since AB2-009 and AB2-010 did not show enhanced signaling characteristics from AB2-008 in the luciferase assay, they were not subjected to Cripto binding assay.

[00194] While the Smad-2/3 luciferase, Smad-2 phosphorylation, and Cripto reporter assays indicate that AB2-008, AB2-009, and AB2-010 signal through the activin pathway and function very similarly to activin-βA, these assays only show function in an *in vitro* setting. Therefore, more physiologically relevant experiments are required to prove that these activin/BMP-2 chimeras will elicit a biological response similar activin-βA. One classical method used

to test for proper activin function is a follicle stimulating hormone (FSH) release assay. Rat anterior pituitary cells are known to release FSH in response to the presence of activin in both *in vivo* and *in vitro* experiments. Therefore, rat anterior pituitary cells were exposed to increasing amounts of activin-βA or the activin/BMP-2 chimeras and FSH release was measured by radioimmunoassay. All three activin/BMP-2 chimeras showed a dose dependent increase in FSH release similar to activin-βA. The amount of FSH release stimulated by the chimeras was decreased in the presence of increasing amounts of Inhibin. Combined with the *in vitro* assay results, the FSH release assay confirms that AB2-008, AB2-009, and AB2-010 possess the complete activin-βA functional characteristics.

[00195] The chimeras were also tested to check for any additional signaling properties. BMP-2 is already used as a therapeutic agent for certain bone treatments and having chimeras with altered BMP-2 function may prove beneficial. To test if any of the activin/BMP-2 chimeras displayed unique signaling characteristics, a similar experiment to the activin-βA functional assay was performed. Here, a whole cell luciferase reporter assay sensitive to Smad-1/5 activation, the known BMP-2 signaling pathway, was used rather than a reporter sensitive to Smad-2/3 activation. Monitoring the luciferase response in a dose dependant manner, a number of activin/BMP-2 chimeras exhibit interesting traits. These activin/BMP-2 chimeras were identified and classified into 3 groups: Those with upregulated or 'super' BMP-2 activity; those with insensitivity to Noggin, a BMP-2 antagonist; or those with both 'super' BMP-2 activity and insensitivity to Noggin. Activin/BMP-2 chimeras 1b2a3b4b5a6a (AB2-004), 1b2b3b4b5a6a (AB2-011), 1b2b3b4b5b6a (AB2-012), and 1b2a3b4b5b6a (AB2-015) all fall into category of enhanced BMP-2 activity with Noggin insensitivity. In the Smad-1 luciferase assay, these ligands activate the reporter at the same level as $BMP\text{-}2_{wt}$ using 10x less protein (*i.e.*, 10-fold higher activity). Grouped into the category of upregulated BMP-2 activity is 1b2b3b4b5a6b (AB2-013). This chimera shows the same 10-fold increase in activity as AB2-004,-011,-012,-015, but the signal is decreased down to background levels upon the addition of Noggin,

similar to BMP-2_{wt}. Chimera, 1b2a3b4b5a6b (AB2-014), fell into the final category of ligands with normal BMP-2 signaling but with insensitivity. AB2-014 activates the luciferase reporter to the same level as BMP-2_{wt} but its signal cannot be blocked by the addition Noggin. AB2-008 was also tested to see if it activated the Smad-1 pathway in addition to activating the Smad-2 pathway. AB2-008 did not show any Smad-1 activation, even up to levels of 1µg/ml. This result confirms that AB2-008 is a specific activin mimic and does not exhibit non-specific signaling characteristics.

[00196] With the success of the AB2-008 chimera which refolds efficiently and possesses activin-like signaling characteristics, the 1b section was examined as a general tool to improve the refolding of other currently non-refoldable TGF-β ligands. As mentioned before, the 1b section is 30 a.a. long and comprises the N-terminus of BMP-2 as well as the residues forming the first beta strand of finger 1 (Figure 7a). Based on analysis of the ternary structure of BMP-2/BMPRIa/ActRII, the majority of the residues found in section 1b do not form any contacts with either the Type I or Type II receptors. Indeed, of the few residues which do generate contacts with the Type I receptor, Val-26, Gly-27, and Trp-28, the tryptophan is invariant throughout the entire TGF-β superfamily, while the Gly-27 participates in a backbone interaction, and the valine is predominantly a non-polar amino acid at this position throughout the TGF-β superfamily. Based on this, it is possible that the 1b region, while not critical for contributing to ligand-receptor affinity and specificity, is very helpful in proper disulfide bond formation during the chemical refolding process. Therefore, the 1b section was cloned into the additional TGF-β ligands BMP-7, BMP-9, and GDF-8. As with the activin/BMP-2 chimeras, the 1b chimeras were expressed in an *E. coli* expression system and the inclusion bodies isolated to high purity.

[00197] **Smad-1 Luciferase Assays in C2C12 Cells.** Smad1-dependent luciferase assays were performed using techniques known in the art. In brief, C2C12 myoblast cells are cultured in Dulbecco's minimum essential medium (DMEM) + 5% FBS supplemented with L-Glutamine and antibiotics. For luciferase reporter assays, cells were trypsinized, washed twice with PBS and plated into 48-well plates

with DMEM + 0.1% FBS. Twenty four hours later, cells were transfected with -1147Id1-luciferase construct containing the Smad binding sites (Id1-Luc), a Smad1 expression construct, and a CAGGS-LacZ plasmid by using Fugene6 (Roche) according to the manufacturer's instruction and cells were stimulated with increasing amounts of BMP-2_{ma} or the various activin/BMP-2 chimeras added 24 hours post transfection. Luciferase activity was measured 24 hours after stimulation with ligands and the values were normalized for transfection efficiency by using beta-galactosidase activity. The activity of the luciferase reporter is expressed in fold-induction relative to control values that are obtained by using -927Id1-luciferase that lacks Smad binding domains (Id1-Luc mut). To test for the ability of Noggin to attenuate the Smad1 signaling of the ligands, the luciferase assays were repeated as described above, with a set dose of Noggin included in the assay.

[00198] Smad-2 Luciferase Assays in HEK293 Cells. HEK293T cells were seeded into 24-well plates coated with polylysine at a density of 150,000 cells/well. After 24 h cells were transfected overnight with a mixture of A3 Lux (25 ng) and β-galactosidase (25 ng) reporter plasmids, the transcription factor FAST2 (50 ng), and empty pCDNA3 vector (400 ng) using Perfectin® transfection reagent (GenLantis) according to the manufacturer's recommendations. Then the cells were treated with increasing doses of activin- βA or activin/BMP-2 chimeras for 16-24 h. The cells were harvested in ice-cold lysis buffer (1% Triton X-100 in 25 mM glycylglycine, 4 nM EGTA, 15 mM $MgSO_4$ containing 1 mM dithiothreitol) and assayed for luciferase and β-galactosidase activities using standard methods. To assess the ability of the activin/BMP-2 chimeras to bind known TGF-β co-receptors, the HEK293T cells were treated with increasing doses of activin-βA or activin/BMP-2 chimeras in the presence or absence of transfected Cripto for 16-24 h (mouse Cripto construct was a generous gift from Malcolm Whitman (Department of Cell Biology, Harvard Medical School, Boston, MA)). Activity was then measures as previously described.

[00199] Follicle Stimulating Hormone (FSH) Release from Rat Interior Pituitary Cells. The assay was performed as previously described in the art. Briefly, freshly isolated cells from male

Sprague-Dawley rat interior pituitaries from several animals were combined and seeded into 96-well plates at a density of 50,000 cells/well in βPJ medium supplemented with 2% fetal bovine serum and appropriate growth factors. After 24 h cells were treated with increasing doses of activin-βA or activin/BMP-2 chimeras (0-40 nM). After 72 h, media were harvested and the concentration of the secreted FSH was determined by radioimmunoassay.

[00200] Surface Plasmon Resonance (BIAcore) Affinity Studies. The affinity of the ligands to BMPRIa, ActRII, and ActRIIb was monitored by using a Biacore 3000 (GE Healthcare) and the data were analyzed by using BIAevaluation software ver. 4.1 (GE Healthcare). Using primary amine coupling, receptor ECDs were immobilized on a CM5 chip. The receptors were immobilized independently on flow cells 2-4 for 10 minutes at a flow rate of 5 µL/min and a concentration of 20 µM in 10 mM Na acetate, pH 4.0. Flow cell 1 was left blank, no immobilized protein, as a negative control. The experiments were performed at a flow rate of 50 µL/min in 20 mM Tris-HCl pH 7.9, 250 mM NaCl, 0.36% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.005% Tween-20. A minimum of five concentrations, plus a zero concentration, were run per sample for kinetic analysis and the data were fit by using a global 1:1 Langmuir binding with mass transfer.

Example 2

[00201] Synthesis of the BMP Heterodimer Ligand. The crystal structure of BMP2-BMPRIa-ActRIIb has shown that each receptor molecule do not associate extracellularly and have 4 distinct ligand-receptor interface. This suggested that a heterodimer would have 2 distinct type I interfaces and 2 distinct type II interfaces. To characterize functional and other aspects of the BMP ligand recombinant heterodimers were synthesized. The purity of the protein was verified by sodium dodecyl sulfate-polyacrlamide gel electrophoresis (SDS-PAGE). Figure 1a illustrates the migration of the BMP2/BMP6 heterodimer as a single band under non-reducing (lane 1) and as two distinct bands under reducing conditions (lane 3) on SDS-PAGE. The two distinct bands correlate to the two different monomer species (about 13 and 15 kDa respectively), of the BMP2/BMP6 heterodimer. This evidence is further supported with surface-

enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) data. Three separate, purified samples of the BMP2 and BMP6 homodimers and the BMP2/BMP6 heterodimer were assayed on SELDI-TOF-MS. As Figure 1b demonstrates, the three samples each correspond to their predicted mass with no other contaminating species. These assays indicate that a pure BMP2/BMP6 heterodimer was generated.

[00202] **BMP Heterodimer Activity *in vitro*.** To assay the interactions between the BMP2/BMP6 heterodimer and type I and type II TGF-beta receptor ECDs, surface plasmon resonance was utilized to measure the *in vitro* affinity. The TGF-beta receptors were immobilized to a chip and the TGF-beta ligands were flowed over the surface while monitoring the interactions. Table 3 summarizes the ligands tested and the varying affinities for the type I and type II receptor ECDs. In the case of the BMP2/BMP6 heterodimer, it adopts the greater affinity from each of its BMP2 and BMP6 monomer subunits. As shown, the BMP2/BMP6 heterodimer has similar affinity for the type I receptor as the BMP2 homodimer. However, instead of adopting the type II receptor ECD affinity from the BMP2 subunit, the BMP2/BMP6 heterodimer has an affinity similar to the BMP6 homodimer for the type II receptor. This indicates that the high affinity for the type II receptor ECD is contributed by the BMP6 monomer subunit while the high affinity for the type I receptor ECD is contributed by the BMP2 monomer subunit.

Table 3

Ligand Affinity Data from BIAcore Analysis				
Ligand	**Receptor BMPR-Ia**		**Receptor ActRIIb**	
	k_{off} [1/s]/k_{on}[1/M*s]	K_D [nM]	k_{off} [1/s]/k_{on}[1/M*s]	K_D [nM]
BMP2	$1.11 \times 10^{-3}/8.52 \times 10^{5}$	1.31	$2.57 \times 10^{-2}/6.68 \times 10^{5}$	38.5
BMP6	$9.37 \times 10^{-3}/1.50 \times 10^{5}$	62.8	$1.82 \times 10^{-3}/2.73 \times 10^{5}$	6.68
BMP2/BMP6	$1.05 \times 10^{-3}/1.03 \times 10^{6}$	1.02	$8.58/1.32 \times 10^{9}$	6.52

Ligand affinity data from BIAcore experiments. The BIAcore data is shown as the dissociation rate, k_{off}, and the association rate, k_{on}, based on a global fit using the kinetic model 1:1 Langmuir binding with mass transfer. The binding constant K_D is calculated as k_{off}/k_{on}. The receptors were immobilized to the chip surface, with the ligands flowed over the surface.

[00203] To examine if receptor ECD affinity of the BMP2/BMP6 heterodimer correlates with signaling activity, a luciferase

reporter assay was used. Using the C2C12 mouse myoblast cell line, the BMP ligands were quantitatively tested for the ability to activate a Smad1 dependent reporter gene. BMP2, BMP6 and BMP2/BMP6, all showed dose-dependent reporter activation. The BMP2/BMP6 heterodimer ligand showed further greater activation of the reporter gene than the BMP2 or BMP6 homodimer counterparts (Figure 2). Up to 22-fold and 400-fold less BMP2/BMP6 was required to activate the reporter gene to an equivalent level as compared to the BMP2 and BMP6 homodimers respectively. Given that BMP2/BMP6 heterodimer has a high affinity to both type I and type II receptor ECDs, the results suggest that increased affinity to receptor correlates with level of intracellular signaling activity.

[00204] To further characterize activities of BMP2/BMP6 heterodimer, an *ex vivo* assay using chick limb bud mesenchyme cells in micromass culture was used. Primary cultured limb bud mesenchymal cells undergo chondrogenesis in a BMP-dependent manner, and this system allows the characterization of homo and heterodimer in a biological process. Figure 3 displays micrograph images of the staining of the chondrogenic nodules whose formation is known to be stimulated by BMPs. The extent of chondrogenesis is quantitated by measuring dye bound to the chondrogenic nodule. Similar to the reporter assay, a dose-dependent activation of chondrogenesis of limb bud mesenchymal cells by different BMP ligands. A unique aspect of this assay is that BMP6 has a slightly higher activity than BMP2, while its activity in the Smad-1-dependent reporter activation was significantly lower than that of BMP2. This likely involves Type II receptor-initiated distinct signaling such as the p38 pathway. Greater activity of BMP2/BMP6 heterodimer to activate chondrogenesis in this system was observed. BMP2/BMP6 activated chondrogenesis to the similar level of BMP2 and BMP6 homodimer at 10-fold concentration. The heterodimer ligand also induces a higher maximum response at the same concentration. This assay allows for the correlation of not only higher ligand-receptor affinity to higher signaling activity but extends this observation to an increase in biological activity.

[00205] The data with ligand-receptor-ECD affinity, *in vitro* and *ex vivo* assays have demonstrated that a functional asymmetric

BMP2/BMP6 dimer was generated. The asymmetric nature of the BMP2/BMP6 heterodimer allows for the manipulation of specific TGF-beta receptor sites of the ligand. Figure 5 displays the specific mutagenesis of the BMP2/BMP6 heterodimer and the quantification of the ability of the different mutant variants to activate a Smad1 dependent reporter gene using the same system as described above (all factors are at 1nM concentration). The quantified values are displayed as a percentage fold activation compared to the BMP2/BMP6 wild type heterodimer with no mutagenesis (normalized to 100% activation of the reporter gene). The BMP2/BMP6 heterodimer ligands with point mutations to only one of the two type I receptor interfaces with the two type II receptor interfaces intact (Figure 5, samples d and e), are able to activate the reporter gene, although between 20-80% compared to the BMP2/BMP6 wild type heterodimer. In contrast, the BMP2/BMP6 heterodimer with point mutations to only one of the two type II receptor interfaces with the two type I interfaces intact (Figure 5, sample f), can not activate the reporter gene. The BMP2/BMP6 heterodimer ligands with point mutations disrupting one each of the two type I receptor and type II receptor interfaces (Figure 5, samples g and h), can not activate the reporter gene. These results demonstrate that 2 type II sites are required for signaling activity, while only 1 type I site was sufficient for signaling. The difference of signaling activity between two ligands having 1 type I site mutation (d and e in Figure 5) illustrates that Smad1 activation directly correlates with affinity of a type I site and type I receptor (Table 3).

[00206] In further studies with the non-signaling BMP2/BMP6 heterodimer mutants, the ability of the ligands to bind receptor ECD was accessed. Despite the inability of the ligand with only one active type II receptor site to activate the reporter gene (Figure 5, sample f), this ligand is still able to bind type II receptor ECD under native-PAGE conditions. Figure 6 illustrates a type II receptor ECD saturation assay in which the receptor ECD is run on the native-PAGE both in the absence and presence of ligand. In comparing the lane with five micrograms of type II receptor ECD and no factor to the lane with ligand containing two active type II receptor binding sites, the intensity of the band is diminished by

9.0-fold, indicating that the receptor ECD has been incorporated in a ligand-receptor complex. This represents about half of the receptor ECD incorporated into a ligand-receptor complex when comparing the intensity of three micrograms receptor ECD run alone. In the next lane, a ligand with only one active type II receptor site shows an increase in the band intensity by 3.3-fold compared to the ligand with two active type II receptor sites. And in the final lane, a ligand with no active type II receptor sites increases the band intensity by 1.8-fold compared to the ligand with one active type II receptor site. As expected, the BMP2/BMP6 ligand with one active type II receptor site falls in between the ligand with two active type II receptor sites and that with no active type II receptor sites. The single intact type II receptor interface on the mutated BMP2/BMP6 heterodimer is still able to bind one type II TGF-beta receptor ECD, indicating that the extracellular signaling complex can assemble. The inability of the mutated ligand to signal lies further downstream from signaling complex assembly at the cell surface. This assay provides proof for an independent binding model of ligand-receptor complex formation where a single receptor ECD can bind to one of the four receptor sites on the ligand regardless of the affinity or functionality of the other three receptor sites on the ligand.

[00207] The results from the analysis of the recombinant BMP heterodimers prove that a purified homogeneous heterodimer sample can be synthesized (Figure 1). The data further demonstrate that recombinant BMP heterodimers can be expressed in *E.* coli as inclusion bodies, refolded, and purified at a scalable level.

