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(54) Title: METHODS FOR DOSING AN ACTIVIN-ACTRIIA ANTAGONIST AND MONITORING OF TREATED PATIENTS

FIGURE 15. Effect of ActRIIA-mFc on chemotherapy-induced anemia in mice

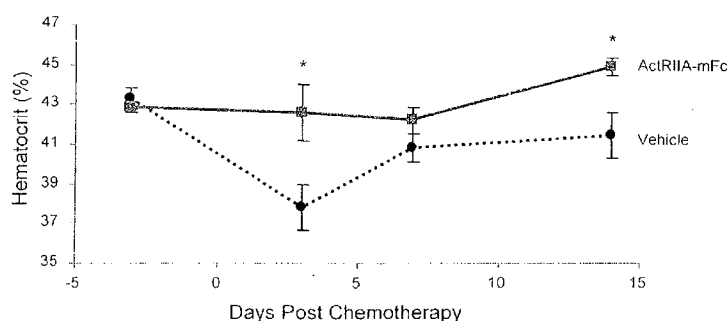


FIGURE 15

(57) Abstract: In certain aspects, the present invention provides methods for dosing a patient with an activin-ActRIIa antagonist and methods for managing patients treated with an activin-ActRIIa antagonist. In certain aspects, the methods involve measuring one or more hematologic parameters in a patient.

METHODS FOR DOSING AN ACTIVIN-ACTRIIA ANTAGONIST AND MONITORING OF TREATED PATIENTS

RELATED APPLICATIONS

- 5 This application claims the benefit of U.S. Provisional Application No. 61/133,354, filed on June 26, 2008, the specification of which is incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

- 10 The transforming growth factor-beta (TGF-beta) superfamily contains a variety of growth factors that share common sequence elements and structural motifs. These proteins are known to exert biological effects on a large variety of cell types in both vertebrates and invertebrates. Members of the superfamily perform important functions during embryonic development in pattern formation and tissue specification and can influence a variety of
- 15 differentiation processes, including adipogenesis, myogenesis, chondrogenesis, cardiogenesis, hematopoiesis, neurogenesis, and epithelial cell differentiation. The family is divided into two general branches: the BMP/GDF and the TGF-beta/Activin/BMP10 branches, whose members have diverse, often complementary effects. By manipulating the activity of a member of the TGF-beta family, it is often possible to cause significant
- 20 physiological changes in an organism. For example, the Piedmontese and Belgian Blue cattle breeds carry a loss-of-function mutation in the GDF8 (also called myostatin) gene that causes a marked increase in muscle mass. Grobet et al., Nat Genet. 1997, 17(1):71-4. Furthermore, in humans, inactive alleles of GDF8 are associated with increased muscle mass and, reportedly, exceptional strength. Schuelke et al., N Engl J Med 2004, 350:2682-8.
- 25 Changes in muscle, bone, cartilage and other tissues may be achieved by agonizing or antagonizing signaling that is mediated by an appropriate TGF-beta family member. It is an object of the present disclosure to provide alternative methods for administering modulators of the TGF-beta superfamily to patients.

SUMMARY OF THE INVENTION

In part, the disclosure relates to methods for administering activin antagonists, as well as ActRIIa antagonists (collectively, "activin-ActRIIa antagonists"), to patients in a manner that is appropriate given the effects that such antagonists can have on a variety of tissues, including red blood cells. In part, the disclosure demonstrates that activin-ActRIIa antagonists can increase red blood cell and hemoglobin levels and also increase bone density. This dual effect has particular advantages in patients that have both anemia and bone loss, such as many cancer patients (where anemia and bone loss can be a consequence of the tumor or a consequence of irradiation or chemotherapy), patients with osteoporosis and patients with renal failure. In particular, the disclosure demonstrates that a soluble form of ActRIIa acts as an inhibitor of activin and, when administered in vivo, increases red blood cell levels. While soluble ActRIIa may affect red blood cell levels through a mechanism other than activin antagonism, the disclosure nonetheless demonstrates that desirable therapeutic agents may be selected on the basis of activin antagonism or ActRIIa antagonism or both. Such agents are referred to collectively as activin-ActRIIa antagonists. As described herein, and in published patent applications WO/2009/038745, WO/2008/100384, WO/2008/094708, WO/2008/076437, WO/2007/062188 and WO/2006/012627, activin-ActRIIa antagonists also have a variety of other therapeutic uses including, for example, promoting bone growth, decreasing FSH levels, treating multiple myeloma and treating breast cancer. In certain instances, when administering an activin-ActRIIa antagonists for promoting bone growth or treating breast cancer, it may be desirable to monitor the effects on red blood cells during administration of an activin-ActRIIa antagonists, or to determine or adjust the dosing of an activin-ActRIIa antagonists, in order to reduce undesired effects on red blood cells. For example, excessive increases in red blood cell levels, hemoglobin levels, or hematocrit levels may cause increases in blood pressure or other undesirable side effects. It may also be desirable to restrict dosing of activin-ActRIIa antagonists to patients who have appropriate hematologic parameters. For example, it may be desirable to limit dosing to only those patients who have a hemoglobin level below normal (e.g., below 12 g/dL, below 11 g/dL, below 10 g/dL or below 9 g/dL or lower).

Therefore, in certain embodiments, the disclosure provides methods for managing a patient that has been treated with, or is a candidate to be treated with, an activin-ActRIIa antagonist, including, for example, activin-binding ActRIIa polypeptides, anti-activin

antibodies, anti-ActRIIa antibodies, activin- or ActRIIa-targeted small molecules and aptamers, and nucleic acids that decrease expression of activin or ActRIIa, by monitoring in the patient one or more hematologic parameters that correlate with an increase in red blood cell levels, such as, for example, red blood cell levels, blood pressure, or iron stores.

5 In certain aspects, the disclosure provides polypeptides comprising a soluble, activin-binding ActRIIa polypeptide that binds to activin. ActRIIa polypeptides may be formulated as a pharmaceutical preparation comprising the activin-binding ActRIIa polypeptide and a pharmaceutically acceptable carrier. The activin-binding ActRIIa polypeptide may bind to activin with a K_D less than 1 micromolar or less than 100, 10 or 1 nanomolar. Optionally, the
10 activin-binding ActRIIa polypeptide selectively binds activin versus GDF11 and/or GDF8, and optionally with a K_D that is at least 10-fold, 20-fold or 50-fold lower with respect to activin than with respect to GDF11 and/or GDF8. While not wishing to be bound to a particular mechanism of action, it is expected that this degree of selectivity for activin inhibition over GDF11/GDF8 inhibition in ActRIIa-Fc accounts for effects on bone or
15 erythropoiesis without a consistently measurable effect on muscle. In many embodiments, an ActRIIa polypeptide will be selected for causing less than 15%, less than 10% or less than 5% increase in muscle at doses that achieve desirable effects on red blood cell levels. In other embodiments, the effect on muscle is acceptable and need not be selected against. The composition may be at least 95% pure, with respect to other polypeptide components, as
20 assessed by size exclusion chromatography, and optionally, the composition is at least 98% pure. An activin-binding ActRIIa polypeptide for use in such a preparation may be any of those disclosed herein, such as a polypeptide having (i.e. comprising) an amino acid sequence selected from SEQ ID NOs: 2, 3, 7, 12 or 13, or having (i.e. comprising) an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97% or 99% identical to an amino acid
25 sequence selected from SEQ ID NOs: 2, 3, 7, 12 or 13. An activin-binding ActRIIa polypeptide may include a functional fragment of a natural ActRIIa polypeptide, such as one comprising at least 10, 20 or 30 amino acids of a sequence selected from SEQ ID NOs: 1-3 or a sequence of SEQ ID NO: 2, lacking the C-terminal 10 to 15 amino acids (the "tail").

A soluble, activin-binding ActRIIa polypeptide may include one or more alterations in
30 the amino acid sequence (e.g., in the ligand-binding domain) relative to a naturally occurring ActRIIa polypeptide. Examples of altered ActRIIa polypeptides are provided in WO 2006/012627, pp. 59-60 and pp. 55-58, respectively, which is incorporated by reference

herein, and throughout U.S. Patent Application Serial No. 12/012,652, incorporated by reference herein. The alteration in the amino acid sequence may, for example, alter glycosylation of the polypeptide when produced in a mammalian, insect or other eukaryotic cell or alter proteolytic cleavage of the polypeptide relative to the naturally occurring ActRIIa polypeptide.

An activin-binding ActRIIa polypeptide may be a fusion protein that has, as one domain, an ActRIIa polypeptide, (e.g., a ligand-binding portion of an ActRIIa) and one or more additional domains that provide a desirable property, such as improved pharmacokinetics, easier purification, targeting to particular tissues, etc. For example, a domain of a fusion protein may enhance one or more of in vivo stability, in vivo half life, uptake/administration, tissue localization or distribution, formation of protein complexes, multimerization of the fusion protein, and/or purification. An activin-binding ActRIIa fusion protein may include an immunoglobulin Fc domain (wild-type or mutant) or a serum albumin or other polypeptide portion that provides desirable properties such as improved pharmacokinetics, improved solubility or improved stability. In a preferred embodiment, an ActRIIa-Fc fusion comprises a relatively unstructured linker positioned between the Fc domain and the extracellular ActRIIa domain. This unstructured linker may be an artificial sequence of 1, 2, 3, 4 or 5 amino acids or a length of between 5 and 15, 20, 30, 50 or more amino acids that are relatively free of secondary structure, or a mixture of both. A linker may be rich in glycine and proline residues and may, for example, contain a single sequence of threonine/serine and glycines or repeating sequences of threonine/serine and glycines (e.g., TG₄ (SEQ ID NO: 15) or SG₄ (SEQ ID NO: 16) singlets or repeats). A fusion protein may include a purification subsequence, such as an epitope tag, a FLAG tag, a polyhistidine sequence, and a GST fusion. Optionally, a soluble ActRIIa polypeptide includes one or more modified amino acid residues selected from: a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, an amino acid conjugated to a lipid moiety, and an amino acid conjugated to an organic derivatizing agent. A pharmaceutical preparation may also include one or more additional compounds such as a compound that is used to treat a bone disorder or a compound that is used to treat anemia. Preferably, a pharmaceutical preparation is substantially pyrogen free. In general, it is preferable that an ActRIIa protein be expressed in a mammalian cell line that mediates suitably natural glycosylation of the ActRIIa protein so as to diminish the likelihood

of an unfavorable immune response in a patient. Human and CHO cell lines have been used successfully, and it is expected that other common mammalian expression systems will be useful.