[00208] The data obtained from the surface plasmon resonance affinity studies (Table 3), shows that a BMP heterodimer is more potent *in vivo* and *in vitro* than the BMP homodimers. As compared to the homodimer counterparts, the BMP2/BMP6 heterodimer has the higher affinity receptor sites from each of its covalently linked monomer subunits. The BMP2/BMP6 heterodimer has a high affinity type I receptor site comparable with the BMP2 homodimer and a high affinity type II receptor site comparable with the BMP6 homodimer. Each of these homodimer ligands secondary receptor sites have lower affinity for the respective receptor compared to their primary receptor

sites. This heterodimer ligand-receptor affinity data, for the first time, provides clear evidence for the mechanism of the high potency TGF-beta heterodimer ligands compared to their homodimer counterparts. With high affinity for both type I and type II receptor ECDs, the TGF-beta signaling complex can more readily assemble and remain assembled as the cell surface. The augmented affinity of the BMP heterodimer correlates directly to increased signaling in the whole cell reporter assays (Figure 2). Additionally, the *ex vivo* data from the mesenchyme cell assays (Figures 3 and 4) demonstrates the ability of the BMP2/BMP6 heterodimer to be able to induce a response at a lower concentration and to a higher maximum than its homodimer counterparts. This data directly supports the correlation between increased lignad-receptor affinity, signaling activity, and biological activity.

[00209] While the high signaling activity of the BMP heterodimer was readily achieved in the whole cell reporter system (Figure 2), elucidating the requirements of the TGF-beta signaling complex and mechanism of activation proved much more difficult. The BMP heterodimer constructs with just one type I receptor site and two type II receptor sites, were still able to activate the reporter gene, although to a lesser extent compared to the fully functional BMP heterodimer (Figure 5). However, the BMP heterodimer constructs with two type I receptor sites and only one type II receptor site failed to activate the reporter gene (Figure 5). This inconsistency between the number of required active type I and type II receptor sites on the ligand can not be readily explained. The data demonstrating complex formation under native-PAGE conditions (Figure 6), illustrates that the type II receptor ECD can bind to the mutated heterodimer with only one active type II receptor interface. This indicates that the problem is not with signaling complex formation between ligand and receptor ECD at the cell surface. The complex with only one type II receptor forms as readily as the complex with one type I receptor, yet the complex with only one type II receptor does not initiate downstream signaling.

[00210] The data suggest that a type II receptor kinase phosphorylates it's partner type II receptor rather than itself, and such "cross-phosphorylation" is the molecular nature of the

autophosphorylation. Alternatively, physical association of intracellular kinase domain between two type II receptor is required for "auto phosphorylation", although ECD does not associate one another. This initial step of the signaling cascade cannot occur in the absence of one type II receptor, and thus, no signal is transduced. The ability of some of the mutant BMP heterodimer ligands to form an active signaling complex with only one type I receptor present (Figure 5), occurs because the two type II receptor kinases in the signaling complex are able to dimerize, autophosphorylate, and then transphosphorylate the single type I receptor kinase. No dimer of the type I receptor kinases is required because the kinase domain is simply transphosphorylated by the autophosphorylated dimer of type II receptor kinases.

[00211] The disclosure shows a truly independent ligand-receptor ECD binding model for the signaling complex formation at the cell surface. As stated above, mutated BMP2/BMP6 heterodimer ligand with only one active type II receptor site can still bind a single type II receptor ECD (Figure 6). This evidence provides grounds for the assertion of the signaling complex's ability to form regardless of the affinity or functionality of the four individual receptor sites of the ligand. If each receptor site on the ligand is able to bind in this independent fashion, another layer of complexity is added to the TGF-beta signaling complex formation. With about forty genes encoding for ligands and many functional ligands possible because of heterodimers an intricate signaling mechanism must exist. With the ability to only signal through twelve receptors, this ligand driven signaling mechanism must rely on the affinity of ligand's individual receptor sites to the different receptors. This is only possible if each of the four receptor sites on the ligand act independently to recruit a receptor into the signaling complex. In order to elicit different biological functions through the same twelve receptors, the independent and individual affinities of each of the four receptor sites on the ligand is the key factor in fine tuning the biological response. In this point of view, a role of specific ligand (homodimer or heterodimer) is to assemble distinct set of type I and type II receptors with distinct affinity, which in turn,

generate different level of signaling and complexity of TGF-b signaling.

[00212] The scalable generation of a novel BMP2/BMP6 construct with high activity *in vitro* and *ex vivo* has far reaching implications. This molecule served as the basis to determine the assembly of the TGF-beta superfamily ligand-receptor signaling complex and to demostrate the direct correlation between ligand-receptor affinity, signaling activity, and biological activity. The differences in affinity between ligand and receptor are crucial and the asymmetric heterodimer ligand signaling adds further complexity to the biological activity of the TGF-beta molecules. the study with the BMP heteromider illustrates how each ligand-receptor interaction contributes to the activity of the TGF-beta superfamily.

[00213] **Generation of heterodimer.** The mature domains of human BMP2 (residues 1-110) and human BMP6 (residues 1-132) were expressed in *E. coli* as inclusion bodies. Mutations to the wild type ligand sequences were based on previously published findings which disrupt the ligand-receptor interfaces (Keller *et al.*, 2004; Kirsch *et al.*, 2000). The expressed inclusion bodies were isolated, purified, and refolded. The refolded BMP2 and BMP6 homodimers, and BMP2/BMP6 heterodimer were purified using a HiTrap heparin column (GE Healthcare) and reversed phase chromatography (GraceVydac). The ligands were lyophilized and re-suspended in 10mM sodium acetate pH 4.0. The ECDs of human BMPRIa (residues 1-129) and mouse ActRIIb (residues 1-98) were expressed in *E. coli* as thioredoxin fusion proteins. Mouse ActRII-ECD (residues 1-102) was expressed and purified from a *P. pastoris* expression system.

[00214] **Surface plasmon resonance (BIAcore) affinity studies.** The affinity of the ligands to BMPRIa, ActRII, and ACTRIIb was monitored by using a Biacore 3000 (GE Healthcare) and the data were analyzed by using BIAevaluation software ver. 4.1 (GE Healthcare). Using primary amine coupling, receptor ECDs were immobilized on a CM5 chip. The receptors were immobilized independently on flow cells 2-4 for 10 minutes at a flow rate of 5 µL/min and a concentration of 20 µM in 10 mM sodium acetate, pH 4.0. Flow cell 1 was left blank with no immobilized protein as a negative control. The experiments were performed at a flow rate of 50 µL/min in 20 mM Tris-HCl, pH

7.9, 250 mM NaCl, 0.36% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 0.005% Tween-20. At least five concentrations, plus a zero concentration, were run per sample for kinetic analysis and the data were fit by using a global 1:1 Langmuir binding with mass transfer.

[00215] Luciferase reporter assays. Smad1-dependent luciferase assays were performed. In brief, C2C12 cells are cultured in Dulbecco's minimum essential medium (DMEM) + 5% FBS supplemented with L-glutamine and antibiotics. For luciferase reporter assays, cells were trypsinized, washed twice with PBS, and plated into 48-well plates with DMEM + 0.1% FBS. Twenty-four hours later, cells were transfected with -1147Id1-luciferase construct containing the Smad binding sites (Id1-Luc) (Nakashima *et al.*, 2001), a Smad-1 expression construct, and a CAGGS-LacZ plasmid using Fugene6 (Roche) according to the manufacturer's instruction. Luciferase activity was measured 24h after stimulation with ligands, and the values were normalized for transfection efficiency using beta-galactosidase activity. The activity of the luciferase reporter is expressed in fold induction relative to control values that are obtained using -927Id1-luciferase that lacks Smad1 binding domains (Id1-Luc mut).

[00216] **Chick Limb Bud Micromass assays.** Chick embryos at Hamburger-Hamilton stage 23-24 (Hamburger and Hamilton, 1951) were collected in Hanks solution containing Ca2+ and Mg2+, and distal 1/3 part of limb buds were dissected out. Ectodermal sheets were removed by trypsinization (0.5% in Hanks solution with Ca2+ and Mg2+) on ice for 30 min, and then mesenchymal tissues were recovered and incubated in Ca^{2+} and Mg^{2+}-free Hanks solution at 37°C for 15 min. The mesenchyme cells were dissociated into single cells by pipetting in OptiMEM medium (Invitrogen) containing 1% FBS. Cultures were seeded into 96-well plates at 4 x 10^5 cells/well. After 1 hour, media containing each ligand was added. Fresh media with ligands was changed daily, and cells were analyzed for chondrogenesis by Alcian blue staining to visualize cartilage nodule and quantification of chondrogenesis as described (Wada *et al.*, 2003).

[00217] **Native-PAGE Ligand-Receptor ECD Complex Formation.** Five micrograms of purified ActRII-ECD alone and with ten micrograms of BMP2/BMP6, BMP2/BMP6 with a single active type II receptor

interface, or BMP2 with no active type II receptor interfaces was loaded onto a native-PAGE gel in 50 mM Tris-HCl, pH 7.9, 700 mM NaCl, and 1.8% CHAPS. The Coomassie Brilliant Blue (Bio-Rad) stained gel was analyzed using the "Integrated Density" function with NIH ImageJ software (Abramoff *et al.*, 2004).

Example 3

[00218] (1) Development of Stem cell media (Valera *et al.*, 2010) using AB2-008. Culturing human embryonic stem cells (hESC) in feeder free conditions requires the use of complex formulation media to maintain pluripotency. Unfortunately, commercial media are very expensive since the growth factors required for the media are difficult to produce in mammalian cells. We have used mTeSR1 formulation to derive a new medium (CIVA medium or mCIVA) for culturing human embryonic stem (hES) cells, and deriving and culturing induced pluripotent stem (iPS) cells (see Figure 2). CIVA medium substitutes TGFβ1 in mTeSR1 for AB2-008, a new chimeric protein with similar activity to Activin-A. hES cells cultured in this medium on matrigel coating maintain pluripotent morphology for more than 20 passages without karyotypic abnormalities. These cells are also positive for the pluripotency markers TRA-1-60 and SSEA-4, and differentiate in response to BMP-2 treatment. iPS cells cultured in this medium also retain morphological characteristics of pluripotency and expression of pluripotency markers. This new CIVA medium is also suitable to derivate iPS cells from human foreskin fibroblasts. CIVA medium has all the properties desired of other commercial media for hES cells but can be formulated for considerably less cost than currently available media. Figure 2. Development of mCIVA formulation using H9 hES cell line.

[00219] Figure 9 shows H9 hES cells cultured in mCIVA using different concentrations of AB2-008 in the absence or presence of human FGF2. A. Differentiated H9 cells after 3 passages (1 ng/mL, AB2-008; no FGF2). B. Differentiated H9 cells after 3 passages (10 ng/mL, AB2-008; no FGF2). C. Differentiated H9 cells after 11 passages (100 ng/mL, AB2-008; no FGF2). D. Differentiated H9 cells after 12 passages (1 ng/mL, AB2-008; 100 ng/mL, FGF2). E. H9 cells after 13 passages (10 ng/mL, AB2-008; 100 ng/mL, FGF2). F.

Differentiated H9 cells after 9 passages (100 ng/mL, AB2-008; 100 ng/mL, FGF2). Differentiated cells are denoted by arrows.

[00220] (2) Mineralization Data of AB2-004, AB2-011, AB2-015 (Yoon *et al.*, 2010). Von Kossa staining was used to monitor the development of pre-osteoblast cell line (MC3T3-E1) for extracellular mineralization by Ca deposition. AB2-004 and AB2-011 show dramatic increase of the stain of nearly 10 times or more intensity. AB2-015 shows the most increase but past 7 days period (not shown here). Control group (A) shows no appreciable mineralization, whereas the control ligand (BMP2) shows modest increase of Ca flux and deposition as shown in Row B. of Figure 10.

[00221] (3) Adult rat regeneration by AB2-004 (in collaboration) P3 digit of Adult rat was severed. Either BMP2 or AB2-004 soaked in an agarose gel bead were added at the sight of sugery. Bone regrowth was monitored. BMP2-treated tissue shows no bone recovery. AB2-004-treated digit shows full recovery.

[00222] (4) Smad2-based signaling (luciferase) assay by AB2-008, AB2-009, AB2-010 (Allendorph *et al.*, 2010). AB2-008, AB2-009, and AB2-010 activate the Smad2 pathway in a manner nearly undistinguishable from activin-βA. Potency for the chimeras is slightly reduced compared to activin-βA, from 5 to 20-fold (see Figure 11).

[00223] (5) Phospho-Smad-2 Assays by AB2-008, AB2-009, and AB2-010. Activin-βA specifically phosphorylates Smad-2 and not Smad-1. This is in contrast to BMP-2 where we see specific phosphorylation of Smad-1 and not Smad-2. AB2-008, AB2-009, and AB2-010 display the same Smad-2 phosphorylation pattern as activin-βA. This confirms all three ligands stimulate the activin-βA signaling pathway in a manner similar to activin-βA (see Figure 11).

[00224] (6) Follicle stimulating hormone release by AB2-008, AB2-009, and AB2-010. When activin-βA is added to rat interior pituitary cells, it causes a dose dependent release of Follicle stimulating hormone (FSH). The activin-βA induced release of FSH can be blocked by the addition of the antagonist Inhibin. Similar to activin-βA, AB2-008, AB2-009, and AB2-010 cause the release of FSH and this stimulation is decreased in the presence of Inhibin.

[00225] **(7) Co-receptor binding with Cripto by AB2-008.** Addition of Cripto reduces both activin-βA and AB2-008 signaling by comparable levels. These data indicate that AB2-008 possesses ability to bind Cripto, activin-βAco-receptor, confirming the functional similarity between AB2-008 and activin-βA.

[00226] **(8) AB2-008 and Activin-βA Receptor affinity**

[00227] AB2-008 shows the receptor binding profile similar to that of activin-βA, which has high affinity to ActRII and none for BMPRIa. Further, the two ligands have nearly the same binding affinity. AB2-008 is ~1.7 weaker than activin-βA.

Table 4. Receptor affinity of Activin-βA versus AB2-008

Receptor Affinity Data from BIAcore Experiments

Ligand	BMPRIa-ECD		ActRII-ECD	
	k_{off}[1/s]/ k_{on}[1/M*s]	K_D [nM]	k_{off}[1/s]/ k_{on}[1/M*s]	K_D [nM]
Activin-βA	No Binding	N.A.	$7.16x10^{-4}/3.52x10^{6}$	0.203
AB2-008	No Binding	N.A.	$8.24x10^{-4}/2.39x10^{6}$	0.344

The receptors were immobilized to the chip surface with the ligands flowed over the surface. The data were fit to a kinetic model (1:1 Langmuir binding with mass transfer) in which K_D is calculated as k_{off}/k_{on}. The table reports data from a single trial. No Binding indicates that the interaction was not detectable.

[00228] **(9) Signaling activity of AB2-011, AB2-012, and AB2-015 by Smad-1 pathway.** AB2-004, AB2-011, AB2-012, and AB2-015 activate the Smad-1 pathway more potently than BMP-2. This activation is 3 to 8-fold higher than BMP-2 (Figure 14).

[00229] **(10) Receptor binding affinity of AB2-004, AB2-011, AB2-012, and AB2-015.** AB2-004, AB2-011, AB2-012, and AB2-015 show similar binding affinity to ActRII as activin-βA. This is ~100 fold higher than the binding BMP-2 has for the same receptor. The type I receptor binding for the AB chimeras ranges from near BMP-2 levels (AB2-004) to activin-βA levels (no binding to BMPRIa for AB2-015).

Table 5. Receptor binding affinity of AB2-004, AB2-011, AB2-012, and AB2-015

Receptor Affinity Data from BIAcore Experiments				
Ligand	BMPRIa-ECD		ActRII-ECD	
	k_{off}[1/s]/ k_{on}[1/M*s]	K_D [nM]	k_{off}[1/s]/ k_{on}[1/M*s]	K_D [nM]
BMP-2	$7.54x10^{-4}/3.60x10^{5}$	2.09	$4.05x10^{-2}/1.10x10^{6}$	36.8
Activin-βA	No Binding	N.A.	$7.16x10^{-4}/3.52x10^{6}$	0.203
AB2-004	$1.85x10^{-2}/1.13x10^{6}$	16.4	$1.87x10^{-4}/4.91x10^{5}$	0.381
AB2-011	$1.93x10^{-2}/4.07x10^{5}$	47.4	$3.39x10^{-4}/6.46x10^{5}$	0.525
AB2-015	No Binding	N.A.	$3.71x10^{-4}/1.61x10^{6}$	0.230
AB2-012	$8.48x10^{-2}/1.61x10^{5}$	526	$1.60x10^{-3}/3.39x10^{6}$	0.472

The receptors were immobilized to the chip surface with the ligands flowed over the surface. The data were fit to a kinetic model (1:1 Langmuir binding with mass transfer) in which K_D is calculated as k_{off}/k_{on}. The table reports data from a single trial. No Binding indicates that the interaction was not detectable.

[00230] (11) Noggin insensitivity of AB2-004, AB2-011, AB2-012, and AB2-015. Noggin suppresses the signaling acitivity by directly complexing with the ligand, and rendering it unable to bind its own receptors for signaling. In contrast to BMP-2 which is blocked to near background levels in the presence of Noggin, the higher signaling of AB2-004, AB2-011, and AB2-015 are not inhibited by Noggin. AB2-012 is partially insensitive to Noggin Inhibition, with a ~50% decrease in signaling with the addition of Noggin (Figure 15). This property makes them particularly powerful in their cellular signaling ability *in vivo*, including bone regeneration.

[00231] (12) Production Efficiency

Table 6. Production efficiency of refolding from E. coli inclusion body.

Construct	Dimer Yield	Rating
1b2b3b4b5b6a	>10%	+++
1b2b3b4b5a6a	>10%	+++
1b2b3b4b5a6b	>10	+++
1b2b3b4a5a6a	5%	+++
1b2b3b4a5b6b	2%	+
1b2b3b4a5a6b	3%	+
1b2b3b4a5b6a	3%	+
1b2b3a4a5a6a	5%	++
1b2b3a4a5a6b	6%	++
1b2b3a4a5b6b	5%	++

1b2b3a4a5b6a	**7%**	**++**
1b2b3a4b5b6b	**3%**	**+**
1b2b3a4b5b6a	**6%**	**++**
1b2b3a4b5a6a	**4%**	**+**
1b2b3a4b5a6b	**5%**	**++**
1b2a3a4a5a6a	**>10%**	**+++**
1b2a3a4a5a6b	**>10%**	**+++**
1b2a3a4a5b6b	**9%**	**++**
1b2a3a4a5b6a	**>10%**	**+++**
1b2a3a4b5b6b	**>10%**	**+++**
1b2a3a4b5b6a	**4%**	**+**
1b2a3a4b5a6b	**1%**	**-**
1b2a3a4b5a6a	**2%**	**+**
1b2a3b4b5b6b	**>10%**	**+++**
1b2a3b4b5b6a	**>10%**	**+++**
1b2a3b4b5a6a	**9%**	**++**
1b2a3b4b5a6b	**>10%**	**+++**
1b2a3b4a5a6a	**4%**	**+**
1b2a3b4a5b6a	**4%**	**+**
1b2a3b4a5b6b	**4%**	**+**
1b2a3b4a5a6b	**1%**	**-**
1b2a3a4a5a6a L66V/V67I	**4%**	**+**
1b(1a_II)2a3a4a5a6a	**3%**	**+**

[00232] (13) Receptor binding affinity of BMP2/BMP6 Heterodimer (Isaacs *et al.*, 2010). BMP2/BMP6 heterodimer has the binding characteristics of BMP2 for type I receptor BMPRIa and BMP6 for the type II receptor ActRIIb. Maintaining high affinity for each receptor type by the heterodimer ligand makes BMP2/BMP6 heterodimer stronger in signaling activities than its homodimeric couterparts, BMP2 and BMP6 homodimers (Table 7).

Table 7. Receptor binding affinity measured by Surface plasmon resonance.

Ligand	**Receptor BMPRIa**		**Receptor ActRIIb**	
	k_{off} [1/s]/k_{on}[1/M*s]	**K_D [nM]**	**k_{off} [1/s]/k_{on}[1/M*s]**	**K_D [nM]**
BMP2	**$1.11 \times 10^{-3}/8.52 \times 10^{5}$**	**1.31**	**$2.57 \times 10^{-2}/6.68 \times 10^{5}$**	**38.5**
BMP6	**$9.37 \times 10^{-3}/1.50 \times 10^{5}$**	**62.8**	**$1.82 \times 10^{-3}/2.73 \times 10^{5}$**	**6.68**
BMP2/BMP6	**$1.05 \times 10^{-3}/1.03 \times 10^{6}$**	**1.02**	**$8.61 \times 10^{-3}/1.32 \times 10^{6}$**	**6.52**

[00233] (14) SMAD-1 Signaling activity of BMP2/BMP6 Heterodimer. The BMP2/BMP6 heterodimer is much more active than either BMP2 or BMP6 alone. BMP2/BMP6 has an EC50 that is at least an order of magnitude higher than BMP2 or BMP6 alone. Further, the maximal response reached by BMP2/BMP6 is higher than the combination of maximum signal reached by BMP2 and BMP6 alone.

[00234] (15) Chick Limb Bud Micromass assays for BMP2/BMP6 heterodimer. BMP2/BMP6 induces chondrogenesis more potently than either BMP2 or BMP6 homodimers. In chick limb bud mesenchyme cell micromass culture chrondogenesis assays, after three days we see that BMP2/BMP6 induces chondrogenesis at both lower concentrations and to a higher level than either BMP2 or BMP6.

Example 4

[00235] Description of subdomains (building blocks) for generating Designer Ligand. In order to create the chimeras, a first step was deciding where to make the borders for each of the segments. The chimera library has been constructed using activin-βA and BMP-2 as two sequence sources. To design the cut-off regions (Junction) for the sections to make the activin/BMP-2 (AB) chimera, a structure-guided approach combined with protein sequence alignment was used. Initially, the 3-dimensional crystal structures of activin-βA (Harrington *et al.*, 2006) and BMP-2 (Allendorph *et al.*, 2006) were inspected structurally. From this analysis, the ligands were loosely divided into 6 distinct sections (see Figure 7 for segments 1 through 6). The exact segment junctions were ultimately determined following a protein sequence alignment of the two ligands to minimize any sequence changes of either protein sequence as a result of joining the Junction. Further, the segmental boundaries were chosen to be located in structural regions away from receptor binding sites.

[00236] Detailed descriptions of Junctions: Between segments 1 and 2 (Junction 1): Focusing on the boundary of segment 1 and segment 2, we found a 10-residue region that is highly conserved between BMP-2 and activin-βA. Indeed, 8 of the 10 residues are identical and the other two are very conservative differences. This

area is located in the tip region of Finger 1 and depending of the ligand, makes or is predicted to make limited contacts with either receptor type. Based on the ternary crystal structure of BMP-2/BMPRIa/ActRII (Allendorph *et al.*, 2006), only Val-26, Gly-27, and Trp-28 (BMP-2 numbering) generate contacts with the type I receptor. Of these three residues, only Val-26 is different between the ligands, but it is a very conservative change since the corresponding residue in activin-βA is Ile-23. Since the residues in this region are very similar and not involved in receptor binding, it makes for a good boundary point for segment 1 and 2.

[00237] Between segments 2 and 3 (Junction 2): Moving to the boundary region between segments 2 and 3, another good area for our boundary cut-off can be found. Here, a 4-residue sequence that is identical between activin-βA and BMP-2 exists. When the ligands are properly folded, this region is located in the center of the dimer, with both cyteines participating in the cystine knot. This is advantageous because the residues here are buried from the surface of the ligands and do not participate in any ligand-receptor interactions.

[00238] Between segments 4 and 5 (Junction 4): Similar to the segment 2/3 boundary, the segment 4/5 boundary is situated in an excellent location for the cut-off. Here, we find a 5-residue region of sequence identity and, as with the segment 2/3 boundary, this region is buried at the center of the ligand dimer. The 2 cysteine residues participate in both the cystine knot as well as the inter-monomer disulfide bond. Again, this location prevents the residues in this region from participating in receptor binding interactions.

[00239] Between segments 5 and 6 (Junction 5): To extend the design of BMP-2 and activin-βA chimeras, other boundary regions have been chosen to facilitate generating RASCH constructs using all members of the TGF-β superfamily. Along with sharing structural architecture, the TGF-β superfamily ligands seem to have certain regions in their protein sequences that are highly conserved. Interestingly, these regions coincide with the boundary regions chosen for making the BMP-2 and activin-βA chimeras. For example, in the boundary region of 4 and 5, most ligands share 3 out of the 4

residues that define the boundary domain. This high degree of similarity, coupled with these regions being isolated from the receptor binding sites, indicates RASCH as the universal strategy to create a library of Designer Ligands with new functionalities.

[00240] Between segments 3 and 4 ((Junction 3): The boundary between segments 3 and 4 is subject to structural variability between different subfamilies, in which ligand-receptor assembly mechanism can differ substantially. In that regard, segments 3 and 4 can be treated as one segmental piece such that two segments will be derived from the common parental strand to preserve their structural integrity.

[00241] The structural similarity among all TGF-beta superfamily ligands forms the rational basis for designing chimeric protein by exchanging (swapping) related segments of the sequences known to carry out certain functionality such as molecular recognition. Protein engineering of Antibody chain, or more specifically of antibody fragment (Fab), will be a prime example where the basic structural scaffold is built on the Core architecture of the light- and heavy chain sequences, for which six variable loops, three from each of the two chains, are responsible for the role of epitope-binding specificity. In the similar vein, the TGF-beta superfamily ligands share their structural framework as a butterfly-like architecture. A portion(s) of the sequence segments functionally equivalent to variable loop regions of Antibody can then be 'implanted' to transfer recognition specificity from one ligand to another. Our design principle distinguishes itself from the aforementioned 'functional transfer by sequence implantation'. The new chimeric library is created on the basis of structural feasibility of each subdomain as defined by each Junction. The junctions between the various domains of the TGF-beta family members used to generate the chimeras of the disclosure provide useful building blocks of the chimera library. By this reasoning, Junctions 1, 2, 4, and 5 are well defined to be broadly applicable to all TGF-beta superfamily members, whereas Junction 3 is not broadly applicable. The application of Junction 3 in the chimera design depends on the target sequences, in which case subdomain segments 3 and 4 can be treated instead as one segment in designing

the chimera library. The approach maximizes the chance of producing such protein products that are foldable, for which functional characterization will then follow.

[00242] Table of additional sequences:

TGF-β Ligand	DNA Sequence	Protein Sequence
BMP-2	caagccaaacacaaacagcggaaacgccttaagtccagctgtaagagacacccttgtacgtggacttcagtgacgtggggtggaatgactggattgtggctcccccggggtatcacgccttttactgccacggagaatgccctttcctctggctgatcatctgaactccactaatcatgccattgttcagacgttggtcaactctgttaactctaagattcctaaggcatgctgtgtcccgacagaactcagtgctatctcgatgctgtaccttgacgagaatgaaaaggttgtattaaagaactatcaggacatggttgtggagggttgtgggtgtcgc (SEQ ID NO:1)	QAKHKQRKRLKSSCKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIVQTLVNSVNSKIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCR (SEQ ID NO:2)
BMP-3 (osteogenin)	cagtggattgaacctcggaattgcgccaggagatacctcaaggtagactttgcagatattggctggagtgaatggattatctcccccaagtcctttgatgcctattattgctctggagcatgccagttccccatgccaaagtctttgaagccatcaaatcatgctaccatccagagtatagtgagagctgtgggggtcgttcctgggattcctgagccttgctgtgtaccagaaaagatgtcctcactcagtattttattctttgatgaaaataagaatgtagtgcttaaagtataccctaacatgacagtagagtcttgcgcttgcaga (SEQ ID NO:42)	QWIEPRNCARRYLKVDFADIGWSEWIISPKSFDAYYCSGACQFPMPKSLKPSNHATIQSIVRAVGVVPGIPEPCCVPEKMSSLSILFFDENKNVVLKVYPNMTVESCACR(SEQ ID NO:43)
BMP-4(BMP-2b)	agccctaagcatcactcacagcgggccaggaagaagaataagaactgccggcgccactcgctctatgtggacttcagcgatgtgggctggaatgactggattgtggccccaccaggctaccaggccttctactgccatggggactgccccttccactggctgaccacctcaactcaaccaaccatgccattgtgcagaccctggtcaattctgtcaattccagtatccccaaagcctgttgtgtgcccactgaactgagtgccatctccatgctgtacctggatgagtagataaggtggtactgaaaaattatcaggagatggtagtagagggatgtgggtgccgc (SEQ ID NO:44)	SPKHHSQRARKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAIVQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR(SEQ ID NO:45)
BMP-5	gcagccaacaaacgaaaaaatcaaaaccgcaataaatccagctctcatcaggactcctccagaatgtccagtgttggagattataacacaagtgagcaaaaacaagcctgtaagaagcacgaactctatgtgagcttccgggatctgggatggcaggactggattatagcaccagaaggatacgctgcatttttattgtgatggagaatgttcttttccacttaacgcccatatgaatgccaccaaccacgctatagttcagactctggttcatctgatgtttcctgaccacgtaccaaagccttgttgtgctccaaccaaattaaatgccatctctgttctgtactttgatgacagctccaatgtcattttgaaaaaatatagaaatatggtagtacgctcatgtggctgccac(SEQ ID NO:46)	AANQNRNKSSSHQDSSRMSSVGDYNTSEQKQACKKHELYVSFRDLGWQDWIIAPEGYAAFYCDGECSFPLNAHMNATNHAIVQTLVHLMFPDHVPKPCCAPTKLNAISVLYFDDSSNVILKKYRNMVVRSCGCH (SEQ ID NO:47)
BMP-6(Vgr-1)	caacagagtcgtaatcgctctacccagtcccaggacgtggcgcgggtctccagtgcttcagattacaacagcagtgaattgaaaacagcctgcaggaagcatgagctgtatgtgagtttccaagacctgggatggcaggactggatcattgcacccaagggctatgctgccaattactgtgatggagaatgctccttcccactcaacgcacacatgaatgcaaccaaccacgcgattgtgcagaccttggttcaccttatgaaccccgagtatgtccccaaaccgtgctgtgcgccaactaagctaaatgccatctcggttctttactttgatgacaactccaatgtcattctgaaaaaatacaggaatatggttgtaagagcttgtggatgccac	QQSRNRSTQSQDVARVSSASDYNSSELKTACRKHELYVSFQDLGWQDWIIAPKGYAANYCDGECSFPLNAHMNATNHAIVQTLVHLMNPEYVPKPCCAPTKLNAISVLYFDDNSNVILKKYRNMVVRACGCH (SEQ ID NO:49)

	(SEQ ID NO:48)	
BMP-7(OP-1)	tccacggggagcaaacagcgcagccagaaccgctccaagac gcccaagaaccaggaagccctgcggatggccaacgtggcag agaacagcagcagcgaccagaggcaggcctgtaagaagcac gagctgtatgtcagcttccgagacctgggctggcaggactggat catcgcgcctgaaggctacgccgcctactactgtgaggggagg tgtgccttccctctgaactcctacatgaacgccaccaaccacgc catcgtgcagacgctggtccacttcatcaacccggaaacggtg cccaagccctgctgtgcgcccacgcagctcaatgccatctccgt cctctacttcgatgacagctccaacgtcatcctgaagaaatacag aaacatggtggtccgggcctgtggctgccac (SEQ ID NO:50)	STGSKQRSQNRSKTPKNQE ALRMANVAENSSSDQRQA CKKHELYVSFRDLGWQDW IIAPEGYAAYYCEGECAFPL NSYMNATNHAIVQTLVHFI NPETVPKPCCAPTQLNAISV LYFDDSSNVILKKYRNMVV RACGCH (SEQ ID NO:51)
BMP-8(OP-2)	gcagtgaggccactgaggaggaggcagccgaagaaaagcaa cgagctgccgcaggccaaccgactcccagggatctttgatgac gtccacggctcccacggccggcaggtctgccgtcggcacgag ctctacgtcagcttccaggacctcggctggctggactgggtcat cgctccccaaggctactcggcctattactgtgagggggagtgct ccttcccactggactcctgcatgaatgccaccaaccacgccatc ctgcagtccctggtgcacctgatgatgccagacgcagtcccca aggcgtgctgtgcacccaccaagctgagcgccacctctgtgct ctactatgacagcagcaacaatgtcatcctgcgcaagcaccgc aacatggtggtcaaggcctgcggctgccac (SEQ ID NO:52)	AVRPLRRRQPKKSNELPQA NRLPGIFDDVHGSHGRQVC RRHELYVSFQDLGWLDWVI APQGYSAYYCEGECSFPLD SCMNATNHAILQSLVHLMK PNAVPKACCAPTKLSATSV LYYDSSNNVILRKHRNMVV KACGCH (SEQ ID NO:53)
BMP-9(GDF-2)	agcgccggggctggcagccactgtcaaaagacctccctgcgg gtaaacttcgaggacatcggctgggacagctggatcattgcacc caaggagtatgaagcctacgagtgtaagggcggctgcttcttcc ccttggctgacgatgtgacgccgacgaaacacgctatcgtgca gaccctggtgcatctcaagttccccacaaaggtgggcaaggcc tgctgtgtgcccaccaaactgagccccatctccgtcctctacaag gatgacatgggggtgcccaccctcaagtaccattacgagggca tgagcgtggcagagtgtgggtgcaggtag (SEQ ID NO:54)	SAGAGSHCQKTSLRVNFED IGWDSWIIAPKEYEAYECK GGCFFPLADDVTPTKHAIV QTLVHLKFPTKVGKACCVP TKLSPISVLYKDDMGVPTL KYHYEGMSVAECGCR (SEQ ID NO:55)
BMP-10	aacgccaaaggaaactactgtaagaggaccccgctctacatcg acttcaaggagattgggtgggactcctggatcatcgctccgcct ggatacgaagcctatgaatgccgtggtgtttgtaactacccctg gcagagcatctcacacccacaaagcatgcaattatccaggcctt ggtccacctcaagaattcccagaaagcttccaaagcctgctgtg tgcccacaaagctagagcccatctccatcctctatttagacaaag gcgtcgtcacctacaagtttaaatacgaaggcatggccgtctcc gaatgtggctgtaga (SEQ ID NO:56)	NAKGNYCKRTPLYIDFKEIG WDSWIIAPPGYEAYECRGV CNYPLAEHLTPTKHAIIQAL VHLKNSQKASKACCVPTKL EPISILYLDKGVVTYKFKYE GMAVSECGCR (SEQ ID NO:57)
BMP-15(GDF-9b)	caagcagatggtatctcagctgaggttactgcctcttcctcaaaa catagcgggcctgaaaataaccagtgttccctccaccctttccaa atcagcttccgccagctgggttgggatcactggatcattgctccc cctttctacaccccaaactactgtaaaggaacttgtctccgagtac tacgcgatggtctcaattcccccaatcacgccattattcagaacct tatcaatcagttggtggaccagagtgtcccccggccctcctgtgt cccgtataagtatgttccaattagtgtccttatgattgaggcaaatg ggagtattttgtacaaggagtatgagggtat gattgctgagtcttgtacatgcaga (SEQ ID NO:58)	QADGISAEVTASSSKHSGPE NNQCSLHPFQISFRQLGWD HWIIAPPFYTPNYCKGTCLR VLRDGLNSPNHAIIQNLINQ LVDQSVPRPSCVPYKYVPIS VLMIEANGSILYKEYEGMIA ESCTCR (SEQ ID NO:59)