As described herein, ActRIIa proteins designated ActRIIa-Fc have desirable properties, including selective binding to activin versus GDF8 and/or GDF11, high affinity ligand binding and serum half life greater than two weeks in animal models and in human patients. In certain embodiments the invention provides ActRIIa-Fc polypeptides and pharmaceutical preparations comprising such polypeptides and a pharmaceutically acceptable excipient.

In certain aspects, the disclosure provides nucleic acids encoding a soluble activin-binding ActRIIa polypeptide. An isolated polynucleotide may comprise a coding sequence for a soluble, activin-binding ActRIIa polypeptide, such as described above. For example, an isolated nucleic acid may include a sequence coding for an extracellular domain (e.g., ligand-binding domain) of an ActRIIa and a sequence that would code for part or all of the transmembrane domain and/or the cytoplasmic domain of an ActRIIa, but for a stop codon positioned within the transmembrane domain or the cytoplasmic domain, or positioned between the extracellular domain and the transmembrane domain or cytoplasmic domain. For example, an isolated polynucleotide may comprise a full-length ActRIIa polynucleotide sequence such as SEQ ID NO: 4 or a partially truncated version of ActRIIa, such as a nucleic acid comprising the nucleic acid sequence of SEQ ID NO:5, which corresponds to the extracellular domain of ActRIIa. An isolated polynucleotide may further comprise a transcription termination codon at least six hundred nucleotides before the 3'-terminus or otherwise positioned such that translation of the polynucleotide gives rise to an extracellular domain optionally fused to a truncated portion of a full-length ActRIIa. A preferred nucleic acid sequence for ActRIIa is SEQ ID NO:14. Nucleic acids disclosed herein may be operably linked to a promoter for expression, and the disclosure provides cells transformed with such recombinant polynucleotides. Preferably the cell is a mammalian cell such as a CHO cell.

In certain aspects, the disclosure provides methods for making a soluble, activin-binding ActRIIa polypeptide. Such a method may include expressing any of the nucleic acids (e.g., SEQ ID NO: 4, 5 or 14) disclosed herein in a suitable cell, such as a Chinese hamster ovary (CHO) cell or human cell. Such a method may comprise: a) culturing a cell under conditions suitable for expression of the soluble ActRIIa polypeptide, wherein said cell is

transformed with a soluble ActRIIa expression construct; and b) recovering the soluble ActRIIa polypeptide so expressed. Soluble ActRIIa polypeptides may be recovered as crude, partially purified or highly purified fractions. Purification may be achieved by a series of purification steps, including, for example, one, two or three or more of the following, in any order: protein A chromatography, anion exchange chromatography (e.g., Q sepharose), hydrophobic interaction chromatography (e.g., phenylsepharose), size exclusion chromatography, and cation exchange chromatography. Soluble ActRIIa polypeptides may be formulated in liquid or solid (e.g., lyophilized) forms.

In certain aspects, the disclosure provides a method for dosing a patient with an activin-ActRIIa antagonist, comprising dosing the patient in amounts and at intervals selected so as to reduce the risk of causing a rise in hemoglobin levels greater than 0.5 g/dL, 1 g/dl or 1.5 g/dL in two weeks.

In certain aspects, the disclosure provides a method for administering an ActRIIa-Fc fusion protein to a patient, comprising administering the ActRIIa fusion protein no more frequently than once per 60 days, once per 90 days, or once per 120 days. In certain embodiments, the patient may be a patient in need of bone growth or a patient suffering from or at risk for developing breast cancer or multiple myeloma, or a patient in need of having decreased FSH.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the purification of ActRIIa-hFc expressed in CHO cells. The protein purifies as a single, well-defined peak as visualized by sizing column (left panel) and Coomassie stained SDS-PAGE (right panel) (left lane: molecular weight standards; right lane: ActRIIa-hFc).

Figure 2 shows the binding of ActRIIa-hFc to activin and GDF-11, as measured by BiaCore™ assay.

Figure 3 shows the effects of ActRIIa-hFc on red blood cell counts in female non-human primates. Female cynomolgus monkeys (four groups of five monkeys each) were treated with placebo or 1mg/kg, 10 mg/kg or 30 mg/kg of ActRIIa-hFc on day 0, day 7, day 14 and day 21. Figure 3A shows red blood cell (RBC) counts. Figure 3B shows hemoglobin

levels. Statistical significance is relative to baseline for each treatment group. At day 57, two monkeys remained in each group.

Figure 4 shows the effects of ActRIIa-hFc on red blood cell counts in male non-human primates. Male cynomolgus monkeys (four groups of five monkeys each) were
5 treated with placebo or 1 mg/kg, 10 mg/kg or 30 mg/kg of ActRIIa-hFc on day 0, day 7, day 14 and day 21. Figure 4A shows red blood cell (RBC) counts. Figure 4B shows hemoglobin levels. Statistical significance is relative to baseline for each treatment group. At day 57, two monkeys remained in each group.

Figure 5 shows the effects of ActRIIa-hFc on reticulocyte counts in female non-
10 human primates. Cynomolgus monkeys (four groups of five monkeys each) were treated with placebo or 1 mg/kg, 10 mg/kg or 30 mg/kg of ActRIIa-hFc on day 0, day 7, day 14 and day 21. Figure 5A shows absolute reticulocyte counts. Figure 5B shows the percentage of reticulocytes relative to RBCs. Statistical significance is relative to baseline for each group. At day 57, two monkeys remained in each group.

Figure 6 shows the effects of ActRIIa-hFc on reticulocyte counts in female non-
15 human primates. Cynomolgus monkeys (four groups of five monkeys each) were treated with placebo or 1 mg/kg, 10 mg/kg or 30 mg/kg of ActRIIa-hFc on day 0, day 7, day 14 and day 21. Figure 6A shows absolute reticulocyte counts. Figure 6B shows the percentage of reticulocytes relative to RBCs. Statistical significance is relative to baseline for each group. At day 57, two monkeys remained in each group.

Figure 7 shows results from the human clinical trial described in Example 5, where the area-under-curve (AUC) and administered dose of ActRIIa-hFc have a linear correlation, regardless of whether ActRIIa-hFc was administered intravenously (IV) or subcutaneously (SC).

Figure 8 shows a comparison of serum levels of ActRIIa-hFc in patients administered
25 IV or SC.

Figure 9 shows bone alkaline phosphatase (BAP) levels in response to different dose levels of ActRIIa-hFc. BAP is a marker for anabolic bone growth.

Figure 10 depicts the median change from baseline of hematocrit levels from the
30 human clinical trial described in Example 5. ActRIIa-hFc was administered intravenously (IV) at the indicated dosage.

Figure 11 depicts the median change from baseline of hemoglobin levels from the human clinical trial described in Example 5. ActRIIa-hFc was administered intravenously (IV) at the indicated dosage.

5 Figure 12 depicts the median change from baseline of RBC (red blood cell) count from the human clinical trial described in Example 5. ActRIIa-hFc was administered intravenously (IV) at the indicated dosage.

Figure 13 depicts the median change from baseline of reticulocyte count from the human clinical trial described in Example 5. ActRIIa-hFc was administered intravenously (IV) at the indicated dosage.

10 Figure 14 shows an alignment of human ActRIIA and ActRIIB with the residues that are deduced herein, based on composite analysis of multiple ActRIIB and ActRIIA crystal structures to directly contact ligand (the ligand binding pocket) indicated with boxes.

Figure 15 shows the effect of ActRIIA-mFc on hematocrit in a mouse model of chemotherapy-induced anemia. Data are means $\pm$ SEM. *, $P < 0.05$ vs. vehicle at same time point. A single dose of ActRIIA-mFc before chemotherapy prevented the decline in hematocrit level otherwise observed after administration of the chemotherapeutic paclitaxel.

Figure 16 shows the dose-dependent effect of ActRIIA-mFc on hematocrit in a mouse model of chemotherapy-induced anemia. Data are means $\pm$ SEM. **, $P < 0.01$; ***, $P < 0.001$ vs. vehicle at same time point. Two weeks after paclitaxel administration, ActRIIA-mFc treatment increased hematocrit level as a function of dose number.

Figure 17 shows the effect of ActRIIA-mFc on hematocrit in a partially nephrectomized (NEPHX) mouse model of chronic kidney disease. Data are means $\pm$ SEM. *, $P < 0.05$ vs. vehicle at same time point. ActRIIA-mFc treatment prevented the decline in hematocrit level otherwise observed at 4 weeks and produced a beneficial trend in hematocrit at 8 weeks.

DETAILED DESCRIPTION OF THE INVENTION

1. Overview

The transforming growth factor-beta (TGF-beta) superfamily contains a variety of growth factors that share common sequence elements and structural motifs. These proteins are known to exert biological effects on a large variety of cell types in both vertebrates and invertebrates. Members of the superfamily perform important functions during embryonic development in pattern formation and tissue specification and can influence a variety of differentiation processes, including adipogenesis, myogenesis, chondrogenesis, cardiogenesis, hematopoiesis, neurogenesis, and epithelial cell differentiation. The family is divided into two general branches: the BMP/GDF and the TGF-beta/Activin/BMP10 branches, whose members have diverse, often complementary effects. By manipulating the activity of a member of the TGF-beta family, it is often possible to cause significant physiological changes in an organism. For example, the Piedmontese and Belgian Blue cattle breeds carry a loss-of-function mutation in the GDF8 (also called myostatin) gene that causes a marked increase in muscle mass. Grobet et al., Nat Genet. 1997, 17(1):71-4. Furthermore, in humans, inactive alleles of GDF8 are associated with increased muscle mass and, reportedly, exceptional strength. Schuelke et al., N Engl J Med 2004, 350:2682-8.

Activins are dimeric polypeptide growth factors that belong to the TGF-beta superfamily. There are three principal activin forms (A, B, and AB) that are homo/heterodimers of two closely related β subunits ($\beta_A\beta_A$, $\beta_B\beta_B$, and $\beta_A\beta_B$, respectively). The human genome also encodes an activin C and an activin E, which are primarily expressed in the liver, and heterodimeric forms containing β_C or β_E are also known. In the TGF-beta superfamily, activins are unique and multifunctional factors that can stimulate hormone production in ovarian and placental cells, support neuronal cell survival, influence cell-cycle progress positively or negatively depending on cell type, and induce mesodermal differentiation at least in amphibian embryos (DePaolo et al., 1991, Proc Soc Exp Biol Med. 198:500-512; Dyson et al., 1997, Curr Biol. 7:81-84; Woodruff, 1998, Biochem Pharmacol. 55:953-963). Moreover, erythroid differentiation factor (EDF) isolated from the stimulated human monocytic leukemic cells was found to be identical to activin A (Murata et al., 1988, PNAS, 85:2434). It has been suggested that activin A promotes erythropoiesis in the bone marrow. In several tissues, activin signaling is antagonized by its related heterodimer, inhibin. For example, during the release of follicle-stimulating hormone (FSH) from the

pituitary, activin promotes FSH secretion and synthesis, while inhibin prevents FSH secretion and synthesis. Other proteins that may regulate activin bioactivity and/or bind to activin include follistatin (FS), follistatin-related protein (FSRP) and α_2 -macroglobulin.

5 TGF- β signals are mediated by heteromeric complexes of type I and type II serine/threonine kinase receptors, which phosphorylate and activate downstream Smad proteins upon ligand stimulation (Massagué, 2000, Nat. Rev. Mol. Cell Biol. 1:169-178). These type I and type II receptors are transmembrane proteins, composed of a ligand-binding extracellular domain with cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine specificity. Type I receptors are essential for signaling; and type II
10 receptors are required for binding ligands and for expression of type I receptors. Type I and II activin receptors form a stable complex after ligand binding, resulting in phosphorylation of type I receptors by type II receptors.

Two related type II receptors (ActRII), ActRIIa and ActRIIb, have been identified as the type II receptors for activins (Mathews and Vale, 1991, Cell 65:973-982; Attisano et al.,
15 1992, Cell 68: 97-108). Besides activins, ActRIIa and ActRIIb can biochemically interact with several other TGF- β family proteins, including BMP7, Nodal, GDF8, and GDF11 (Yamashita et al., 1995, J. Cell Biol. 130:217-226; Lee and McPherron, 2001, Proc. Natl. Acad. Sci. 98:9306-9311; Yeo and Whitman, 2001, Mol. Cell 7: 949-957; Oh et al., 2002, Genes Dev. 16:2749-54). ALK4 is the primary type I receptor for activins, particularly for
20 activin A, and ALK-7 may serve as a receptor for activins as well, particularly for activin B.

As demonstrated herein, a soluble ActRIIa polypeptide (sActRIIa), which shows substantial preference in binding to activin A as opposed to other TGF-beta family members, such as GDF8 or GDF11, is effective to increase red blood cell levels in vivo. While not wishing to be bound to any particular mechanism, it is expected that the effect of sActRIIa is
25 caused primarily by an activin antagonist effect, given the very strong activin binding (picomolar dissociation constant) exhibited by the particular sActRIIa construct used in these studies. Regardless of mechanism, it is apparent from this disclosure that ActRIIa-activin antagonists increase red blood cell levels in rodents, monkeys and humans. It should be noted that hematopoiesis is a complex process, regulated by a variety of factors, including
30 erythropoietin, G-CSF and iron homeostasis. The terms "increase red blood cell levels" and "promote red blood cell formation" refer to clinically observable metrics, such as hematocrit,

red blood cell counts and hemoglobin measurements, and are intended to be neutral as to the mechanism by which such changes occur.

The data reported herein with respect to non-human primates are reproducible in mice, rats and humans as well, and therefore, this disclosure provides methods for using ActRIIa polypeptides and other activin-ActRIIa antagonists to promote red blood cell production and increase red blood cell levels in mammals ranging from rodents to humans.

In addition to stimulating red blood cell levels, activin-ActRIIa antagonists are useful for a variety of therapeutic applications, including, for example, promoting bone growth (see PCT Publication No. WO2007/062188, which is hereby incorporated by reference in its entirety), and treating breast cancer (see PCT Application No. PCT/US2008/001429, which is hereby incorporated by reference in its entirety). In certain instances, when administering an activin-ActRIIa antagonists for the purpose of increasing bone or treating breast cancer, it may be desirable to reduce or minimize or otherwise monitor effects on red blood cells. In some instances, a dual effect on blood cells and bone or other tissue will be desirable, but it is generally recognized that pharmaceutically promoted increases in red blood cells, even up to a level that is typically considered normal, can have adverse effects on patients, and thus are often monitored or managed with care. By monitoring various hematologic parameters in patients being treated with, or who are candidates for treatment with, an activin-ActRIIa antagonist, appropriate dosing (including amounts and frequency of administration) may be determined based on an individual patient's needs, baseline hematologic parameters, and purpose for treatment. Furthermore, therapeutic progress and effects on one or more hematologic parameters over time may be useful in managing patients being dosed with an activin-ActRIIa antagonist by facilitating patient care, determining appropriate maintenance dosing (both amounts and frequency), etc.

Activin-ActRIIa antagonists include, for example, activin-binding soluble ActRIIa polypeptides, antibodies that bind to activin (particularly the activin A or B subunits, also referred to as β A or β B) and disrupt ActRIIa binding, antibodies that bind to ActRIIa and disrupt activin binding, non-antibody proteins selected for activin or ActRIIa binding (see e.g., WO/2002/088171, WO/2006/055689, and WO/2002/032925 for examples of such proteins and methods for design and selection of same), randomized peptides selected for activin or ActRIIa binding, often affixed to an Fc domain. Two different proteins (or other moieties) with activin or ActRIIa binding activity, especially activin binders that block the

type I (e.g., a soluble type I activin receptor) and type II (e.g., a soluble type II activin receptor) binding sites, respectively, may be linked together to create a bifunctional binding molecule. Nucleic acid aptamers, small molecules and other agents that inhibit the activin-ActRIIa signaling axis are included as activin-ActRIIa antagonists. Various proteins have
5 activin-ActRIIa antagonist activity, including inhibin (i.e., inhibin alpha subunit), although inhibin does not universally antagonize activin in all tissues, follistatin (e.g., follistatin-288 and follistatin-315), FSRP, FLRG, activin C, alpha(2)-macroglobulin, and an M108A (methionine to alanine change at position 108) mutant activin A. Generally, alternative forms of activin, particularly those with alterations in the type I receptor binding domain can bind to
10 type II receptors and fail to form an active ternary complex, thus acting as antagonists. Additionally, nucleic acids, such as antisense molecules, siRNAs or ribozymes that inhibit activin A, B, C or E, or, particularly, ActRIIa expression, can be used as activin-ActRIIa antagonists. The activin-ActRIIa antagonist to be used may exhibit selectivity for inhibiting activin-mediated signaling versus other members of the TGF-beta family, and particularly
15 with respect to GDF8 and GDF11.

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and in the specific context where each term is used. Certain terms are discussed below or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the invention and
20 how to make and use them. The scope or meaning of any use of a term will be apparent from the specific context in which the term is used.

“About” and “approximately” shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typically, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more
25 preferably within 5% of a given value or range of values.

Alternatively, and particularly in biological systems, the terms “about” and “approximately” may mean values that are within an order of magnitude, preferably within 5-fold and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term “about” or “approximately”
30 can be inferred when not expressly stated.

The methods of the invention may include steps of comparing sequences to each other, including wild-type sequence to one or more mutants (sequence variants). Such comparisons typically comprise alignments of polymer sequences, e.g., using sequence alignment programs and/or algorithms that are well known in the art (for example, BLAST, FASTA and MEGALIGN, to name a few). The skilled artisan can readily appreciate that, in such alignments, where a mutation contains a residue insertion or deletion, the sequence alignment will introduce a “gap” (typically represented by a dash, or “A”) in the polymer sequence not containing the inserted or deleted residue.

“Homologous,” in all its grammatical forms and spelling variations, refers to the relationship between two proteins that possess a “common evolutionary origin,” including proteins from superfamilies in the same species of organism, as well as homologous proteins from different species of organism. Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

The term “sequence similarity,” in all its grammatical forms, refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin.

However, in common usage and in the instant application, the term “homologous,” when modified with an adverb such as “highly,” may refer to sequence similarity and may or may not relate to a common evolutionary origin.

2. ActRIIa Polypeptides

In certain aspects, the present invention relates to ActRIIa polypeptides. As used herein, the term “ActRIIa” refers to a family of activin receptor type IIa (ActRIIa) proteins from any species and variants derived from such ActRIIa proteins by mutagenesis or other modification. Reference to ActRIIa herein is understood to be a reference to any one of the currently identified forms. Members of the ActRIIa family are generally transmembrane proteins, composed of a ligand-binding extracellular domain with a cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine kinase activity.

The term "ActRIIa polypeptide" includes polypeptides comprising any naturally occurring polypeptide of an ActRIIa family member as well as any variants thereof (including mutants, fragments, fusions, and peptidomimetic forms) that retain a useful activity. See, for example, WO/2006/012627. For example, ActRIIa polypeptides include polypeptides derived from the sequence of any known ActRIIa having a sequence at least about 80% identical to the sequence of an ActRIIa polypeptide, and optionally at least 85%, 90%, 95%, 97%, 99% or greater identity. For example, an ActRIIa polypeptide of the invention may bind to and inhibit the function of an ActRIIa protein and/or activin. An ActRIIa polypeptide may be selected for activity in promoting red blood cell formation in vivo. Examples of ActRIIa polypeptides include human ActRIIa precursor polypeptide (SEQ ID NO: 1) and soluble human ActRIIa polypeptides (e.g., SEQ ID NOs: 2, 3, 7 and 12).