GDF-1	gacgccgaacccgtgttgggcggcggccccgggggcgcttgt cgcgcgcggcggctgtacgtgagcttccgcgaggtgggctgg caccgctgggtcatcgcgccgcgcggcttcctggccaactact gccagggtcagtgcgcgctgcccgtcgcgctgtcggggtccg gggggccgccggcgctcaaccacgctgtgctgcgcgcgctca tgcacgcggccgccccgggagccgccgacctgccctgctgc gtgcccgcgcgcctgtcgcccatctccgtgctcttctttgacaac agcgacaacgtggtgctgcggcagtatgaggacatggtggtg gacgagtgcggctgccgc (SEQ ID NO:60)	DAEPVLGGGPGGACRARRL YVSFREVGWHRWVIAPRGF LANYCQGQCALPVALSGSG GPPALNHAVLRALMHAAA PGAADLPCCVPARLSPISVL FFDNSDNVVLRQYEDMVV DECGC (SEQ ID NO:61)
GDF-3(Vgr-2)	gcagccatccctgtccccaagctttcttgtaagaacctctgccac cgtcaccagctattcattaacttccgggacctgggttggcacaag tggatcattgcccccaaggggttcatggcaaattactgccatgg agagtgtcccttctcactgaccatctctctcaacagctccaattat gctttcatgcaagccctgatgcatgccgttgacccagagatccc ccaggctgtgtgtatccccaccaagctgtctcccatttccatgctc taccaggacaataatgacaatgtcattctacgacattatgaagac atggtagtcgatgaatgtgggtgtggg (SEQ ID NO:62)	AAIPVPKLSCKNLCHRHQLF INFRDLGWHKWIIAPKGFM ANYCHGECPFSLTISLNSSN YAFMQALMHAVDPEIPQA VCIPTKLSPISMLYQDNNDN VILRHYEDMVVDECGCG (SEQ ID NO:63)
GDF-5(BMP-14)	gccccactggccactcgccagggcaagcgacccagcaagaa ccttaaggctcgctgcagtcggaaggcactgcatgtcaacttca aggacatgggctgggacgactggatcatcgcaccccttgagta cgaggctttccactgcgaggggctgtgcgagttcccattgcgct cccacctggagcccacgaatcatgcagtcatccagaccctgat gaactccatggaccccgagtccacaccacccacctgctgtgtg cccacgcggctgagtcccatcagcatcctcttcattgactctgcc aacaacgtggtgtataagcagtatgaggacat ggtcgtggagtcgtgtggctgcagg (SEQ ID NO:64)	APLATRQGKRPSKNLKARC SRKALHVNFKDMGWDDWI IAPLEYEAFHCEGLCEFPLR SHLEPTNHAVIQTLMNSMD PESTPPTCCVPTRLSPISILFI DSANNVVYKQYEDMVVES CGCR (SEQ ID NO:65)
GDF-6(BMP-13)	acggccttcgccagtcgccatggcaagcggcacggcaagaag tccaggctacgctgcagcaagaagcccctgcacgtgaacttca aggagctgggctgggacgactggattatcgcgcccctggagta cgaggcctatcactgcgagggtgtatgcgacttcccgctgcgct cgcacctggagcccaccaaccacgccatcatccagacgctgat gaactccatggaccccggctccaccccgcccagctgctgcgtg cccaccaaattgactcccatcagcattctatacatcgacgcggg caataatgtggtctacaagcagtacgaggacatggtggtggagt cgtgcggctgcagg (SEQ ID NO:66)	TAFASRHGKRHGKKSRLRC SKKPLHVNFKELGWDDWII APLEYEAYHCEGVCDFPLR SHLEPTNHAIIQTLMNSMDP GSTPPSCCVPTKLTPISILYID AGNNVVYKQYEDMVVESC GCR(SEQ ID NO:67)
GDF-7(BMP-12)	acggcgttggccgggacgcggacatcgcagggcagcggcg ggggcgcgggccggggccacgggcgcaggggccggagcc gctgcagccgcaagccgttgcacgtggacttcaaggagctcgg ctgggacgactggatcatcgcgccgctggactacgaggcgtac cactgcgagggcctttgcgacttccctttgcgttcgcacctcgag cccaccaaccatgccatcattcagacgctgctcaactccatggc accagacgcggcgccggcctcctgctgtgtgccagcgcgcct cagccccatcagcatcctctacatcgacgccgccaacaacgttg tctacaagcaatacgaggacatggtggtggaggcctgcggctg cagg (SEQ ID NO:68)	TALAGTRTSQGSGGGAGRG HGRRGRSRCSRKPLHVDFK ELGWDDWIIAPLDYEAYHC EGLCDFPLRSHLEPTNHAII QTLLNSMAPDAAPASCCVP ARLSPISILYIDAANNVVYK QYEDMVVEACGCR (SEQ ID NO:69)
GDF-8(Myostatin)	gattttggtcttgactgtgatgagcactcaacagaatcacgatgct gtcgttaccctctaactgtggattttgaagcttttggatgggattgg attatcgctcctaaaagatataaggccaattactgctctggagagt gtgaatttgtatttttacaaaaatatcctcatactcatctggtacacc aagcaaaccccagaggttcagcaggcccttgctgtactcccac aaagatgtctccaattaatatgctatattttaatggcaaagaacaa ataatatatgggaaaattccagcgatggtagtagaccgctgtgg	DFGLDCDEHSTESRCCRYP LTVDFEAFGWDWIIAPKRY KANYCSGECEFVFLQKYPH THLVHQANPRGSAGPCCTP TKMSPINMLYFNGKEQIIYG KIPAMVVDRCGCS (SEQ ID NO:71)

	gtgctca (SEQ ID NO:70)	
GDF-9	ggtcaggaaactgtcagttctgaattgaagaagcccttgggccc agcttccttcaatctgagtgaatacttcagacaatttcttcttcccca aaatgagtgtgagctccatgactttagacttagctttagtcagctg aagtgggacaactggattgtggctccgcacaggtacaaccctc gatactgtaaaggggactgtccaagggcagttggacatcggtat ggctctccagttcacaccatggtacagaacatcatctatgagaa gctggactcctcagtgccaagaccgtcatgtgtacctgccaaat acagccccttgagtgttttgaccattgagcccgatggctcaattg cctataaagagtacgaagatatgatagctacaaagtgcacctgt cgt (SEQ ID NO:72)	GQETVSSELKKPLGPASFNL SEYFRQFLLPQNECELHDFR LSFSQLKWDNWIVAPHRYN PRYCKGDCPRAVGHRYGSP VHTMVQNIIYEKLDSSVPRP SCVPAKYSPLSVLTIEPDGSI AYKEYEDMIATKCTCR (SEQ ID NO:73)
GDF-10(BMP-3b)	aagacgatgcagaaagcccggaggaagcagtgggatgagcc gagggtgtgctcccggaggtacctgaaggtggacttcgcagac atcggctggaatgaatggataatctcaccgaaatcttttgatgcct actactgcgcgggagcatgtgagttccccatgcctaagatcgtt cgtccatccaaccatgccaccatccagagcattgtcagggctgt gggcatcatccctggcatcccagagccctgctgtgttcccgata agatgaactcccttggggtcctcttcctggatgagaat301cgg aatgtggttctgaaggtgtaccccaacatgtccgtggacacctgt gcctgccggtga (SEQ ID NO:74)	KTMQKARRKQWDEPRVCS RRYLKVDFADIGWNEWIISP KSFDAYYCAGACEFPMPKI VRPSNHATIQSIVRAVGIIPG IPEPCCVPDKMNSLGVLFLD ENRNVVLKVYPNMSVDTC ACR (SEQ ID NO:75)
GDF-11(BMP-11)	aacctgggtctggactgcgacgagcactcaagcgagtcccgct gctgccgatatcccctcacagtggactttgaggctttcggctggg actggatcatcgcacctaagcgctacaaggccaactactgctcc ggccagtgcgagtacatgttcatgcaaaaatatccgcatccca tttggtgcagcaggccaatccaagaggctctgctgggccctgtt gtacccccaccaagatgtccccaatcaacatgctctacttcaatg acaagcagcagattatctacggcaagatccctggcatggtggtg gatcgctgtggctgctct (SEQ ID NO:76)	NLGLDCDEHSSESRCCRYP LTVDFEAFGWDWIIAPKRY KANYCSGQCEYMFMQKYP HTHLVQQANPRGSAGPCCT PTKMSPINMLYFNDKQQIIY GKIPGMVVDRCGCS (SEQ ID NO:77)
GDF-15	gcgcgcaacggggaccactgtccgctcgggcccgggcgttgc tgccgtctgcacacggtccgcgcgtcgctggaagacctgggct gggccgattgggtgctgtcgccacgggaggtgcaagtgaccat gtgcatcggcgcgtgcccgagccagttccgggcggcaaacat gcacgcgcagatcaagacgagcctgcaccgcctgaagcccg acacggtgccagcgccctgctgcgtgcccgccagctacaatcc catggtgctcattcaaaagaccgacaccggggtgtcgctccag acctatgatgacttgttagccaaagactgccactgcata (SEQ ID NO:78)	ARNGDDCPLGPGRCCRLHT VRASLEDLGWADWVLSPR EVQVTMCIGACPSQFRAAN MHAQIKTSLHRLKPDTEPA PCCVPASYNPMVLIQKTDT GVSLQTYDDLLAKDCHCI (SEQ ID NO:79)
Nodal	catcacttgccagacagaagtcaactgtgtcggaaggtcaagtt ccaggtggacttcaacctgatcggatggggctcctggatcatct accccaagcagtacaacgcctatcgctgtgagggcgagtgtcc taatcctgttggggaggagtttcatccgaccaaccatgcatacat ccagagtctgctgaaacgttaccagccccaccgagtcccttcca cttgttgtgccccagtgaagaccaagccgctgagcatgctgtat gtggataatggcagagtgctcctagatcaccataaagacatgat cgtggaagaatgtgggtgcctc (SEQ ID NO:80)	HHLPDRSQLCRKVKFQVDF NLIGWGSWIIYPKQYNAYR CEGECPNPVGEEFHPTNHA YIQSLLKRYQPHRVPSTCCA PVKTKPLSMLYVDNGRVLL DHHKDMIVEECGCL (SEQ ID NO:81)

Activin-βA	ggcctggagtgcgacggcaaggtcaacatctgctgtaagaaac agttctttgtcagtttcaaggacatcggctggaatgactggatcat tgctccctctggctatcatgccaactactgcgagggtgagtgcc cgagccatatagcaggcacgtccgggtcctcactgtccttccac tcaacagtcatcaaccactacgcatgcggccatagccccttgc caacctcaaatcgtgctgtgtgcccaccaagctgagacccatgt ccatgttgtactatgatgatggtcaaaacatcatcaaaaaggaca ttcagaacatgatcgtggaggagtgcgggtgctcc (SEQ ID NO:82)	GLECDGKVNICCKKQFFVS FKDIGWNDWIIAPSGYHAN YCEGECPSHIAGTSGSSLSF HSTVINHYRMRGHSPFANL KSCCVPTKLRPMSMLYYDD GQNIIKKDIQNMIVEECGCS (SEQ ID NO:83)
Activin-βB	ggcctggagtgcgatggccggaccaacctctgttgcaggcaac agttcttcattgacttccgcctcatcggctggaacgactggatcat agcacccaccggctactacggcaactactgtgagggcagctgc ccagcctacctggcaggggtccccggctctgcctcctccttcca cacggctgtggtgaaccagtaccgcatgcggggtctgaacccc ggcacggtgaactcctgctgcattcccaccaagctgagcaccat gtccatgctgtacttcgatgatgagtacaacatcgtcaagcggg acgtgcccaacatgattgtggaggagtgcggctgcgcc (SEQ ID NO:84)	GLECDGRTNLCCRQQFFIDF RLIGWNDWIIAPTGYYGNY CEGSCPAYLAGVPGSASSF HTAVVNQYRMRGLNPGTV NSCCIPTKLSTMSMLYFDDE YNIVKRDVPNMIVEECGCA (SEQ ID NO:85)
Activin-βC	ggcatcgactgccaaggagggtccaggatgtgctgtcgacaa gagtttttgtggacttccgtgagattggctggcacgactggatca tccagcctgagggctacgccatgaacttctgcatagggcagtgc ccactacacatagcaggcatgcctggtattgctgcctcctttcac actgcagtgctcaatcttctcaaggccaacacagctgcaggcac cactggaggggctcatgctgtgtacccacggcccggcgccc cctgtctctgctctattatgacagggacagcaacattgtcaagact gacatacctgacatggtagtagaggcctgtgggtgcagt (SEQ ID NO:86)	GIDCQGGSRMCCRQEFFVD FREIGWHDWIIQPEGYAMN FCIGQCPLHIAGMPGIAASF HTAVLNLLKANTAAGTTG GGSCCVPTARRPLSLLYYD RDSNIVKTDIPDMVVEACG CS (SEQ ID NO:87)
Activin-βE	acccccacctgtgagcctgcgacccccttatgttgcaggcgag accattacgtagacttccaggaactgggatggcgggactggat actgcagcccgaggggtaccagctgaattactgcagtgggcag tgccctcccacctggctggcagcccaggcattgctgcctctttc cattctgccgtcttcagcctcctcaaagccaacaatccttggcct gccagtacctcctgttgtgtccctactgcccgaaggcccctctct ctcctctacctggatcataatggcaatgtggtcaagacggatgtg ccagatatggtggtggaggcctgtggctgcagc (SEQ ID NO:88)	TPTCEPATPLCCRRDHYVD FQELGWRDWILQPEGYQLN YCSGQCPPHLAGSPGIAASF HSAVFSLLKANNPWPASTS CCVPTARRPLSLLYLDHNG NVVKTDVPDMVVEACGCS (SEQ ID NO:89)
Inhibin-α	tcaactcccctgatgtcctggccttggtctccctctgctctgcgcc tgctgcagaggcctccggaggaaccggctgcccatgccaact gccacagagtagcactgaacatctccttccaggagctgggctg ggaacggtggatcgtgtaccctcccagtttcatcttccactactgt catggtggttgtgggctgcacatcccaccaaacctgtccttcca gtccctggggctcccccctaccccagcccagccctactccttgct gccaggggcccagccctgctgtgctgctctcccagggaccatg aggcccctacatgtccgcaccacctcggatggaggttactctttc aagtatgagacagtgcccaaccttctcacgcagcactgtgcttgt atc (SEQ ID NO:90)	STPLMSWPWSPSALRLLQR PPEEPAAHANCHRVALNISF QELGWERWIVYPPSFIFHYC HGGCGLHIPPNLSLPVPGAP PTPAQPYSLLPGAQPCCAAL PGTMRPLHVRTTSDGGYSF KYETVPNLLTQHCACI (SEQ ID NO:91)
TGF-β1	gccctggacaccaactattgcttcagctccacggagaagaactg ctgcgtgcggcagctgtacattgacttccgcaaggacctcggct ggaagtggatccacgagcccaagggctaccatgccaacttctg cctcgggccctgccccctacatttggagcctggacacgcagtac agcaaggtcctggccctgtacaaccagcataacccgggcgcct cggcggcgccgtgctgcgtgccgcaggcgctggagccgctg cccatcgtgtactacgtgggccgcaagcccaaggtggagcag ctgtccaacatgatcgtgcgctcctgcaagtgcagc (SEQ	ALDTNYCFSSTEKNCCVRQ LYIDFRKDLGWKWIHEPKG YHANFCLGPCPYIWSLDTQ YSKVLALYNQHNPGASAAP CCVPQALEPLPIVYYVGRKP KVEQLSNMIVRSCKCS (SEQ ID NO:93)

	ID NO:92	
TGF-β2	gctttggatgcggcctattgctttagaaatgtgcaggataattgct gcctacgtccactttacattgatttcaagagggatctagggtgga aatggatacacgaacccaaagggtacaatgccaacttctgtgct ggagcatgcccgtatttatggagttcagacactcagcacagcag ggtcctgagcttatataataccataaatccagaagcatctgcttct ccttgctgcgtgtcccaagatttagaacctctaaccattctctacta cattggcaaaacacccaagattgaacagctttctaatatgattgta aagtcttgcaaatgcagc (SEQ ID NO:94)	ALDAAYCFRNVQDNCCLRP LYIDFKRDLGWKWIHEPKG YNANFCAGACPYLWSSDT QHSRVLSLYNTINPEASASP CCVSQDLEPLTILYYIGKTP KIEQLSNMIVKSCKCS (SEQ ID NO:95)
TGF-β3	gctttggacaccaattactgcttccgcaacttggaggagaactgc tgtgtgcgcccccctctacattgacttccgacaggatctgggctgg aagtgggtccatgaacctaagggctactatgccaacttctgctca ggcccttgcccatacctccgcagtgcagacacaacccacagca cggtgctgggactgtacaacactctgaaccctgaagcatctgcc tcgccttgctgcgtgccccaggacctggagccccctgaccatcct gtactatgttgggaggacccccaaagtggagcagctctccaac atggtggtgaagtcttgtaaatgtagc (SEQ ID NO:96)	ALDTNYCFRNLEENCCVRP LYIDFRQDLGWKWVHEPK GYYANFCSGPCPYLRSADT THSTVLGLYNTLNPEASASP CCVPQDLEPLTILYYVGRTP KVEQLSNMVVKSCKCS (SEQ ID NO:97)

What is claimed is:

1. A recombinant polypeptide comprising:

at least two peptide segments, a first segment of the polypeptide comprising a sequence having at least 80% identity to a first TGF-beta family protein and a second segment comprising a sequence having at least 80% identity to a second TGF-beta family protein or a combination thereof, wherein the segments are operably linked and have activity of at least one of the first or second parental TGF-beta family protein, or activity of new *in vivo* signaling and cellular property.

2. The polypeptide of claim 1, wherein the polypeptide comprises 5 or 6 domains, having the general sequence order of 1-2-3-4-5-6, wherein each domain can be recombined with at least one other domain from at least one other TGF-beta family polypeptide in a sequential order.

3. The polypeptide of claim 2, wherein the polypeptide comprises 5 domains.

4. The polypeptide of claim 2 or 3, wherein domain 3 is only shuffled when at least one other domain is shuffled in a chimeric polypeptide.

5. The polypetpide of claim 2, wherein the domains are as set forth in Table A.

6. The polypeptide of claim 1, wherein the polypeptide comprises an N-terminal segment from BMP-2.

7. The polypeptide of claim 1, wherein the at least two polypeptide segments comprise 6 peptide segments operably linked N- to C-terminal.

8. The polypeptide of claim 7, wherein each of the first and second TGF-beta family proteins have structural similarity and wherein each segment correspondes to a structural motif of the segment.

9. The polypeptide of claim 1, wherein the first TGF-beta family protein is BMP-2 and the second TGF-beta family protein is activin.

10. The polypeptide of claim 7, wherein the first TGF-beta family protein is BMP-2 and the second TGF-beta family protein is activin or other family member.