The human ActRIIa precursor protein sequence is as follows:

MGAAAKLAFVFLISCSSGAILGRSETQECLFFNANWEKDRTNQTGVPEP
 CYGDKDKRRHCFATWKNISGSIEIVKQGCWLDDINCYDRTDCVEKKDSP
 EVYFCCCEGNMCNEKFSYFPEMEVTQPTSNPVTPKPPYYNILLYSLVPL
 MLIAGIVICAFWVYRHHKMAYPPVLVPTQDPGPPPPSPLLGLKPLQLLE
 VKARGRFGCVWKAQLLNEYVAVKIFPIQDKQSWQNEYEVYSLPGMKHEN
 ILQFIGAEKRGTSVDVDLWLITAFHEKGSLSDFLKANVVSWNELCHIAE
 TMARGLAYLHEDIPLKDGHKPAISHRDIKSKNVLLKNNLTACIADFG
 ALKFEAGKSAGDTHGQVGTRRYMAPEVLEGAINFQRDAFLRIDMYAMGL
 VLWELASRCTAADGPVDEYMLPFEEEIGQHPSLEDMQEVVHKKKRPVL
 RDYWQKHAGMAMLCETIEECWDHDAEARLSAGCVGERITQMQRLTNIIT
 TEDIIVTVMTMVTNVDFPPKESL (SEQ ID NO: 1)

The signal peptide is single underlined; the extracellular domain is in bold and the potential N-linked glycosylation sites are double underlined.

The human ActRIIa soluble (extracellular), processed polypeptide sequence is as follows:

ILGRSETQECLFFNANWEKDRTNQTGVPEPCYGD~~DKRRHCFATWKNISG~~
 SIEIVKQGCWLDDINCYDRTDCVEKKDSPEVYFCCCEGNMCNEKFSYFP
 EMEVTQPTSNPVTPKPP (SEQ ID NO: 2)

The C-terminal “tail” of the extracellular domain is underlined. The sequence with the “tail” deleted (a $\Delta 15$ sequence) is as follows:

ILGRSETQECLFFNANWEKDRTNQTVGEPCYGDKDKRRHCFATWKNISG
SIEIVKQGCWLDDINCYDRITDCVEKKDSPEVYFCCCEGNMCNEKFSYFP
EM (SEQ ID NO:3)

The nucleic acid sequence encoding human ActR1a precursor protein is as follows (nucleotides 164-1705 of Genbank entry NM_001616):

ATGGGAGCTGCTGCAAAGTTGGCGTTTGCCGTCTTTCTTATCTCCTGTT
CTTCAGGTGCTATACTTGGTAGATCAGAACTCAGGAGTGTCTTTTCTT
10 TAATGCTAATTGGGAAAAAGACAGAACCAATCAAACCTGGTGTGAACCG
TGTTATGGTGACAAAGATAAACGGCGGCATTGTTTTGCTACCTGGAAGA
ATATTTCTGGTTCATTGAAATAGTGAAACAAGGTTGTTGGCTGGATGA
TATCAACTGCTATGACAGGACTGATTGTGTAGAAAAAAGACAGCCCT
GAAGTATATTTTTGTTGCTGTGAGGGCAATATGTGTAATGAAAAGTTTT
15 CTTATTTTCCAGAGATGGAAGTCACACAGCCCACTTCAAATCCAGTTAC
ACCTAAGCCACCCTATTACAACATCCTGCTCTATTCCCTGGTGCCACTT
ATGTTAATTGCGGGGATTGTCATTTGTGCATTTTGGGTGTACAGGCATC
ACAAGATGGCCTACCCTCCTGTACTTGTTCCTCAACTCAAGACCCAGGACC
ACCCCCACCTTCTCCATTACTAGGGTTGAAACCACTGCAGTTATTAGAA
20 GTGAAAGCAAGGGGAAGATTTGGTTGTGTCTGGAAGCCCAGTTGCTTA
ACGAATATGTGGCTGTCAAAATATTTCCAATACAGGACAAACAGTCATG
GCAAAATGAATACGAAGTCTACAGTTTGCCTGGAATGAAGCATGAGAAC
ATATTACAGTTCATTGGTGCAGAAAACGAGGCACCAGTGTGATGTGG
ATCTTTGGCTGATCACAGCATTTTCATGAAAAGGGTTCACTATCAGACTT
25 TCTTAAGGCTAATGTGGTCTCTTGAATGAACTGTGTCATATTGCAGAA
ACCATGGCTAGAGGATTGGCATATTTACATGAGGATATACCTGGCCTAA
AAGATGGCCACAAACCTGCCATATCTCACAGGGACATCAAAAGTAAAAA
TGTGCTGTTGAAAAACAACCTGACAGCTTGCATTGCTGACTTTGGGTTG
GCCTTAAATTTGAGGCTGGCAAGTCTGCAGGCGATACCCATGGACAGG
30 TTGGTACCCGGAGGTACATGGCTCCAGAGGTATTAGAGGGTGCTATAAA
CTTCAAAGGGATGCATTTTTGAGGATAGATATGTATGCCATGGGATTA
GTCCTATGGGAACCTGGCTTCTCGCTGTACTGCTGCAGATGGACCTGTAG
ATGAATACATGTTGCCATTTGAGGAGGAAATTGGCCAGCATCCATCTCT

TGAAGACATGCAGGAAGTTGTTGTGCATAAAAAAAGAGGCCTGTTTTA
 AGAGATTATTGGCAGAAACATGCTGGAATGGCAATGCTCTGTGAAACCA
 TTGAAGAATGTTGGGATCACGACGCAGAAGCCAGGTATCAGCTGGATG
 TGTAGGTGAAAGAATTACCCAGATGCAGAGACTAACAAATATTATTACC
 5 ACAGAGGACATTGTAACAGTGGTCACAATGGTGACAAATGTTGACTTTC
 CTCCCAAAGAATCTAGTCTATGA (SEQ ID NO: 4)

The nucleic acid sequence encoding a human ActRIIa soluble (extracellular)
 polypeptide is as follows:

ATACTTGGTAGATCAGAACTCAGGAGTGTCTTTTCTTTAATGCTAATT
 10 GGGAAAAAGACAGAACCAATCAAACCTGGTGTGTAACCGTGTTATGGTGA
 CAAAGATAAACGGCGGCATTGTTTTGCTACCTGGAAGAATATTTCTGGT
 TCCATTGAAATAGTGAAACAAGGTGTTGGCTGGATGATATCAACTGCT
 ATGACAGGACTGATTGTGTAGAAAAAAGACAGCCCTGAAGTATATTT
 TTGTTGCTGTGAGGGCAATATGTGTAATGAAAAGTTTTCTTATTTTCCA
 15 GAGATGGAAGTCACACAGCCCACTTCAAATCCAGTTACACCTAAGCCAC
 CC (SEQ ID NO: 5)

In a specific embodiment, the invention relates to soluble ActRIIa polypeptides. As
 described herein, the term "soluble ActRIIa polypeptide" generally refers to polypeptides
 comprising an extracellular domain of an ActRIIa protein. The term "soluble ActRIIa
 20 polypeptide," as used herein, includes any naturally occurring extracellular domain of an
 ActRIIa protein as well as any variants thereof (including mutants, fragments and
 peptidomimetic forms). An activin-binding ActRIIa polypeptide is one that retains the ability
 to bind to activin, including, for example, activin AA, AB, BB, or forms that include a C or E
 subunit. Optionally, an activin-binding ActRIIa polypeptide will bind to activin AA with a
 25 dissociation constant of 1 nM or less. The extracellular domain of an ActRIIa protein binds
 to activin and is generally soluble in physiological conditions, and thus can be termed a
 soluble, activin-binding ActRIIa polypeptide. Examples of soluble, activin-binding ActRIIa
 polypeptides include the soluble polypeptides illustrated in SEQ ID NOs: 2, 3, 7, 12 and 13.
 SEQ ID NO:7 is referred to as ActRIIa-hFc, and is described further in the Examples. Other
 30 examples of soluble, activin-binding ActRIIa polypeptides comprise a signal sequence in
 addition to the extracellular domain of an ActRIIa protein, for example, the honey bee
 mellitin leader sequence (SEQ ID NO: 8), the tissue plasminogen activator (TPA) leader (SEQ

ID NO: 9) or the native ActRIIa leader (SEQ ID NO: 10). The ActRIIa-hFc polypeptide illustrated in SEQ ID NO:13 uses a TPA leader.

A general formula for an active ActRIIa variant protein is one that comprises amino acids 12-82 of SEQ ID NO: 2, respectively, but optionally beginning at a position ranging from 1-5 or 3-5 and ending at a position ranging from 110-116 or 110-115, respectively, and comprising no more than 1, 2, 5, 10 or 15 conservative amino acid changes in the ligand binding pocket, and zero, one or more non-conservative alterations at positions 40, 53, 55, 74, 79 and/or 82 in the ligand binding pocket. Such a protein may comprise an amino acid sequence that retains greater than 80%, 90%, 95% or 99% sequence identity to the sequence of amino acids 29-109 of SEQ ID NO: 2.

Functionally active fragments of ActRIIa polypeptides can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding an ActRIIa polypeptide. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments that can function as antagonists (inhibitors) of ActRIIa protein or signaling mediated by activin.

Functionally active variants of ActRIIa polypeptides can be obtained by screening libraries of modified polypeptides recombinantly produced from the corresponding mutagenized nucleic acids encoding an ActRIIa polypeptide. The variants can be produced and tested to identify those that can function as antagonists (inhibitors) of ActRIIa protein or signaling mediated by activin. In certain embodiments, a functional variant of the ActRIIa polypeptides comprises an amino acid sequence that is at least 75% identical to an amino acid sequence selected from SEQ ID NOs: 2 or 3. In certain cases, the functional variant has an amino acid sequence at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from SEQ ID NOs: 2 or 3.

Functional variants may be generated by modifying the structure of an ActRIIa polypeptide for such purposes as enhancing therapeutic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified ActRIIa polypeptides when selected to retain activin binding, are considered functional equivalents of the naturally-occurring ActRIIa polypeptides. Modified ActRIIa polypeptides 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. Whether a change in the amino acid sequence of an ActRIIa polypeptide results in a functional homolog can be readily determined by assessing the ability of the variant ActRIIa polypeptide to produce a response in cells in a fashion similar to the wild-type ActRIIa polypeptide.