11. The polypeptide of claim 10, wherein the segments of the BMP-2 protein comprise segment 1: amino acid residue from about 1 to about x_1 of SEQ ID NO:2 ("1b"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:2 ("2b"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:2 ("3b"); segment 4 is from about amino acid residue x_3 to about x_4 of SEQ ID NO:2 ("4b"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:2 ("5b"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:2 ("6b"); and wherein: x_1 is residue 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 of SEQ ID NO:2; x_2 is residue 45, 46, 47, or 48 of SEQ ID NO:2; x_3 is residue 65, 66, 67, or 68 of SEQ ID NO:2; x_4 is residue 76, 77, 78, 79, 80, 81 or 82 of SEQ ID NO:2; x_5 is residue 88, 89, 90, 91, 92, 93, or 94 of SEQ ID NO:2; and x_6 is residue 112, 113, or 114 or SEQ ID NO:2, corresponding to the C-terminus of BMP-2; and

wherein the segments of the activin protein comprise segment 1, amino acid residue from about 1 to about x_1 of SEQ ID NO:5 ("1a"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:5 ("2a"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:5 ("3a"); segment 4 is from about amino acid residue x_3 to about x_4 of SEQ ID NO:5 ("4a"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:5 ("5a"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:5 ("6a"); and wherein: x_1 is residue 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 of SEQ ID NO:5; x_2 is residue 42, 43, 44, or 45 of SEQ ID NO:5; x_3 is residue 61, 62, 63, or 64 of SEQ ID NO:5; x_4 is residue 78, 79, 80, 81, 82, 83 or 84 of SEQ ID NO:5; x_5 is residue 90, 91, 92, 93, 94, 95 or 96 of SEQ ID NO:5; and x_6 is residue 114, 115, or 116 or SEQ ID NO:5; and

wherein the polypeptide has an order of segment 1-segment 2-segment 3-segment 4-segment 5-segment 6.

12. The polypeptide of claim 10, wherein the polypeptide comprises a sequence of segments selected from the group consisting of 1b2b3b4b5b6a; 1b2b3b4b5a6a; 1b2b3b4b5a6b; 1b2b3a4a5a6a; 1b2b3a4a5b6a; 1b2a3a4a5a6a; 1b2a3a4a5a6a L66V/V67I; 1b(1a_II)2a3a4a5a6a; 1b2a3a4a5a6b; 1b2a3a4a5b6b; 1b2a3a4a5b6a; 1b2a3b4b5b6a; 1b2a3b4b5a6a; and 1b2a3b4b5a6b.

13. The polypeptide of claim 1, wherein the polypeptide comprises 80%, 90%, 95%, 98% or 99% identity to a sequence as set forth in SEQ ID NO:7, 9, 11, 13, 15, 17, 19, 1, 23, 25, 2, 29, 31, 33, 35, 37, 39 or 41 and wherein the polypeptide modulates the SMAD or DAXX pathway.

14. The polypeptide of claim 13, wherein the polypeptide comprises a sequence selected from the group consisting of SEQ ID NO:7, 9, 11, 13, 15, 17, 19, 1, 23, 25, 2, 29, 31, 33, 35, 37, 39 and 41.

15. A chimeric TGF-beta family polypeptide comprising a segment of a first TGF-beta family protein operably linked to a segment of a second different TGF-beta family protein to provide a chimeric polypeptide having SMAD or DAXX modulating activity.

16. The polypeptide of claim 15, wherein the first TGF-beta family protein is BMP-2 and the second TGF-beta family protein is activin.

17. The polypeptide of claim 16, wherein the segments of the BMP-2 protein comprise segment 1: amino acid residue from about 1 to about x_1 of SEQ ID NO:2 ("1b"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:2 ("2b"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:2 ("3b"); segment 4 is from about amino acid residue x_3 to about x_4 of SEQ ID NO:2 ("4b"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:2 ("5b"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:2 ("6b"); and wherein: x_1 is residue 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 of SEQ ID NO:2; x_2 is residue 45, 46, 47, or 48 of SEQ ID NO:2; x_3 is residue 65, 66, 67, or 68 of SEQ ID NO:2; x_4 is residue 76, 77, 78, 79, 80,

81 or 82 of SEQ ID NO:2; x_5 is residue 88, 89, 90, 91, 92, 93, or 94 of SEQ ID NO:2; and x_6 is residue 112, 113, or 114 or SEQ ID NO:2, corresponding to the C-terminus of BMP-2; and

wherein the segments of the activin protein comprise segment 1, amino acid residue from about 1 to about x_1 of SEQ ID NO:5 ("1a"); segment 2 is from about amino acid residue x_1 to about x_2 of SEQ ID NO:5 ("2a"); segment 3 is from about amino acid residue x_2 to about x_3 of SEQ ID NO:5 ("3a"); segment 4 is from about amino acid residue x_3 to about x_4 of SEQ ID NO:5 ("4a"); segment 5 is from about amino acid residue x_4 to about x_5 of SEQ ID NO:5 ("5a"); and segment 6 is from about amino acid residue x_5 to about x_6 of SEQ ID NO:5 ("6a"); and wherein: x_1 is residue 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 of SEQ ID NO:5; x_2 is residue 42, 43, 44, or 45 of SEQ ID NO:5; x_3 is residue 61, 62, 63, or 64 of SEQ ID NO:5; x_4 is residue 78, 79, 80, 81, 82, 83 or 84 of SEQ ID NO:5; x_5 is residue 90, 91, 92, 93, 94, 95 or 96 of SEQ ID NO:5; and x_6 is residue 114, 115, or 116 or SEQ ID NO:5; and

wherein the polypeptide has an order of segment 1-segment 2-segment 3-segment 4-segment 5-segment 6.

18. The polypeptide of claim 16, wherein the polypeptide comprises a sequence of segments selected from the group consisting of 1b2b3b4b5b6a; 1b2b3b4b5a6a; 1b2b3b4b5a6b; 1b2b3a4a5a6a; 1b2b3a4a5b6a; 1b2a3a4a5a6a; 1b2a3a4a5a6a L66V/V67I; 1b(1a_II)2a3a4a5a6a; 1b2a3a4a5a6b; 1b2a3a4a5b6b; 1b2a3a4a5b6a; 1b2a3b4b5b6a; 1b2a3b4b5a6a; and 1b2a3b4b5a6b.

19. A polynucleotide encoding a polypeptide of claim 1 or 15.

20. The polynucleotide of claim 19, wherein the polynucleotide comprises sequences from a plurality of TGF-beta family polynucleotides operably linked to encode a functional chimeric polypeptide having SMAD or DAXX modulating activity.

21. A polynucleotide encoding a polypeptide of claim 20.

22. A polynucleotide comprising a sequence selected from the group consisting of 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 24, 26, 28, and 40.

23. A vector comprising a polynucleotide of claim 21 or 22.

24. A host cell comprising the vector of claim 23.

25. A host cell comprising a polynucleotide of claim 21 or 22.

26. A method of producing a chimeric TGF-beta polypeptide comprising

(a) aligning the sequences of at least two TGF-beta family member proteins;

(b) identifying structurally related domains of the at least two family member proteins;

(c) identifying points of cross-over of the at least two TGF-beta proteins comprising regions at either or both ends of the structurally related domains and wherein the regions comprise at least 80%, 95%, 98%, 99% or 100% sequence identity over at least 5 consecutive amino acids;

(d) generating a chimeric TGF-beta polypeptide comprising at least one domain from a first TGF-beta family member protein and at least one domain from a second TGF-beta family member protein wherein the domains are linked at the points of cross-over; and

(e) assaying the chimeric TGF-beta polypeptide for type 1 and type II ligand binding capacity.

27. A chimeric polypeptide produced from the method of claim 26.

28. A method of modulating cell proliferation or activity associated with the Smad or DAXX pathway comprising contacting a cell with a chimeric polypeptide of claim 1, 15 or 27.

29. A method of treating a disease or disorder associated with bone, cartilage, neurological tissue, cardiac tissue, skeletal muscle or endocrine tissue comprising contacting the tissue with a chimeric polypeptide of claim 1, 15, or 27.

30. A method of treating a cell proliferative disease or disorder comprising contacting a cell having the cell proliferative disease or disorder with a chimeric polypeptide of claim 1, 15, or 27.

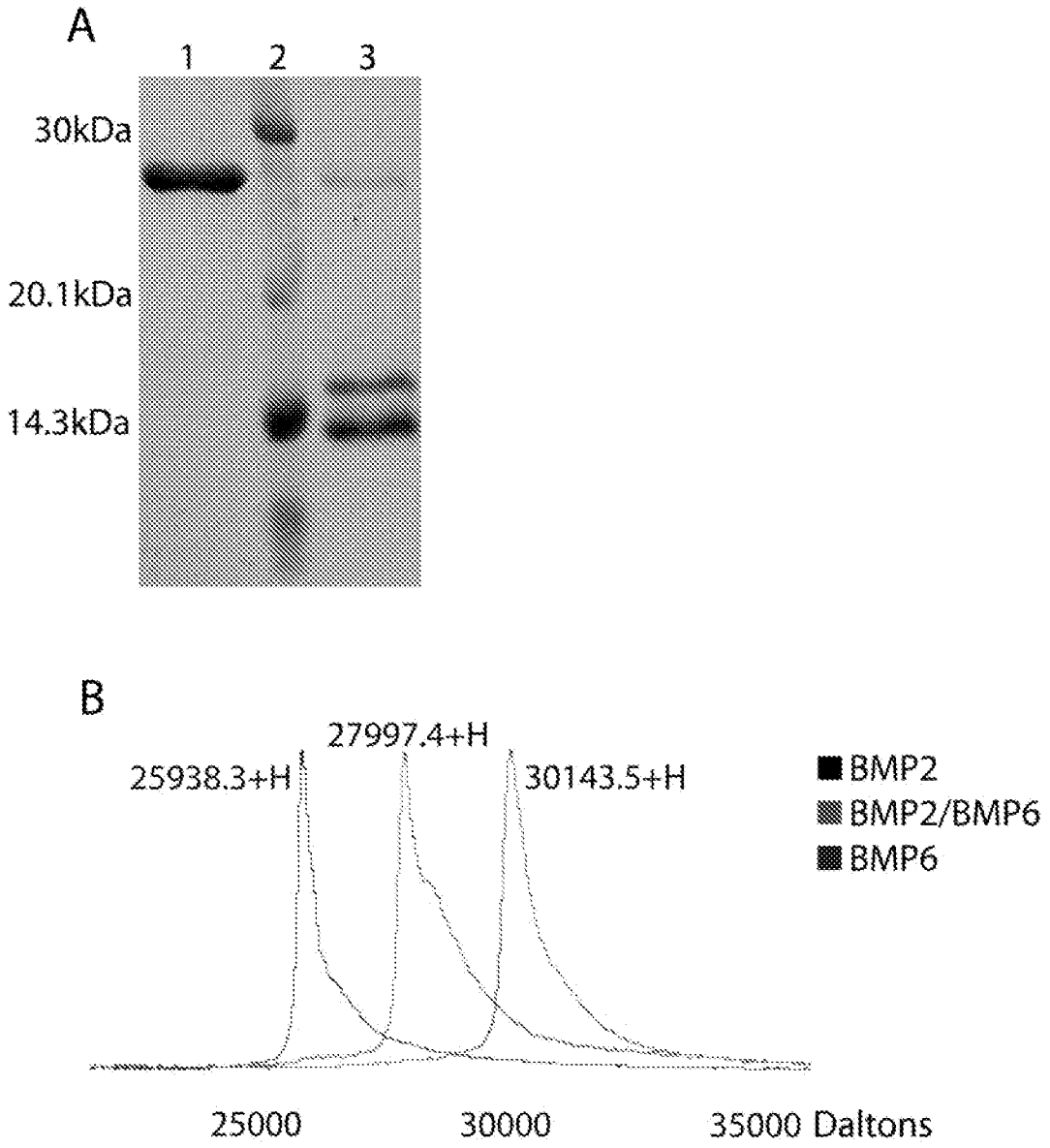
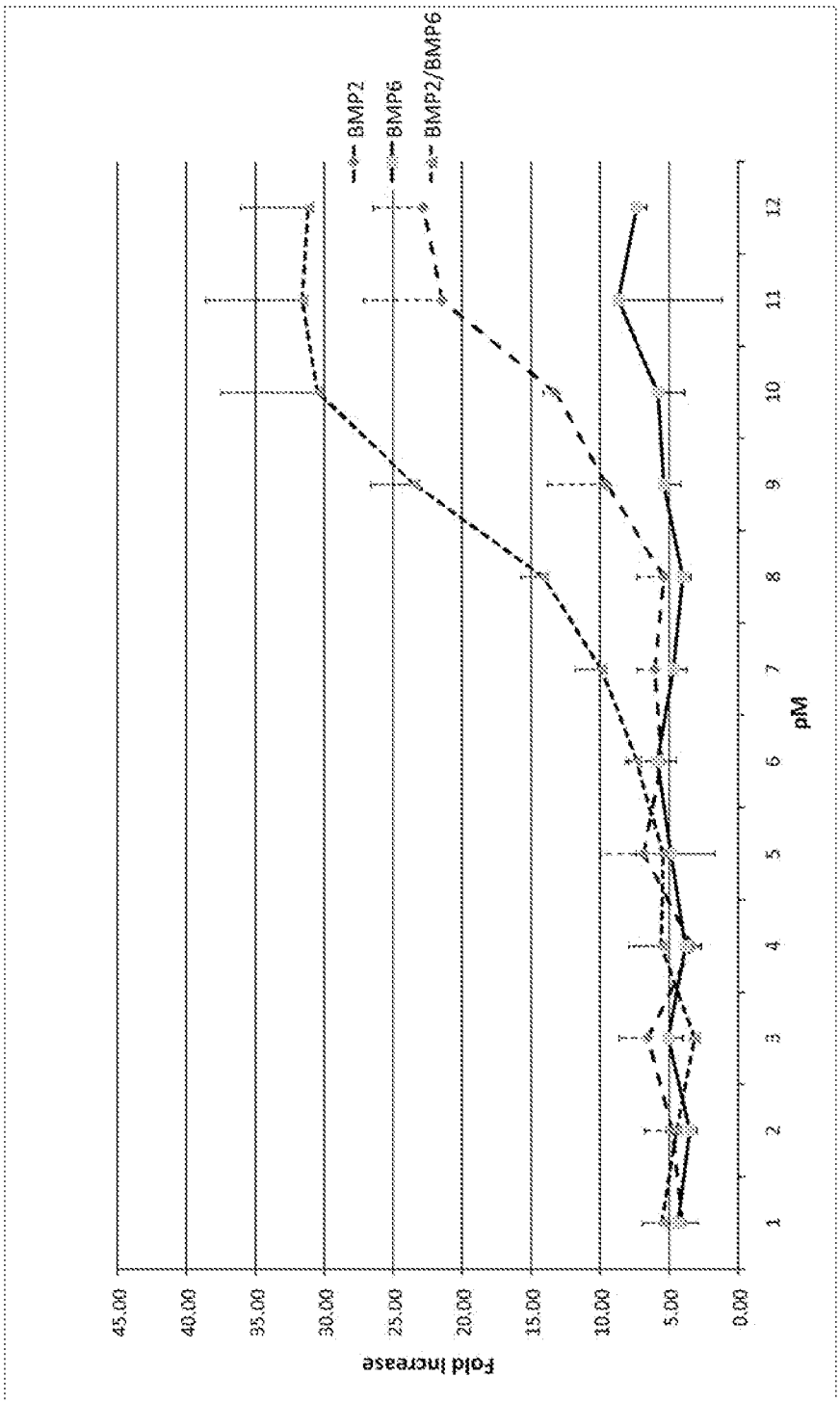

A
1
2
3
30kDa
20.1kDa
14.3kDa
B
27997.4+H
25938.3+H
30143.5+H
BMP2
BMP2/BMP6
BMP6
25000
30000
35000 Daltons

FIGURE 1


BMP2
BMP6
BMP2/BMP6
Fold Increase
45.00
40.00
35.00
30.00
25.00
20.00
15.00
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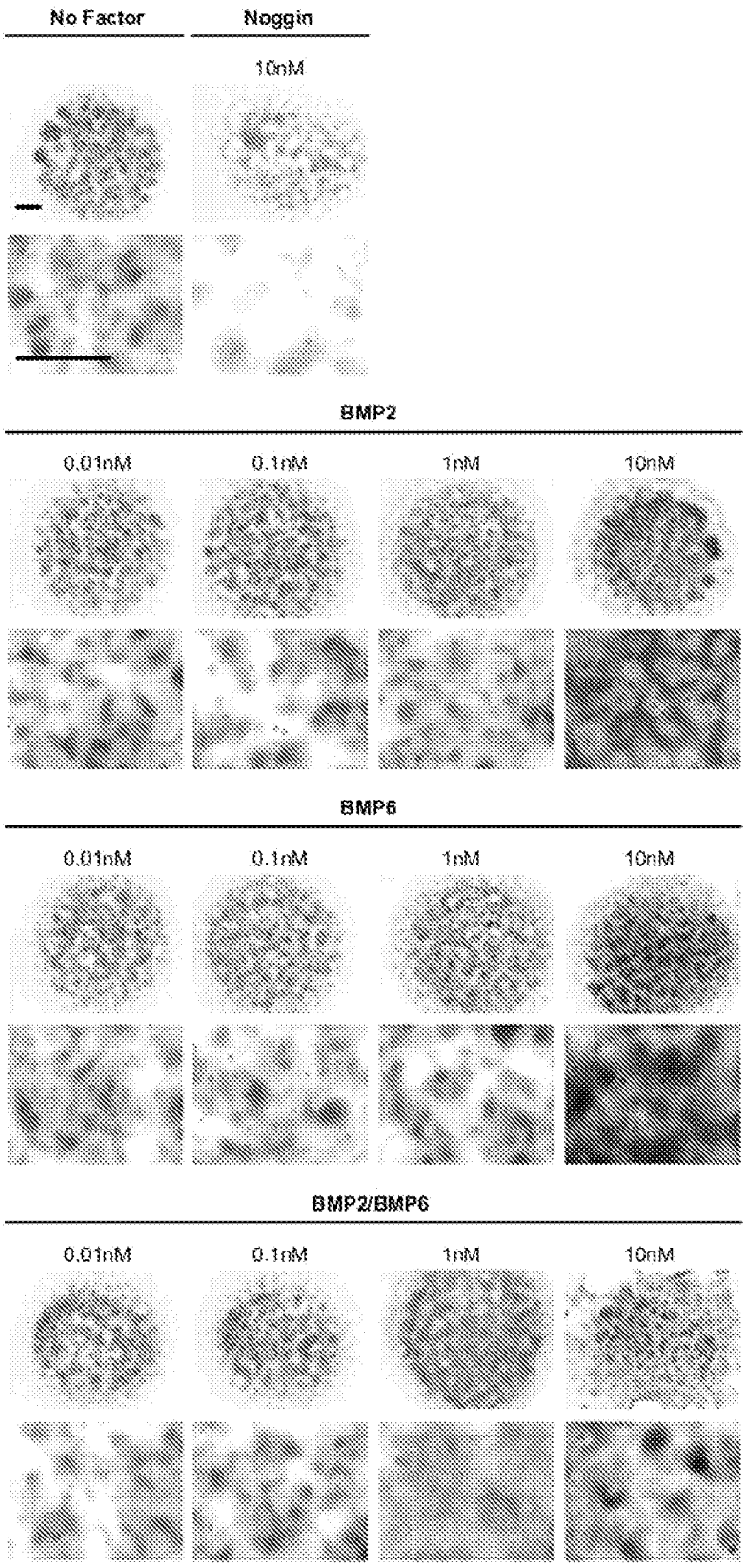
6
7
8
9
10
11
12
pM