In certain embodiments, the present invention contemplates specific mutations of the ActRIIa polypeptides so as to alter the glycosylation of the polypeptide. 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 or asparagine-X-serine (where "X" is any amino acid) which is 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 ActRIIa 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 on an ActRIIa polypeptide is by chemical or enzymatic coupling of glycosides to the ActRIIa 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. Removal of one or more carbohydrate moieties present on an ActRIIa polypeptide may be accomplished chemically and/or enzymatically. Chemical deglycosylation may involve, for example, exposure of the ActRIIa polypeptide 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. Enzymatic cleavage of

carbohydrate moieties on ActRIIa 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 sequence of an ActRIIa polypeptide may be adjusted, as appropriate, depending on the type of expression system used, as mammalian, yeast, insect and plant cells may all introduce
5 differing glycosylation patterns that can be affected by the amino acid sequence of the peptide. In general, ActRIIa proteins for use in humans may be expressed in a mammalian cell line that provides proper glycosylation, such as HEK293 or CHO cell lines, although other mammalian expression cell lines are expected to be useful as well. Other non-mammalian cell lines may be used (e.g., yeast, E. coli, insect cells), and in some cases, such
10 cell lines may be engineered to include enzymes that confer mammalian-type glycosylation patterns on the expressed proteins.

This disclosure further contemplates a method of generating mutants, particularly sets of combinatorial mutants of an ActRIIa polypeptide, as well as truncation mutants; pools of combinatorial mutants are especially useful for identifying functional variant sequences. The
15 purpose of screening such combinatorial libraries may be to generate, for example, ActRIIa polypeptide variants which bind to activin or other ligands. A variety of screening assays are provided below, and such assays may be used to evaluate variants. For example, an ActRIIa polypeptide variant may be screened for ability to bind to an ActRIIa ligand, to prevent binding of an ActRIIa ligand to an ActRIIa polypeptide or to interfere with signaling caused
20 by an ActRIIa ligand.

The activity of an ActRIIa polypeptide or its variants may also be tested in a cell-based or in vivo assay. For example, the effect of an ActRIIa polypeptide variant on the expression of genes involved in hematopoiesis may be assessed. This may, as needed, be performed in the presence of one or more recombinant ActRIIa ligand proteins (e.g., activin),
25 and cells may be transfected so as to produce an ActRIIa polypeptide and/or variants thereof, and optionally, an ActRIIa ligand. Likewise, an ActRIIa polypeptide may be administered to a mouse or other animal, and one or more blood measurements, such as an RBC count, hemoglobin, or reticulocyte count may be assessed.

Combinatorially-derived variants can be generated which have a selective or generally
30 increased potency relative to a naturally occurring ActRIIa polypeptide. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding a wild-type ActRIIa polypeptide. For example, the altered protein can

be rendered either more stable or less stable to proteolytic degradation or other cellular processes which result in destruction of, or otherwise inactivation of a native ActRIIa polypeptide. Such variants, and the genes which encode them, can be utilized to alter ActRIIa polypeptide levels by modulating the half-life of the ActRIIa polypeptides. For instance, a short half-life can give rise to more transient biological effects and, when part of an inducible expression system, can allow tighter control of recombinant ActRIIa polypeptide levels within the cell. In an Fc fusion protein, mutations may be made in the linker (if any) and/or the Fc portion to alter the half-life of the protein.

A combinatorial library may be produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential ActRIIa polypeptide sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential ActRIIa polypeptide nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display).

There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes can then be ligated into an appropriate vector for expression. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al., (1981) *Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al., (1984) *Science* 198:1056; Ike et al., (1983) *Nucleic Acid Res.* 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) *Science* 249:386-390; Roberts et al., (1992) *PNAS USA* 89:2429-2433; Devlin et al., (1990) *Science* 249: 404-406; Cwirla et al., (1990) *PNAS USA* 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, ActRIIa polypeptide variants can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) *Biochemistry* 33:1565-1572; Wang et al., (1994) *J. Biol. Chem.* 269:3095-3099; Balint et al., (1993) *Gene* 137:109-118; Grodberg et al., (1993) *Eur. J. Biochem.* 218:597-

601; Nagashima et al., (1993) J. Biol. Chem. 268:2888-2892; Lowman et al., (1991) Biochemistry 30:10832-10838; and Cunningham et al., (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) Virology 193:653-660; Brown et al., (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al., (1982) Science 232:316); by
5 saturation mutagenesis (Meyers et al., (1986) Science 232:613); by PCR mutagenesis (Leung et al., (1989) Method Cell Mol Biol 1:11-19); or by random mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol Biol 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for
10 identifying truncated (bioactive) forms of ActRIIa polypeptides.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the
15 combinatorial mutagenesis of ActRIIa polypeptides. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was
20 detected. Preferred assays include activin binding assays and activin-mediated cell signaling assays.

In certain embodiments, the ActRIIa polypeptides of the invention may further comprise post-translational modifications in addition to any that are naturally present in the ActRIIa polypeptides. Such modifications include, but are not limited to, acetylation,
25 carboxylation, glycosylation, phosphorylation, lipidation, and acylation. As a result, the modified ActRIIa polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of an ActRIIa polypeptide may be tested as described herein for other ActRIIa polypeptide variants. When an ActRIIa polypeptide is produced in cells by
30 cleaving a nascent form of the ActRIIa polypeptide, post-translational processing may also be important for correct folding and/or function of the protein. Different cells (such as CHO, HeLa, MDCK, 293, WI38, NIH-3T3 or HEK293) have specific cellular machinery and

characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the ActRIIa polypeptides.

In certain aspects, functional variants or modified forms of the ActRIIa polypeptides include fusion proteins having at least a portion of the ActRIIa polypeptides and one or more fusion domains. Well known examples of such fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), or human serum albumin. A fusion domain may be selected so as to confer a desired property. For example, some fusion domains are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt- conjugated resins are used. Many of such matrices are available in "kit" form, such as the Pharmacia GST purification system and the QIAexpressTM system (Qiagen) useful with (HIS₆) fusion partners. As another example, a fusion domain may be selected so as to facilitate detection of the ActRIIa polypeptides. Examples of such detection domains include the various fluorescent proteins (e.g., GFP) as well as "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain preferred embodiments, an ActRIIa polypeptide is fused with a domain that stabilizes the ActRIIa polypeptide in vivo (a "stabilizer" domain). By "stabilizing" is meant anything that increases serum half life, regardless of whether this is because of decreased destruction, decreased clearance by the kidney, or other pharmacokinetic effect. Fusions with the Fc portion of an immunoglobulin are known to confer desirable pharmacokinetic properties on a wide range of proteins. Constant domains from an immunoglobulin, particularly an IgG heavy chain, may also be used as stabilizing domains. Likewise, fusions to human serum albumin can confer desirable properties. Other types of fusion domains that may be selected include multimerizing (e.g., dimerizing,

tetramerizing) domains and functional domains (that confer an additional biological function, such as further stimulation of bone growth).

As a specific example, the present invention provides a fusion protein comprising a soluble extracellular domain of ActRIIa fused to an Fc domain. An example of an IgG1 Fc domain is shown below (SEQ ID NO: 6).

THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD (A) VSHEDPEVKFNWYVDG
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK (A) VSNKALPVPIEKTISKAK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSG
PFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN (A) HYTQKSLSLSPGK*

Optionally, the Fc domain has one or more mutations at residues such as Asp-265, lysine 322, and Asn-434. In certain cases, the mutant Fc domain having one or more of these mutations (e.g., Asp-265 mutation) has reduced ability of binding to the Fcγ receptor relative to a wildtype Fc domain. In other cases, the mutant Fc domain having one or more of these mutations (e.g., Asn-434 mutation) has increased ability of binding to the MHC class I-related Fc-receptor (FcRN) relative to a wildtype Fc domain. Fc domains from IgG2, IgG3 and IgG4 may also be used.

It is understood that different elements of the fusion proteins may be arranged in any manner that is consistent with the desired functionality. For example, an ActRIIa polypeptide may be placed C-terminal to a heterologous domain, or, alternatively, a heterologous domain may be placed C-terminal to an ActRIIa polypeptide. The ActRIIa polypeptide domain and the heterologous domain need not be adjacent in a fusion protein, and additional domains or amino acid sequences may be included C- or N-terminal to either domain or between the domains.

In certain embodiments, the ActRIIa polypeptides of the present invention contain one or more modifications that are capable of stabilizing the ActRIIa polypeptides. For example, such modifications enhance the in vitro half life of the ActRIIa polypeptides, enhance circulatory half life of the ActRIIa polypeptides or reducing proteolytic degradation of the ActRIIa polypeptides. Such stabilizing modifications include, but are not limited to, fusion proteins (including, for example, fusion proteins comprising an ActRIIa polypeptide and a stabilizer domain), modifications of a glycosylation site (including, for example, addition of a glycosylation site to an ActRIIa polypeptide), and modifications of carbohydrate

moiety (including, for example, removal of carbohydrate moieties from an ActRIIa polypeptide). As used herein, the term “stabilizer domain” not only refers to a fusion domain (e.g., Fc) as in the case of fusion proteins, but also includes nonproteinaceous modifications such as a carbohydrate moiety, or nonproteinaceous moiety, such as polyethylene glycol.

5 In certain embodiments, the present invention makes available isolated and/or purified forms of the ActRIIa polypeptides, which are isolated from, or otherwise substantially free of, other proteins. ActRIIa polypeptides will generally be produced by expression from recombinant nucleic acids.

10 3. Nucleic Acids Encoding ActRIIa Polypeptides

 In certain aspects, the invention provides isolated and/or recombinant nucleic acids encoding any of the ActRIIa polypeptides (e.g., soluble ActRIIa polypeptides), including fragments, functional variants and fusion proteins disclosed herein. For example, SEQ ID NO: 4 encodes the naturally occurring human ActRIIa precursor polypeptide, while SEQ ID
15 NO: 5 encodes the processed extracellular domain of ActRIIa. The subject nucleic acids may be single-stranded or double stranded. Such nucleic acids may be DNA or RNA molecules. These nucleic acids may be used, for example, in methods for making ActRIIa polypeptides or as direct therapeutic agents (e.g., in a gene therapy approach).

 In certain aspects, the subject nucleic acids encoding ActRIIa polypeptides are further
20 understood to include nucleic acids that are variants of SEQ ID NO: 4 or 5.

 In certain embodiments, the invention provides isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to SEQ ID NOs: 4 or 5. One of ordinary skill in the art will appreciate that nucleic acid sequences complementary to SEQ ID NOs: 4 or 5, and variants of SEQ ID NOs: 4 or 5 are also within
25 the scope of this invention. In further embodiments, the nucleic acid sequences of the invention can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

 In other embodiments, nucleic acids of the invention also include nucleotide sequences, and the ActRIIa polypeptides encoded by such nucleic acids, that hybridize under
30 highly stringent conditions to the nucleotide sequence designated in SEQ ID NOs: 4 or 5, complement sequence of SEQ ID NOs: 4 or 5 or fragments thereof. As discussed above, one

of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from the nucleic acids as set forth in SEQ ID NOs: 4 or 5 due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

In certain embodiments, the recombinant nucleic acids of the invention may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate to the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences.

Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

In certain aspects of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding an ActRIIa polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the ActRIIa polypeptide. Accordingly, the term regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding an ActRIIa polypeptide. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, RSV promoters, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

A recombinant nucleic acid of the invention can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells,

eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant ActRIIa polypeptide include plasmids and other vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids
5 for expression in prokaryotic cells, such as *E. coli*.

Some mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors
10 are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins
15 in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and in transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A*
20 *Laboratory Manual*, 3rd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 2001). In some instances, it may be desirable to express the recombinant polypeptides by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal
25 containing pBlueBac III).

In a preferred embodiment, a vector will be designed for production of the subject ActRIIa polypeptides in CHO cells, such as a Pcmv-Script vector (Stratagene, La Jolla, Calif.), pcDNA4 vectors (Invitrogen, Carlsbad, Calif.) and pCI-neo vectors (Promega, Madison, Wisc.). As will be apparent, the subject gene constructs can be used to cause
30 expression of the subject ActRIIa polypeptides in cells propagated in culture, e.g., to produce proteins, including fusion proteins or variant proteins, for purification.

This disclosure also pertains to a host cell transfected with a recombinant gene including a coding sequence (e.g., SEQ ID NO: 4 or 5) for one or more of the subject ActRIIa polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, an ActRIIa polypeptide of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present invention further pertains to methods of producing the subject ActRIIa polypeptides. For example, a host cell transfected with an expression vector encoding an ActRIIa polypeptide can be cultured under appropriate conditions to allow expression of the ActRIIa polypeptide to occur. The ActRIIa polypeptide may be secreted and isolated from a mixture of cells and medium containing the ActRIIa polypeptide.

Alternatively, the ActRIIa polypeptide may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art.

The subject ActRIIa polypeptides can be isolated from cell culture medium, host cells, or both, using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, immunoaffinity purification with antibodies specific for particular epitopes of the ActRIIa polypeptides and affinity purification with an agent that binds to a domain fused to the ActRIIa polypeptide (e.g., a protein A column may be used to purify an ActRIIa-Fc fusion). In a preferred embodiment, the ActRIIa polypeptide is a fusion protein containing a domain which facilitates its purification. In a preferred embodiment, purification is achieved by a series of column chromatography steps, including, for example, three or more of the following, in any order: protein A chromatography, Q sepharose chromatography, phenylsepharose chromatography, size exclusion chromatography, and cation exchange chromatography. The purification could be completed with viral filtration and buffer exchange. As demonstrated herein, ActRIIa-hFc protein was purified to a purity of >98% as determined by size exclusion chromatography and >95% as determined by SDS PAGE. This level of purity was sufficient to achieve desirable results in mice, rats, non-human primates and humans.

In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion

of the recombinant ActRIIa polypeptide, can allow purification of the expressed fusion protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified ActRIIa polypeptide (e.g., see Hochuli et al., (1987) *J. Chromatography* 411:177; and Janknecht et al., *PNAS USA* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

4. Alternative Activin and ActRIIa Antagonists

The data presented herein demonstrates that antagonists of activin-ActRIIa signaling can be used to increase red blood cell or hemoglobin levels. Although soluble ActRIIa polypeptides, and particularly ActRIIa-Fc, are preferred antagonists, and although such antagonists may affect red blood cell levels through a mechanism other than activin antagonism (e.g., activin inhibition may be an indicator of the tendency of an agent to inhibit the activities of a spectrum of molecules, including, perhaps, other members of the TGF-beta superfamily, and such collective inhibition may lead to the desired effect on hematopoiesis), other types of activin-ActRIIa antagonists are expected to be useful, including anti-activin (e.g., activin β_A , β_B , β_C and β_E) antibodies, anti-ActRIIa antibodies, antisense, RNAi or ribozyme nucleic acids that inhibit the production of ActRIIa, and other inhibitors of activin or ActRIIa, particularly those that disrupt activin-ActRIIa binding.

An antibody that is specifically reactive with an ActRIIa polypeptide (e.g., a soluble ActRIIa polypeptide) and which either binds competitively to ligand with the ActRIIa

polypeptide or otherwise inhibits ActRIIa-mediated signaling may be used as an antagonist of ActRIIa polypeptide activities. Likewise, an antibody that is specifically reactive with an activin β_A , β_B , β_C or β_E polypeptide, or any heterodimer thereof, and which disrupts ActRIIa binding may be used as an antagonist.

5 By using immunogens derived from an ActRIIa polypeptide or an activin polypeptide, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (see, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the activin or ActRIIa polypeptide, an antigenic fragment
10 which is capable of eliciting an antibody response, or a fusion protein. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an ActRIIa or activin polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or
15 other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

Following immunization of an animal with an antigenic preparation of an activin or ActRIIa polypeptide, antisera can be obtained and, if desired, polyclonal antibodies can be isolated from the serum. To produce monoclonal antibodies, antibody-producing cells
20 (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) *Nature*, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) *Immunology Today*, 4: 72), and the
25 EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an activin or ActRIIa polypeptide and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

30 The term "antibody" as used herein is intended to include whole antibodies, e.g., of any isotype (IgG, IgA, IgM, IgE, etc), and includes fragments or domains of immunoglobulins which are reactive with a selected antigen. Antibodies can be fragmented

using conventional techniques and the fragments screened for utility and/or interaction with a specific epitope of interest. Thus, the term includes segments of proteolytically-cleaved or recombinantly-prepared portions of an antibody molecule that are capable of selectively reacting with a certain protein. Non-limiting examples of such proteolytic and/or recombinant fragments include Fab, F(ab')₂, Fab' , Fv, and single chain antibodies (scFv) containing a V[L] and/or V[H] domain joined by a peptide linker. The scFv's may be covalently or non-covalently linked to form antibodies having two or more binding sites. The term antibody also includes polyclonal, monoclonal, or other purified preparations of antibodies and recombinant antibodies. The term "recombinant antibody", means an antibody, or antigen binding domain of an immunoglobulin, expressed from a nucleic acid that has been constructed using the techniques of molecular biology, such as a humanized antibody or a fully human antibody developed from a single chain antibody. Single domain and single chain antibodies are also included within the term "recombinant antibody".

In certain embodiments, an antibody of the invention is a monoclonal antibody, and in certain embodiments, the invention makes available methods for generating novel antibodies. For example, a method for generating a monoclonal antibody that binds specifically to an ActRIIa polypeptide or activin polypeptide may comprise administering to a mouse an amount of an immunogenic composition comprising the antigen polypeptide effective to stimulate a detectable immune response, obtaining antibody-producing cells (e.g., cells from the spleen) from the mouse and fusing the antibody-producing cells with myeloma cells to obtain antibody-producing hybridomas, and testing the antibody-producing hybridomas to identify a hybridoma that produces a monoclonal antibody that binds specifically to the antigen. Once obtained, a hybridoma can be propagated in a cell culture, optionally in culture conditions where the hybridoma-derived cells produce the monoclonal antibody that binds specifically to the antigen. The monoclonal antibody may be purified from the cell culture.

The adjective "specifically reactive with" as used in reference to an antibody is intended to mean, as is generally understood in the art, that the antibody is sufficiently selective between the antigen of interest (e.g., an activin or ActRIIa polypeptide) and other antigens that are not of interest that the antibody is useful for, at minimum, detecting the presence of the antigen of interest in a particular type of biological sample. In certain methods employing the antibody, such as therapeutic applications, a higher degree of specificity in binding may be desirable. Monoclonal antibodies generally have a greater

tendency (as compared to polyclonal antibodies) to discriminate effectively between the desired antigens and cross-reacting polypeptides. One characteristic that influences the specificity of an antibody:antigen interaction is the affinity of the antibody for the antigen. Although the desired specificity may be reached with a range of different affinities, generally
5 preferred antibodies will have an affinity (a dissociation constant) of about 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} M or less.

In addition, the techniques used to screen antibodies in order to identify a desirable antibody may influence the properties of the antibody obtained. For example, if an antibody is to be used for binding an antigen in solution, it may be desirable to test solution binding. A
10 variety of different techniques are available for testing interaction between antibodies and antigens to identify particularly desirable antibodies. Such techniques include ELISAs, surface plasmon resonance binding assays (e.g., the BiacoreTM binding assay, Biacore AB, Uppsala, Sweden), sandwich assays (e.g., the paramagnetic bead system of IGEN International, Inc., Gaithersburg, Maryland), western blots, immunoprecipitation assays, and
15 immunohistochemistry.