FIGURE 2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Noggin
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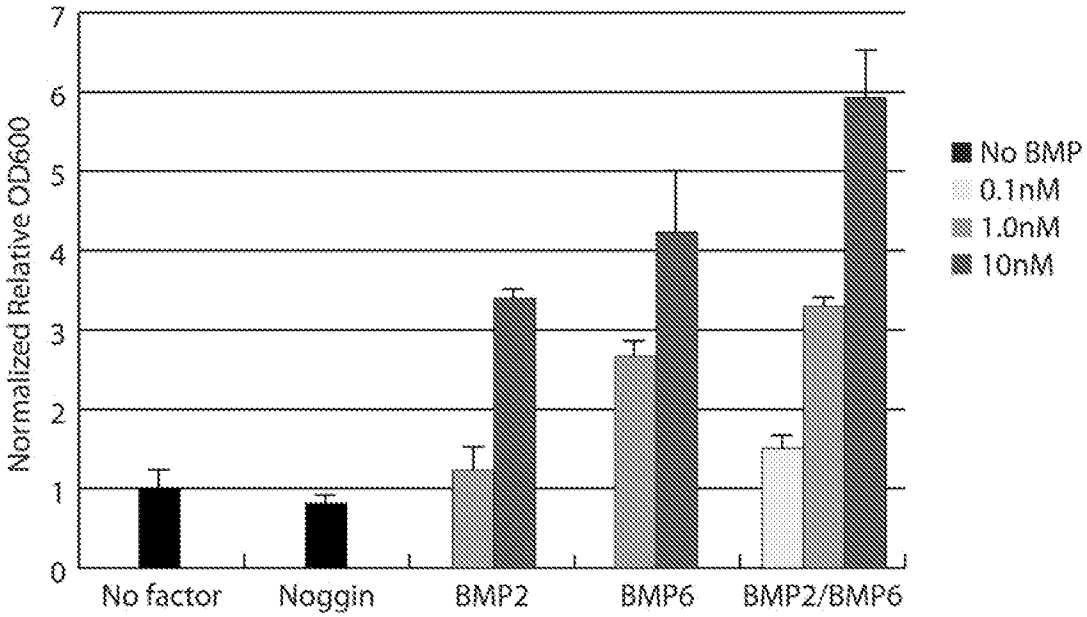
BMP2
0.01nM
0.1nM
1nM
10nM
BMP6
0.01nM
0.1nM
1nM
10nM
BMP2/BMP6
0.01nM
0.1nM
1nM
10nM

FIGURE 3


Normalized Relative OD600
0
1
2
3
4
5
6
7
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Noggin
BMP2
BMP6
BMP2/BMP6
No BMP
0.1nM
1.0nM
10nM

FIGURE 4

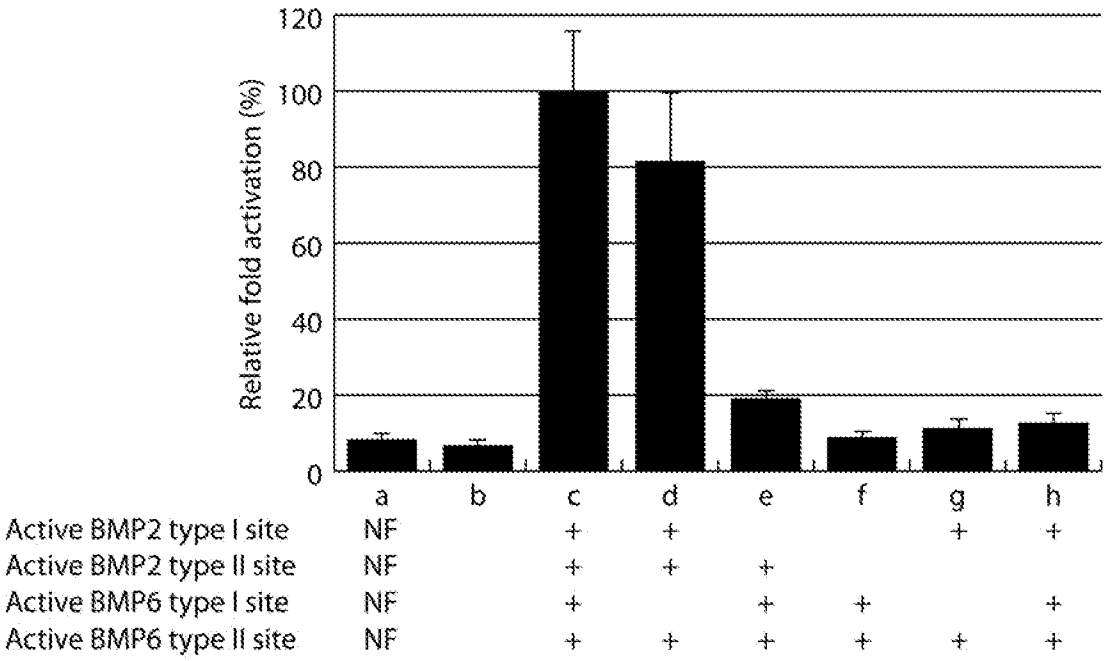
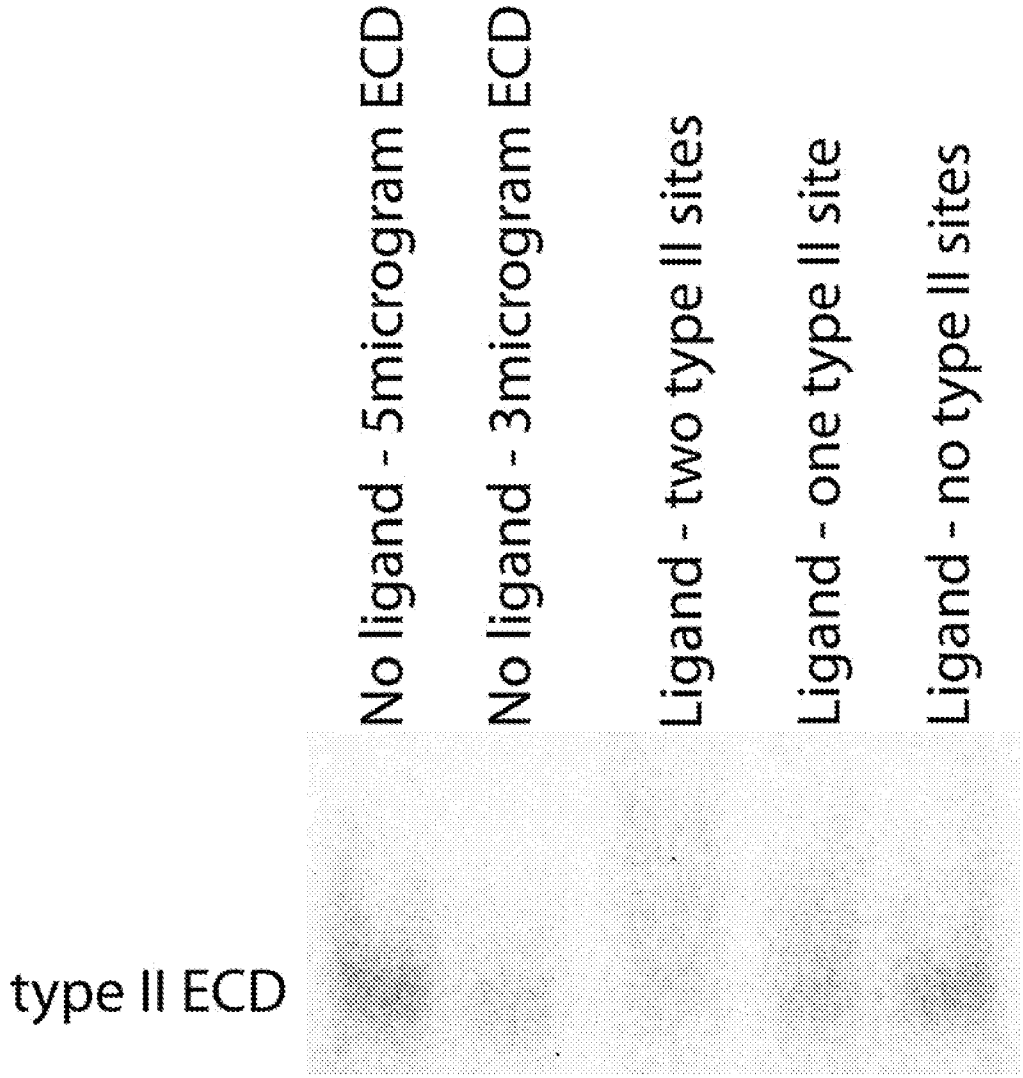

120
100
80
60
40
20
0
Relative fold activation (%)
a
b
c
d
e
f
g
h
Active BMP2 type I site NF + + + +
Active BMP2 type II site NF + + +
Active BMP6 type I site NF + + + +
Active BMP6 type II site NF + + + + + +

FIGURE 5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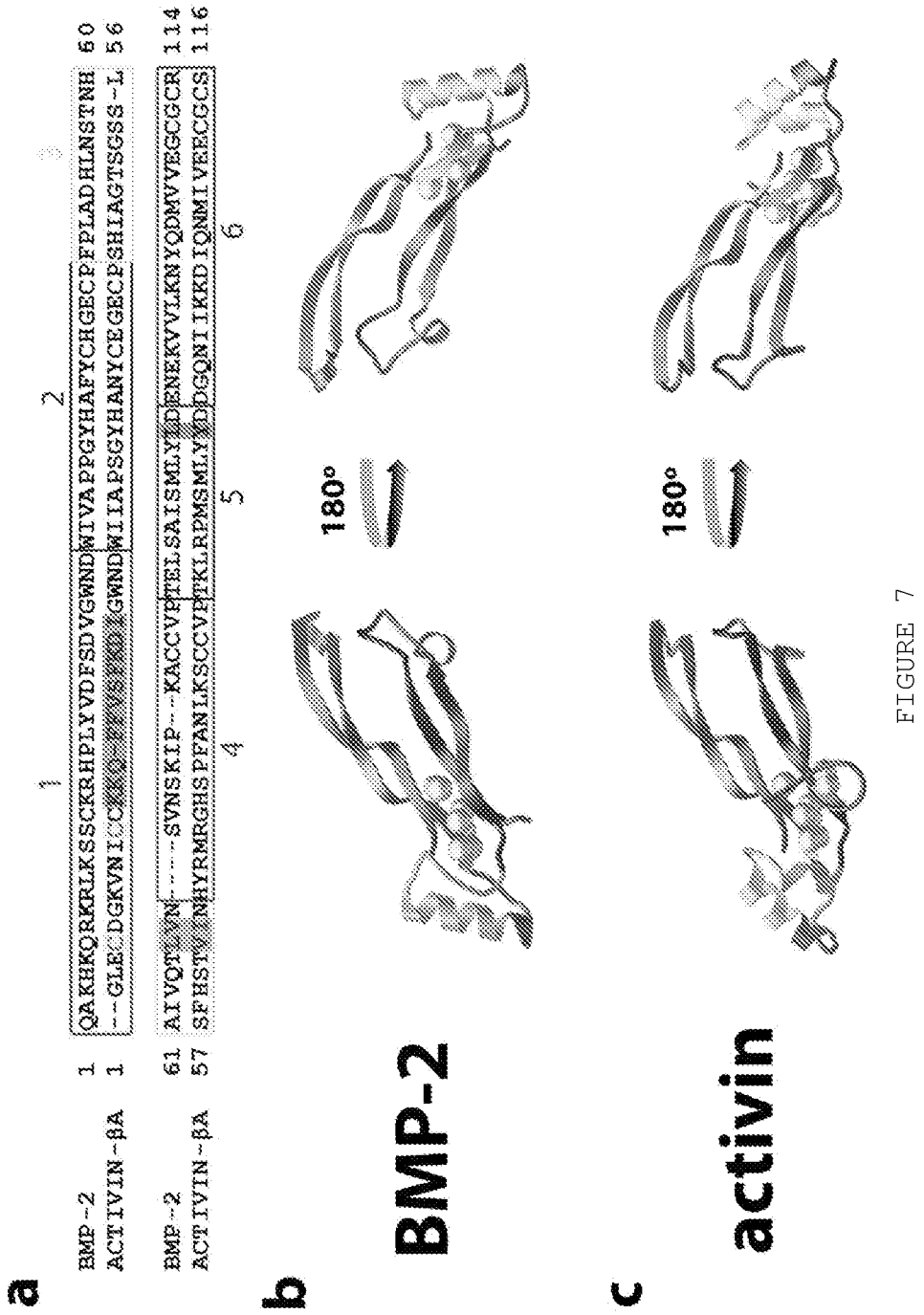
No ligand - 3microgram ECD
Ligand - two type II sites
Ligand - one type II site
Ligand - no type II sites
type II ECD

FIGURE 6


a
1
2
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ACTIVIN-βA 1 --GLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAGTSGSS-L 56
3
4
5
6
BMP-2 61 AIVQTLVN----SVNSKIP--KACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCR 114
ACTIVIN-βA 57 SFHSTVFNHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS 116
b
BMP-2
180°
c
activin
180°