Examples of categories of nucleic acid compounds that are activin or ActRIIa antagonists include antisense nucleic acids, RNAi constructs and catalytic nucleic acid constructs. A nucleic acid compound may be single or double stranded. A double stranded compound may also include regions of overhang or non-complementarity, where one or the
20 other of the strands is single stranded. A single stranded compound may include regions of self-complementarity, meaning that the compound forms a so-called "hairpin" or "stem-loop" structure, with a region of double helical structure. A nucleic acid compound may comprise a nucleotide sequence that is complementary to a region consisting of no more than 1000, no more than 500, no more than 250, no more than 100, or no more than 50, 35, 25, 22, 20, 18 or
25 15 nucleotides of the full-length ActRIIa nucleic acid sequence or activin β_A , β_B , β_C , or β_E nucleic acid sequence. The region of complementarity will preferably be at least 8 nucleotides, and optionally about 18 to 35 nucleotides. A region of complementarity may fall within an intron, a coding sequence or a noncoding sequence of the target transcript, such as the coding sequence portion. Generally, a nucleic acid compound will have a length of about
30 8 to about 500 nucleotides or base pairs in length, and optionally the length will be about 14 to about 50 nucleotides. A nucleic acid may be a DNA (particularly for use as an antisense), RNA or RNA:DNA hybrid. Any one strand may include a mixture of DNA and RNA, as

well as modified forms that cannot readily be classified as either DNA or RNA. Likewise, a double stranded compound may be DNA:DNA, DNA:RNA or RNA:RNA, and any one strand may also include a mixture of DNA and RNA, as well as modified forms that cannot readily be classified as either DNA or RNA. A nucleic acid compound may include any of a variety of modifications, including one or modifications to the backbone (the sugar-phosphate portion in a natural nucleic acid, including internucleotide linkages) or the base portion (the purine or pyrimidine portion of a natural nucleic acid). An antisense nucleic acid compound will preferably have a length of about 15 to about 30 nucleotides and will often contain one or more modifications to improve characteristics such as stability in the serum, in a cell or in a place where the compound is likely to be delivered, such as the stomach in the case of orally delivered compounds and the lung for inhaled compounds. In the case of an RNAi construct, the strand complementary to the target transcript will generally be RNA or modifications thereof. The other strand may be RNA, DNA or any other variation. The duplex portion of double stranded or single stranded "hairpin" RNAi construct will generally have a length of 18 to 40 nucleotides in length and optionally about 21 to 23 nucleotides in length, so long as it serves as a Dicer substrate. Catalytic or enzymatic nucleic acids may be ribozymes or DNA enzymes and may also contain modified forms. Nucleic acid compounds may inhibit expression of the target by about 50%, 75%, 90% or more when contacted with cells under physiological conditions and at a concentration where a nonsense or sense control has little or no effect. Preferred concentrations for testing the effect of nucleic acid compounds are 1, 5 and 10 micromolar. Nucleic acid compounds may also be tested for effects on, for example, red blood cell levels.

In certain embodiments, an activin-ActRIIa antagonist may be a follistatin polypeptide that antagonizes activin bioactivity and/or binds to activin. The term "follistatin polypeptide" includes polypeptides comprising any naturally occurring polypeptide of follistatin as well as any variants thereof (including mutants, fragments, fusions, and peptidomimetic forms) that retain a useful activity, and further includes any functional monomer or multimer of follistatin. Variants of follistatin polypeptides that retain activin binding properties can be identified based on previous studies involving follistatin and activin interactions. For example, WO2008/030367 discloses specific follistatin domains ("FSDs") that are shown to be important for activin binding. As shown below in SEQ ID NOs: 19-21, the N-terminus follistatin domain ("FSND" SEQ ID NO: 19), FSD2 (SEQ ID NO: 20), and to

a lesser extent FSD1 (SEQ ID NO: 21) represent exemplary domains within follistatin important for activin binding. In addition, methods for making and testing libraries of polypeptides are described above in the context of ActRIIa polypeptides and such methods also pertain to making and testing variants of follistatin. Follistatin polypeptides include polypeptides derived from the sequence of any known follistatin having a sequence at least about 80% identical to the sequence of a follistatin polypeptide, and optionally at least 85%, 90%, 95%, 97%, 99% or greater identity. Examples of follistatin polypeptides include the mature follistatin polypeptide or shorter isoforms or other variants of the human follistatin precursor polypeptide (SEQ ID NO: 17) as described, for example, in WO2005/025601.

The human follistatin precursor polypeptide isoform FST344 is as follows:

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTEL
 SKEECCSTGRLSTSWTEEDVNDNTLFKWMI FNGGAPNCIPCKETCENVDC
 GPGKKCRMNKKNKPRCVCAPDCSNITWKG P VCGLDGKTYRNECALLKARC
 KEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAYCVTCNRICPEPA
 SSEQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQC
 TGGKKCLWDFKVGGRGCSLCDELCPDSKSDEPVCASDNATYASECAMKEA
 ACSSGVLLLEVKGHSCN**SISEDTEEEEEEDEDQDYSFPISILEW** (SEQ ID
 NO: 17; NP_037541.1 FOLLISTATIN ISOFORM FST344)

The signal peptide is single underlined; the last 27 residues in bold represent additional amino acids as compared to a shorter follistatin isoform FST317 (NP_006341) below.

The human follistatin precursor polypeptide isoform FST317 is as follows:

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTEL
 SKEECCSTGRLSTSWTEEDVNDNTLFKWMI FNGGAPNCIPCKETCENVDC
 GPGKKCRMNKKNKPRCVCAPDCSNITWKG P VCGLDGKTYRNECALLKARC
 KEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAYCVTCNRICPEPA
 SSEQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQC
 TGGKKCLWDFKVGGRGCSLCDELCPDSKSDEPVCASDNATYASECAMKEA
 ACSSGVLLLEVKGHSGSCN (SEQ ID NO: 18)

The signal peptide is single underlined.

N-terminus follistatin domain (FSND) sequence is as follows:

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWM
IFNGGAPNCIPCK (SEQ ID NO: 19; FSND)

The FSD1 and FSD2 sequences are as follows:

5 ETCENVDCGPGKKCRMNKKNKPRCV (SEQ ID NO: 20; FSD1)
KTCRDVFCPGSSSTCVVDQTNNAYCVT (SEQ ID NO: 21; FSD2)

In other embodiments, an activin-ActRIIa antagonist may be a follistatin-like related gene (FLRG) that antagonizes activin bioactivity and/or binds to activin. The term "FLRG polypeptide" includes polypeptides comprising any naturally occurring polypeptide of FLRG as well as any variants thereof (including mutants, fragments, fusions, and peptidomimetic forms) that retain a useful activity. Variants of FLRG polypeptides that retain activin binding properties can be identified using routine methods to assay FLRG and activin interactions. See, for example, US 6,537,966. In addition, methods for making and testing libraries of polypeptides are described above in the context of ActRIIa polypeptides and such methods also pertain to making and testing variants of FLRG. FLRG polypeptides include polypeptides derived from the sequence of any known FLRG having a sequence at least about 80% identical to the sequence of an FLRG polypeptide, and optionally at least 85%, 90%, 95%, 97%, 99% or greater identity.

The human FLRG precursor polypeptide is as follows:

20 MRPGAPGPLWPLPWGALAWAVGFVSSMSGSGNPAPGGVCWLQQGQEATCSL
VLQTDVTRAECASGNIDTAWSNLTHPGNKINLLGFLGLVHCLPCKDSCD
GVECGPGKACRMLGGRPRCECAPDCSGLPARLQVCGSDGATYRDECELRA
ARCRGHPDLSVMYRGRCRKSCHEHVVCPRPQSCVVDQTGSAHCVVCRAAPC
VPSSPGQELCGNNNVTYISSCHMRQATCFLGRSIGVRHAGSCAGTPEEPP
25 GGESAEEEENFV (SEQ ID NO: 22; NP_005851)

The signal peptide is single underlined.

In certain embodiments, functional variants or modified forms of the follistatin polypeptides and FLRG polypeptides include fusion protein having at least a portion of the follistatin polypeptides or FLRG polypeptides and one or more fusion domains, such as, for example, domains that facilitate isolation, detection, stabilization or multimerization of the polypeptide. Suitable fusion

domains are discussed in detail above with reference to the ActRIIa polypeptides.

In one embodiment, an activin-ActRIIa antagonist is a fusion protein comprising an activin binding portion of a follistatin polypeptide fused to an Fc domain. In another embodiment, an activin-ActRIIa antagonist is a fusion protein comprising
5 an activin binding portion of an FLRG polypeptide fused to an Fc domain.

Follistatin and FLRG have been shown in the literature, and by the applicants with respect to FLRG, to have affinities for Activin A in the picomolar range, indicating that these agents will inhibit activin A signaling to a similar degree as ActRIIa-Fc.

10

5. Exemplary Therapeutic Methods

In certain embodiments, the present invention provides methods for managing a patient that has been treated with, or is a candidate to be treated with, an activin-ActRIIa antagonist by measuring one or more hematologic parameters in the patient. The
15 hematologic parameters may be used to evaluate appropriate dosing for a patient who is a candidate to be treated with an activin-ActRIIa antagonist, to monitor the hematologic parameters during treatment with an activin-ActRIIa antagonist, to evaluate whether to adjust the dosage during treatment with an activin-ActRIIa antagonist, and/or to evaluate an appropriate maintenance dose of an activin-ActRIIa antagonist. If one or more of the
20 hematologic parameters are outside the normal level, dosing with the activin-ActRIIa antagonist may be reduced, delayed or terminated.

Hematologic parameters that may be measured in accordance with the methods provided herein include, for example, red blood cell levels, blood pressure, iron stores, and other agents found in bodily fluids that correlate with increased red blood cell levels, using
25 art recognized methods. Such parameters may be determined using a blood sample from a patient. Increases in red blood cell levels, hemoglobin levels, and/or hematocrit levels may cause increases in blood pressure.

Red blood cell levels may be determined, for example, by determining a red blood cell count, by measuring hemoglobin levels or by measuring hematocrit levels. A red blood
30 cell count may be determined using a commercially available Coulter Counter. The normal range for a red blood cell count is between 4.2-5.9 million cells/cm, although individual

variations should be taken into account. Hemoglobin levels may be determined by lysing the red blood cells, converting the hemoglobin into cyanomethemoglobin and measuring the amount of hemoglobin with a colorimeter. The normal ranges for hemoglobin are 14-18 gm/dl for adult males and 12-16 gm-dl for adult females, although individual variations should be taken into account. Hematocrit (Hct) or packed cell volume (PCV) refers to the ratio of the volume of red blood cells to the volume of whole blood. Hematocrit may be determined, for example, by centrifugation of a blood sample followed by analysis of the layers produced. Normal ranges for hematocrit are approximately 41-51% for men and 35-45% for women, although individual variations should be taken into account.