FIGURE 7

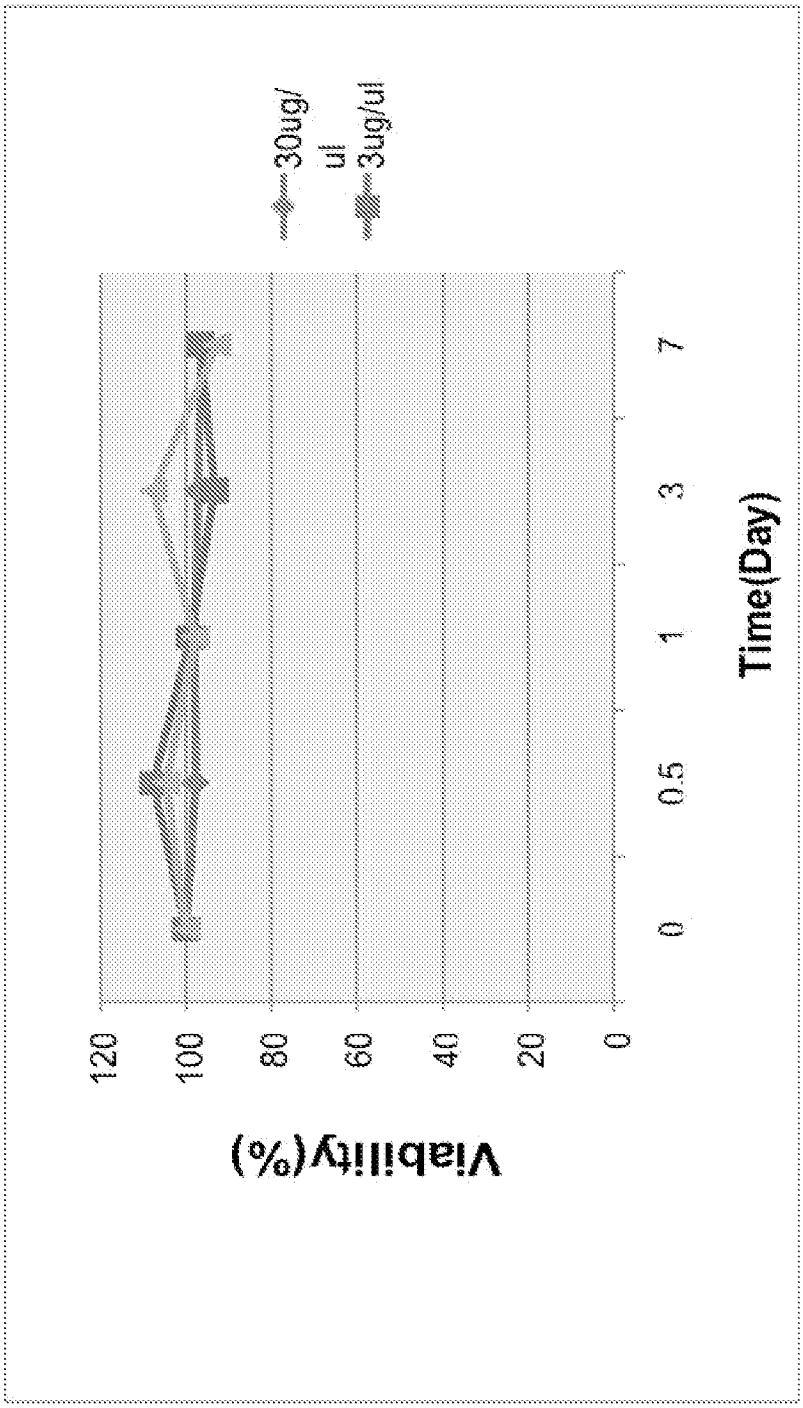
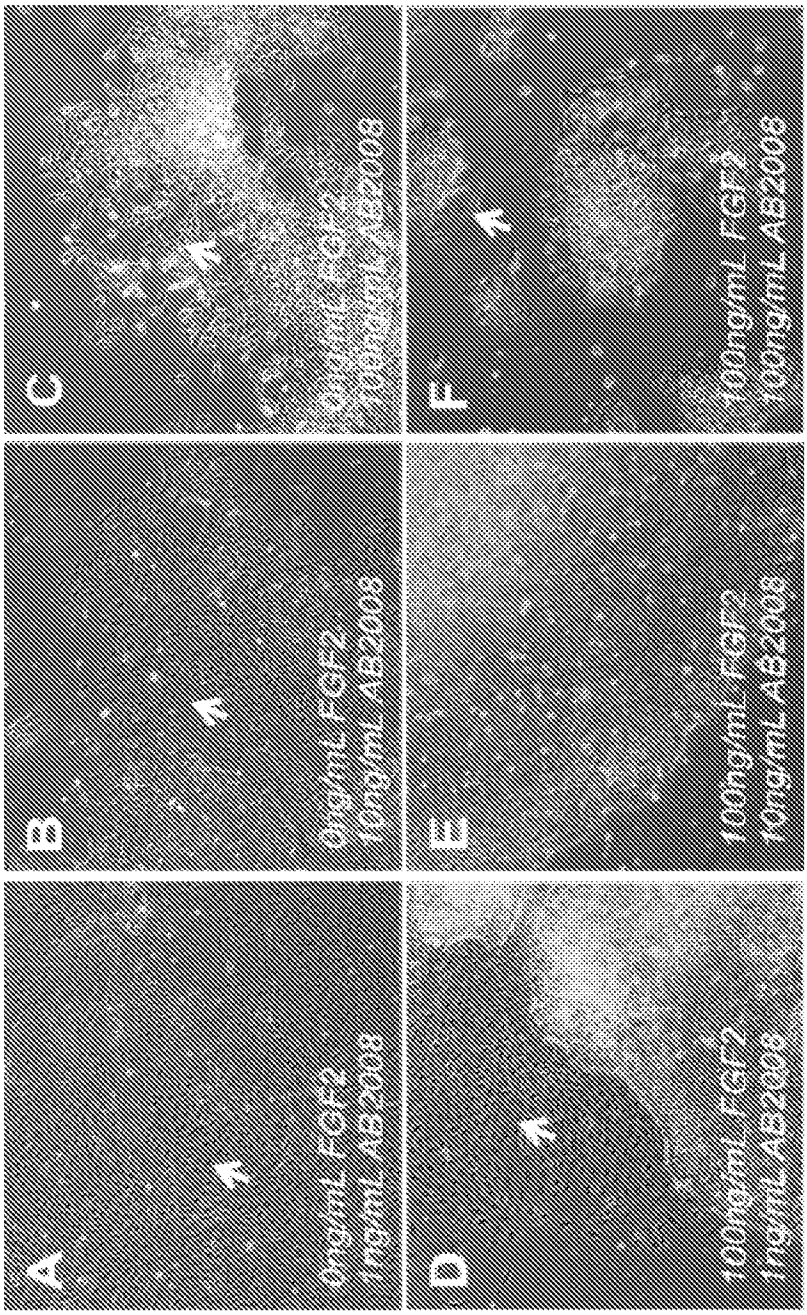


Figure 8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1ng/mL AB2008
B
0ng/mL FGF2
10ng/mL AB2008
C
0ng/mL FGF2
100ng/mL AB2008
D
100ng/mL FGF2
1ng/mL AB2008
E
100ng/mL FGF2
10ng/mL AB2008
F
100ng/mL FGF2
100ng/mL AB2008

FIGURE 9

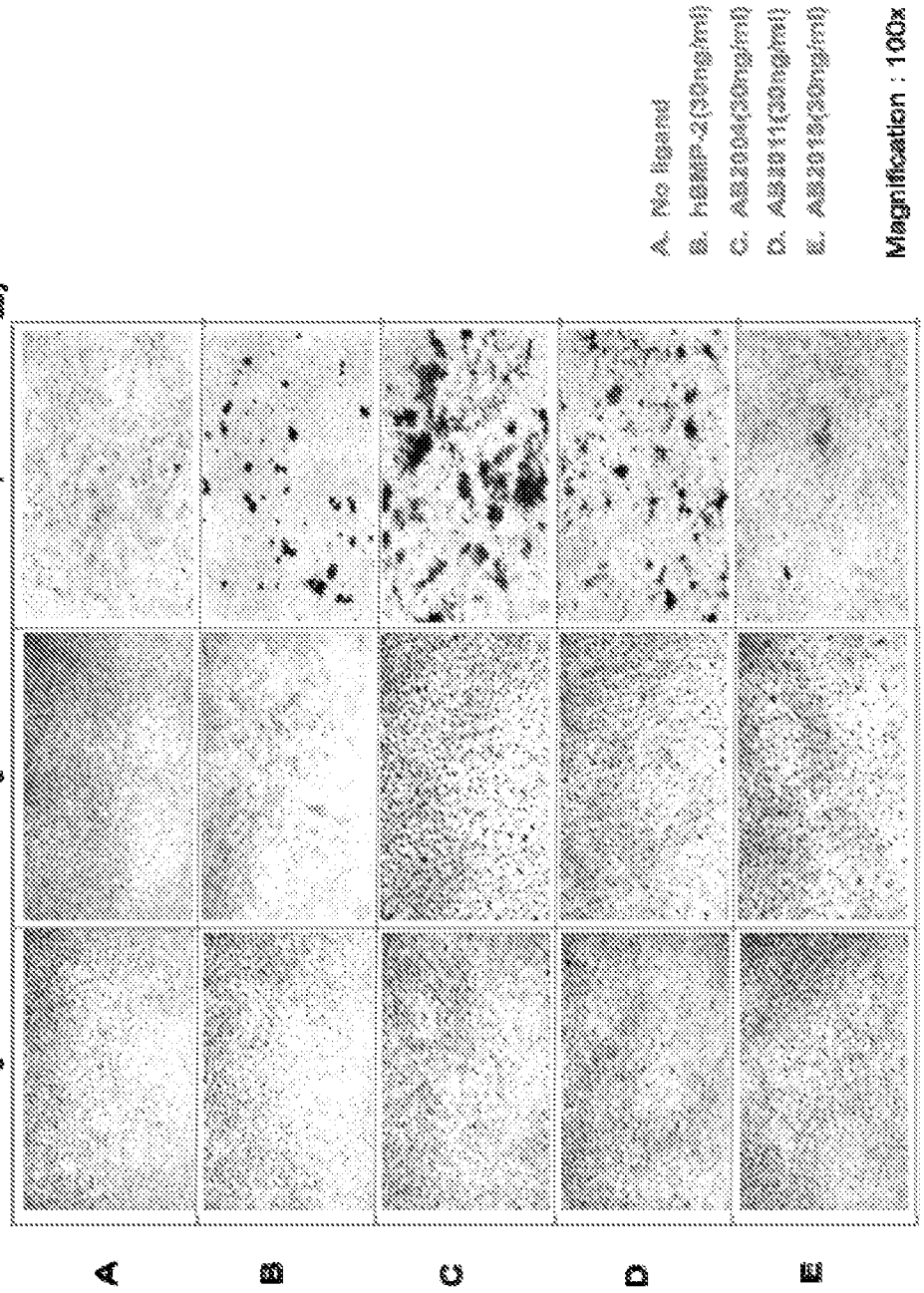
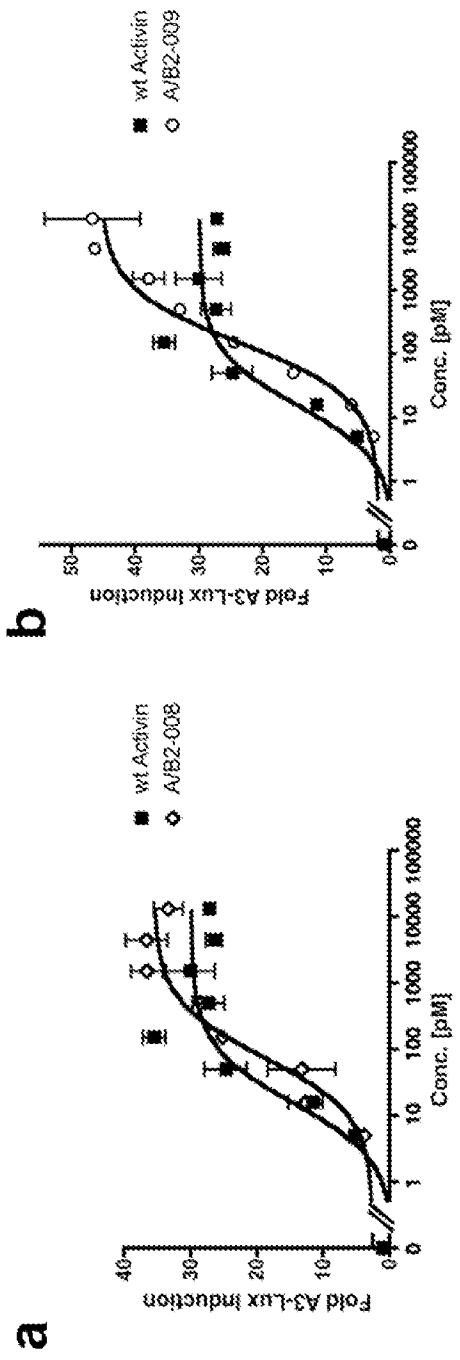

A. No ligand
B. hANP-2(30ng/ml)
C. AB2004(30ng/ml)
D. AB2011(30ng/ml)
E. AB2018(30ng/ml)
Magnification : 100x
day
0
3
7
A
B
C
D
E

FIGURE 10



d

Construct	EC_{50} (pM)	Relative Potency
activin	15.8	1
A/B2-008	76.6	0.21
A/B2-009	149	0.11
A/B2-010	325	0.05

FIGURE 11

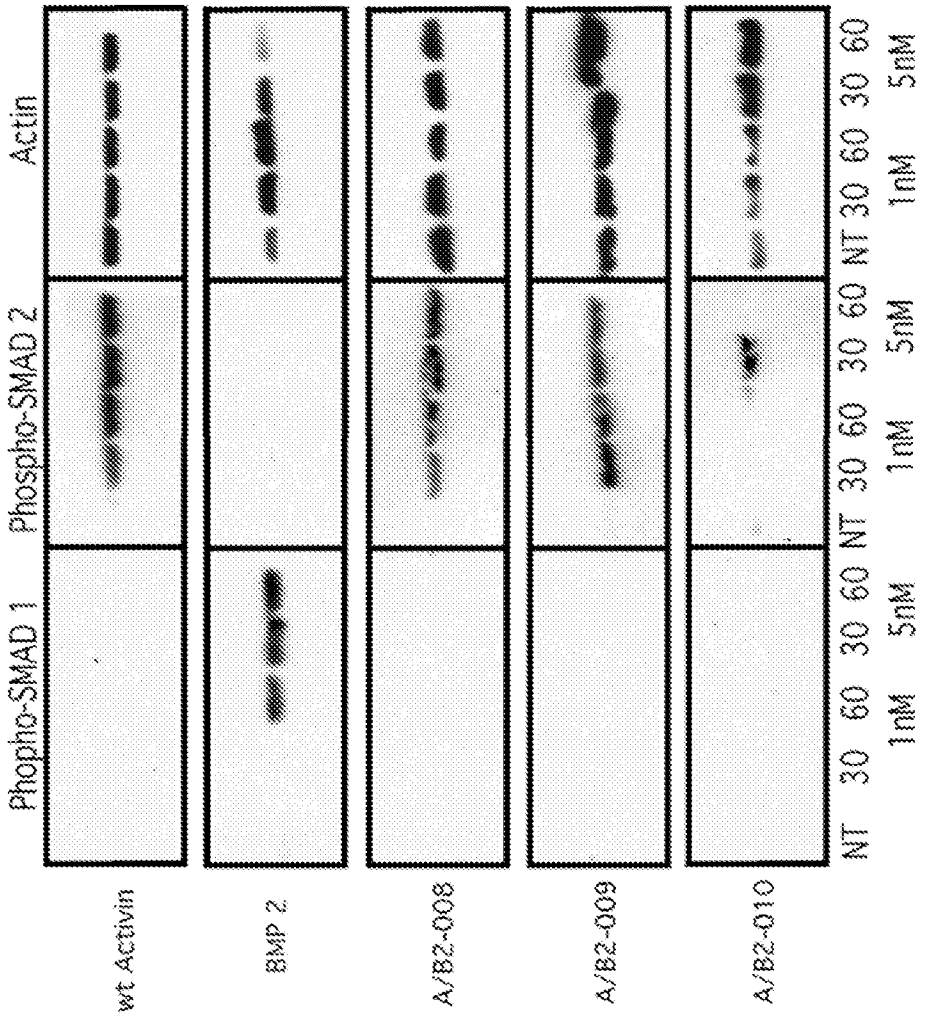

Phopho-SMAD 1
Phospho-SMAD 2
Actin
wt Activin
BMP 2
A/B2-008
A/B2-009
A/B2-010
NT 30 60 30 60 NT 30 60 30 60 NT 30 60 30 60
1nM 5nM 1nM 5nM 1nM 5nM

FIGURE 12

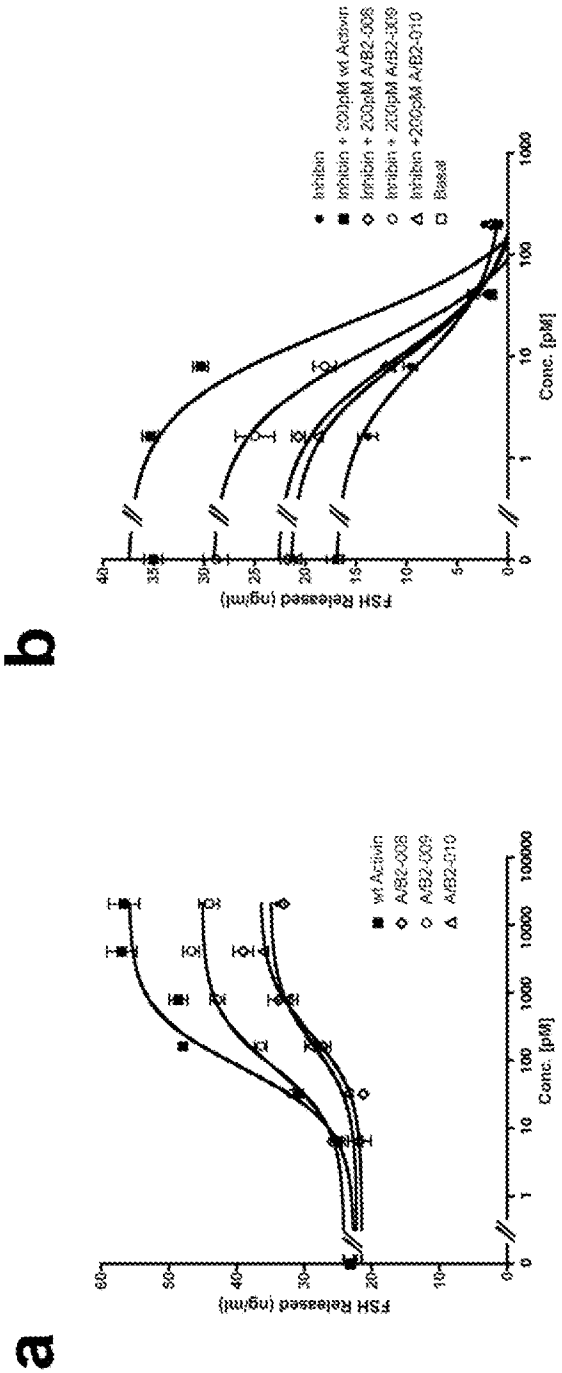

a
FSH Released (ng/ml)
Conc. [pM]
wt Activin
A/B2-008
A/B2-309
A/B2-010
b
FSH Released (ng/ml)
Conc. [pM]
Inhibin
Inhibin + 200pM wt Activin
Inhibin + 200pM A/B2-008
Inhibin + 200pM A/B2-309
Inhibin +200pM A/B2-010
Basal

FIGURE 13

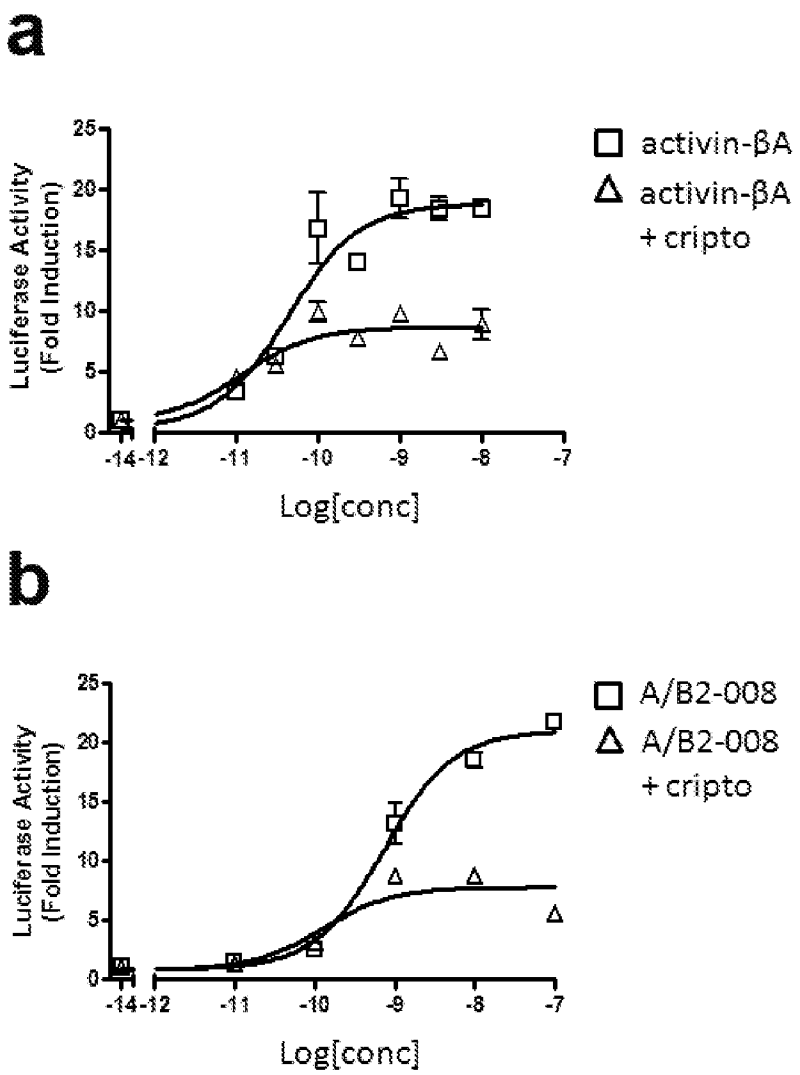

a
Luciferase Activity
(Fold Induction)
25
20
15
10
5
0
-14
-12
-11
-10
-9
-8
-7
Log[conc]
activin-βA
activin-βA
+ cripto
b
Luciferase Activity
(Fold Induction)
25
20
15
10
5
0
-14
-12
-11
-10
-9
-8
-7
Log[conc]
A/B2-008
A/B2-008
+ cripto

FIGURE 14

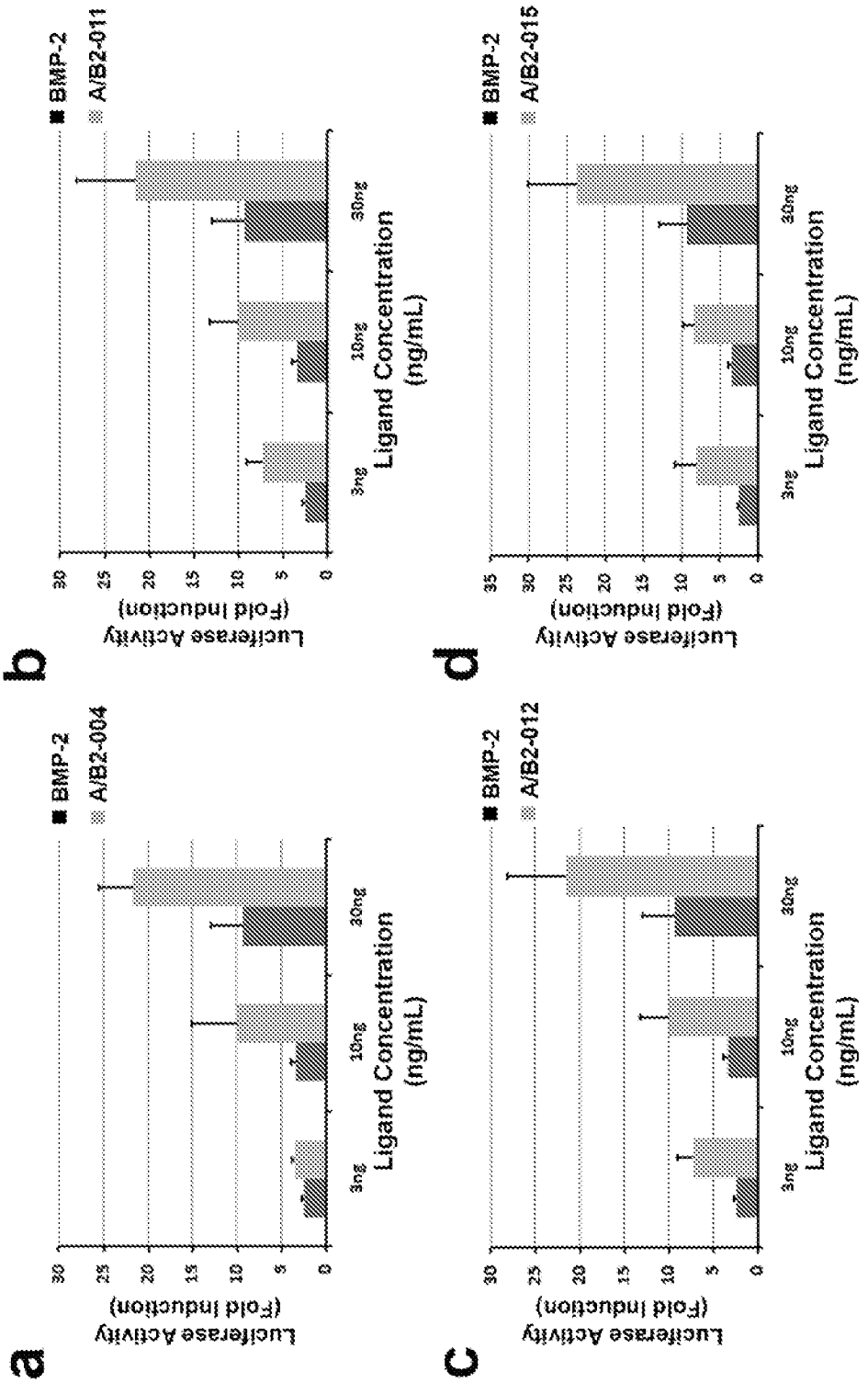

a
b
c
d
BMP-2
A/B2-004
A/B2-011
A/B2-012
A/B2-015
Luciferase Activity
(Fold Induction)
Ligand Concentration
(ng/mL)
3ng
10ng
30ng
0
5
10
15
20
25
30
35

FIGURE 15

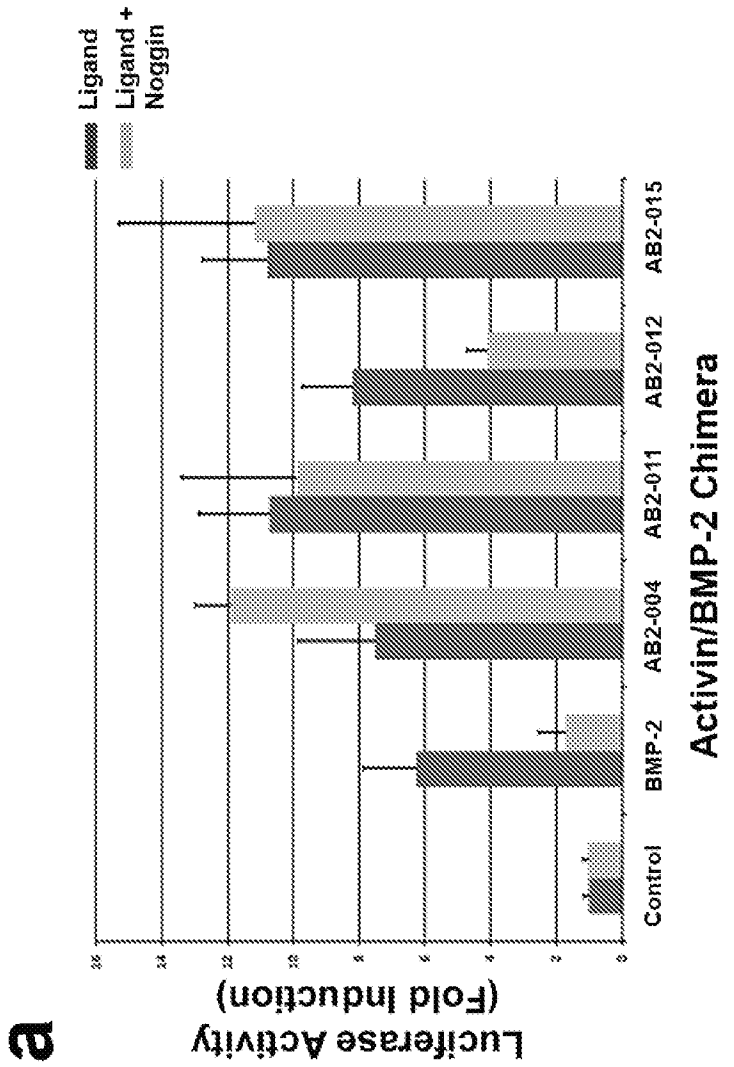

a
Luciferase Activity
(Fold Induction)
Ligand
Ligand + Noggin
Control
BMP-2
AB2-004
AB2-011
AB2-012
AB2-015
Activin/BMP-2 Chimera

FIGURE 16

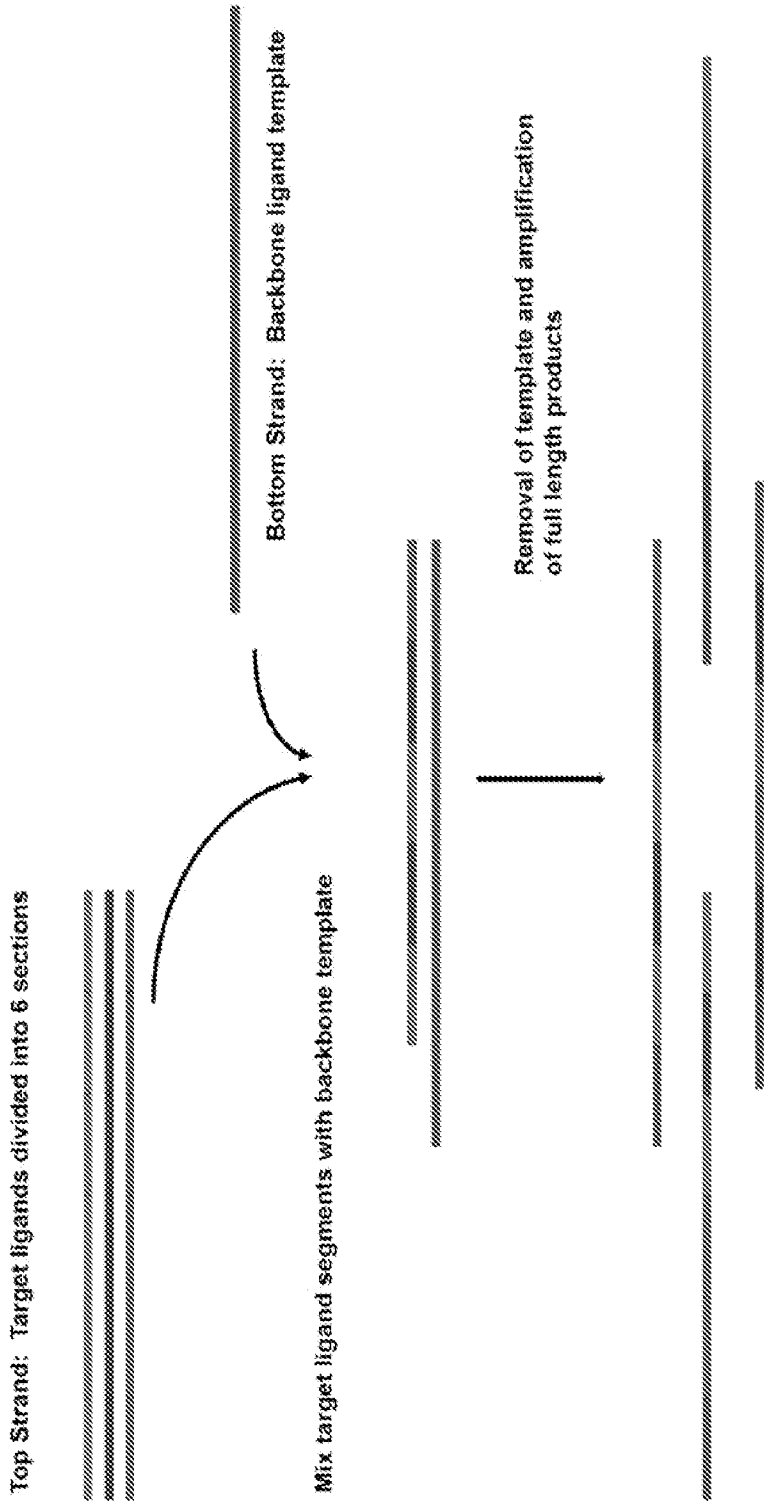

Top Strand: Target ligands divided into 6 sections
Mix target ligand segments with backbone template
Bottom Strand: Backbone ligand template
Removal of template and amplification of full length products
Sequences ready for cloning

FIGURE 17

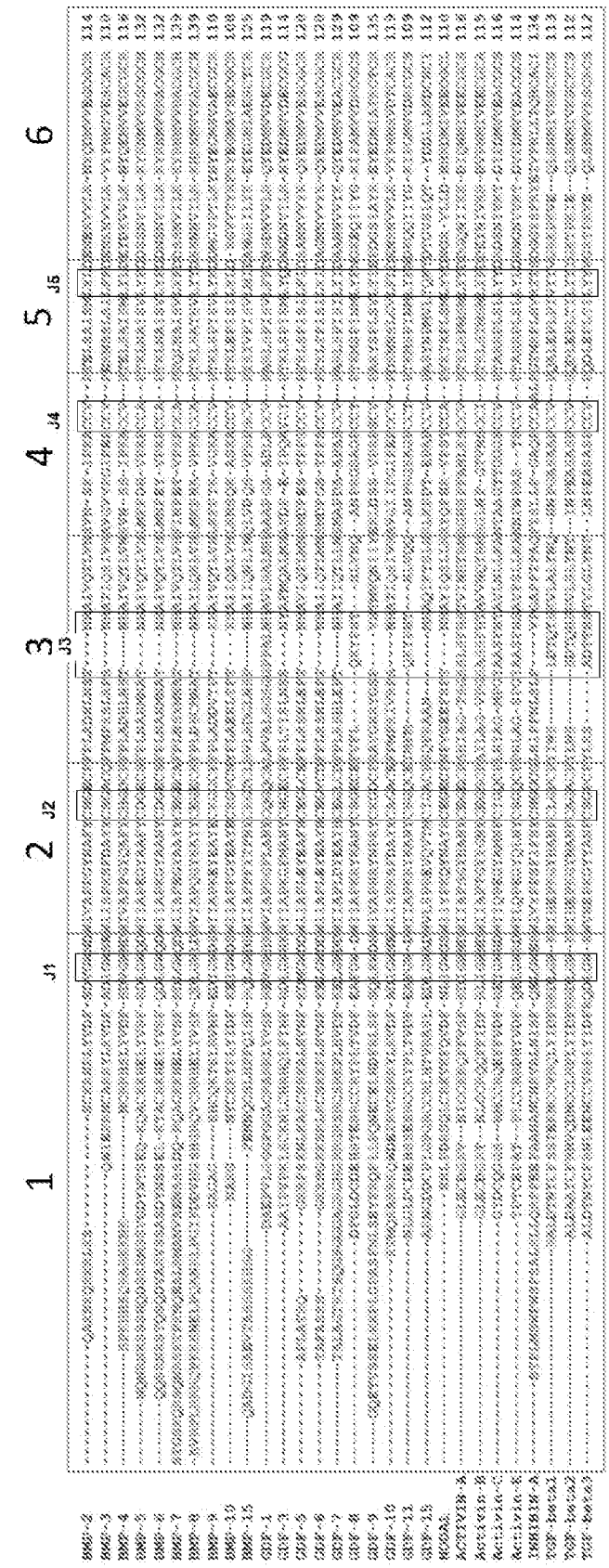


FIGURE 18

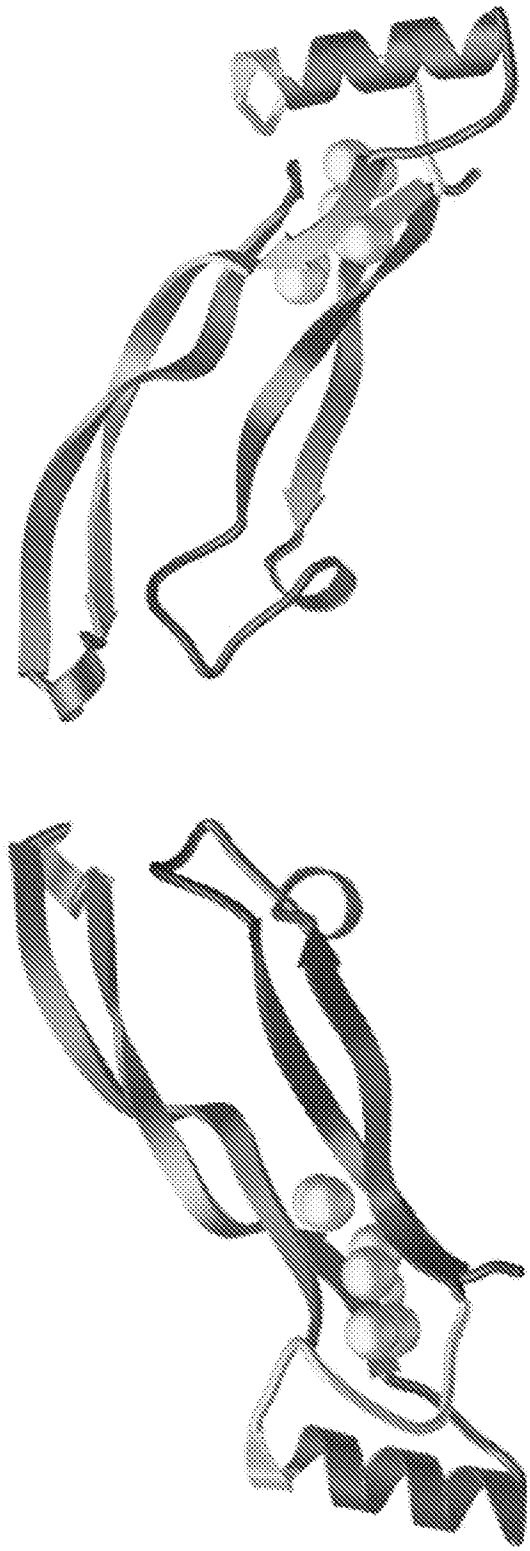


FIGURE 19