Blood pressure, including systolic blood pressure, diastolic blood pressure, or mean arterial blood pressure, may be determined using art recognized methods. Blood pressure is most commonly measured using a sphygmomanometer. Typical values for a resting, healthy adult human are approximately 120 mmHg systolic and 80 mmHg diastolic, although individual variations should be taken into account. Individuals suffering from hypertension typically have a blood pressure ≥ 140 mmHg systolic and ≥ 90 diastolic blood pressure. Individuals having a level above normal but less than 140/90 mmHg are generally referred to as prehypertensive. Additional methods for measuring blood pressure may be found in Pickering et al., Hypertension 45: 142-161 (2005).

Iron stores may be measured using a variety of art recognized techniques including, for example, by determining levels of one or more of the following: serum ferritin (SF), transferrin saturation (TSAT), total iron binding capacity, hemoglobin concentration, zinc protoporphyrin, mean cell volume (MCV), or transferrin receptor in serum. Serum ferritin levels may be determined, for example, using an immunoassay such as an enzyme-linked immunosorbent assay (ELISA) or immunoturbidometry. In normal patients, serum ferritin levels range from 13 to 220 ng/mL, although individual variations should be taken into account. Transferrin saturation levels represent the proportion of transferrin bound to iron and may be determined by dividing serum iron by total iron binding capacity (TIBC). In normal patients, transferrin saturation levels range from 20% to 40%, although individual variations should be taken into account. Serum iron may be determined using colorimetry and is expressed as ug/dl or umol/l. Total iron binding capacity reflects the total capacity of circulating transferrin bound to iron and may be determined using a colorimetric assay to determine the amount of iron that can be bound to unsaturated transferrin in vitro. The

normal range of total iron binding capacity is about 250-450 ug/dl, although individual variations should be taken into account. Additional information about measuring iron stores may be found in the World Health Organization report entitled Assessing the Iron Status of Populations dated April 2004 and in Yamanishi et al., Clinical Chemistry 48: 1565-1570 (2002).

In one embodiment, if one or more hematologic parameters are outside the normal range, or on the high side of normal, in a patient who is a candidate to be treated with an activin-ActRIIa antagonist then onset of administration of the activin-ActRIIa antagonist may be delayed until the hematologic parameters have returned to a normal or acceptable level either naturally or via therapeutic intervention. For example, if a candidate patient is hypertensive or prehypertensive, then the patient may be treated with a blood pressure lowering agent in order to reduce the patient's blood pressure. Any blood pressure lowering agent appropriate for the individual patient's condition may be used including, for example, diuretics, adrenergic inhibitors (including alpha blockers and beta blockers), vasodilators, calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors, or angiotensin II receptor blockers. Blood pressure may alternatively be treated using a diet and exercise regimen. Similarly, if a candidate patient has iron stores that are lower than normal, or on the low side of normal, then the patient may be treated with an appropriate regimen of diet and/or iron supplements until the patient's iron stores have returned to a normal or acceptable level. For patients having higher than normal red blood cell levels and/or hemoglobin levels, then administration of the activin-ActRIIa antagonist may be delayed until the levels have returned to a normal or acceptable level.

In certain embodiments, if one or more hematologic parameters are outside the normal range, or on the high side of normal, in a patient who is a candidate to be treated with an activin-ActRIIa antagonist then the onset of administration may be not be delayed. However, the dosage amount or frequency of dosing of the activin-ActRIIa antagonist may be set at an amount that would reduce the risk of an unacceptable increase in the hematologic parameters arising upon administration of the activin-ActRIIa antagonist. Alternatively, a therapeutic regimen may be developed for the patient that combines an activin-ActRIIa antagonist with a therapeutic agent that addresses the undesirable level of the hematologic parameter. For example, if the patient has elevated blood pressure, then a therapeutic regimen involving administration of an activin-ActRIIa antagonist and a blood pressure

lowering agent may be designed. For a patient having lower than desired iron stores, a therapeutic regimen of an activin-ActRIIa antagonist and iron supplementation may be developed.

5 In one embodiment, baseline parameter(s) for one or more hematologic parameters may be established for a patient who is a candidate to be treated with an activin-ActRIIa antagonists and an appropriate dosing regimen establish for that patient based on the baseline value(s). Alternatively, established baseline parameters based on a patient's medical history could be used to inform an appropriate activin-ActRIIa antagonist dosing regimen for a patient. For example, if a healthy patient has an established baseline blood pressure reading
10 that is above the defined normal range it may not be necessary to bring the patient's blood pressure into the range that is considered normal for the general population prior to treatment with the activin-ActRIIa antagonist. A patient's baseline values for one or more hematologic parameters prior to treatment with an activin-ActRIIa antagonist may also be used as the relevant comparative values for monitoring any changes to the hematologic parameters
15 during treatment with the activin-ActRIIa antagonist.

In certain embodiments, one or more hematologic parameters are measured in patients who are being treated with an activin-ActRIIa antagonist. The hematologic parameters may be used to monitor the patient during treatment and permit adjustment or termination of the dosing with the activin-ActRIIa antagonist or additional dosing with another therapeutic
20 agent. For example, if administration of an activin-ActRIIa antagonist results in an increase in blood pressure, red blood cell level, or hemoglobin level, or a reduction in iron stores, then the dose of the activin-ActRIIa antagonists may be reduced in amount or frequency in order to decrease the effects of the activin-ActRIIa antagonist on the one or more hematologic parameters. If administration or an activin-ActRIIa antagonist results in a change in one or
25 more hematologic parameters that is adverse to the patient, then the dosing of the activin-ActRIIa antagonist may be terminated either temporarily, until the hematologic parameter(s) return to an acceptable level, or permanently. Similarly, if one or more hematologic parameters are not brought within an acceptable range after reducing the dose or frequency of administration of the activin-ActRIIa antagonist then the dosing may be terminated. As an
30 alternative, or in addition to, reducing or terminating the dosing with the activin-ActRIIa antagonist, the patient may be dosed with an additional therapeutic agent that addresses the undesirable level in the hematologic parameter(s), such as, for example, a blood pressure

lowering agent or an iron supplement. For example, if a patient being treated with an activin-ActRIIa antagonist has elevated blood pressure, then dosing with the activin-ActRIIa antagonist may continue at the same level and a blood pressure lowering agent is added to the treatment regimen, dosing with the activin-ActRIIa antagonist may be reduced (e.g., in amount and/or frequency) and a blood pressure lowering agent is added to the treatment regimen, or dosing with the activin-ActRIIa antagonist may be terminated and the patient may be treated with a blood pressure lowering agent.

In certain embodiments, if a patient being treated with an activin-ActRIIa antagonist or a patient who is a candidate for treatment with an activin-ActRIIa antagonist has one or more of the following: a hemoglobin level greater than 12 g/dl, a hemoglobin level greater than 15 g/dl, a blood pressure $\geq 120/80$ mmHg, a blood pressure $\geq 140/90$ mmHg, a transferrin saturation level less than 20%, and/or a ferritin level less than 100 ng/ml, then dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated. As an alternative, or in addition to, reducing, delaying or terminating dosing with activin-ActRIIa antagonist, a therapeutic agent that addresses the undesired level of one or more hematologic parameters (such as a blood pressure lowering agent or an iron supplement) may be administered to the patient.

In one embodiment, the present invention provides a method for dosing a patient with an activin-ActRIIa antagonist by administering to the patient an activin-ActRIIa antagonist in an amount and at a frequency which reduces the risk of causing a rise in hemoglobin levels greater than 1 g/dl over a two week period. The methods may comprise measuring one or more hematologic parameters either before beginning administration of the activin-ActRIIa antagonist and/or during administration of the activin-ActRIIa antagonist. The initial dose of the activin-ActRIIa antagonist may be set so as to reduce the risk of causing a rise in hemoglobin levels greater than 1 g/dl over a two week period. In addition, the dose may be adjusted over time to in order to maintain a reduced risk of causing a rise in hemoglobin levels greater than 1 g/dl in two weeks.

In certain embodiments, the present invention provides a method for administering an ActRIIa-Fc fusion protein to a patient by administering the ActRIIa fusion protein no more frequently than once per 60 days, once per 90 days, or once per 120 days, or 1-6 times per year, 2-6 times per year, 1-5 times per year, 2-5 times per year, 1-4 times per year, 2-4 times per year, 1-3 times per year, or 2-3 times per year. As demonstrated herein, increases in red blood cell levels arising from administration peak around 60 days after administration. At

about 90 days after administration, a significant reduction in red blood cell levels is seen and after about 120 days red blood cell levels return to the baseline level. Accordingly, for patients in which the activin-ActRIIa antagonist is being administered for purposes other than increasing red blood cell levels, it may be desirable to administer subsequent doses of the
5 activin-ActRIIa antagonist after the peak increase in red blood cell levels from the previous dose, or even after red blood cell levels have returned to normal.

In certain embodiments, the invention provides methods for determining dosing and monitoring therapeutic progress with activin-ActRIIa antagonist treatment in patients in which the activin-ActRIIa antagonist is being administered to increase red blood cell levels.

10 The methods involve determining one or more hematologic parameters either prior to beginning dosing with the activin-ActRIIa antagonist and/or during treatment with the activin-ActRIIa antagonist. For example, one or more hematologic parameters may be determined in a patient who is a candidate for administration of an activin-ActRIIa antagonist for increasing blood cell levels to facilitate determination of dosage amount and frequency.

15 One or more hematologic parameters may also be determined in a patient being treated with an activin-ActRIIa antagonist for purposes of increasing red blood cell levels in order to monitor progress of the treatment, facilitate dosing adjustments, and to determine maintenance dosing levels, etc.

In accordance with the methods of the invention, one or more hematologic parameters
20 may be measured at various time points and at varying frequencies as needed for an individual patient based on various factors such as a patient's baseline levels, responsiveness to treatment with an activin-ActRIIa antagonist, general health, age, sex, weight, etc.

Measuring of one or more hematologic parameters may be carried out before and/or during treatment with an activin-ActRIIa antagonist. If conducting multiple measurements of

25 hematologic parameters at various time points, the same set of hematologic parameter(s) need not be measured at each time point. Similarly, the same test for an individual parameter need not be used at each time point. Appropriate hematologic parameters and tests for such parameters may be chosen for an individual taking into account factors specific to the given individual. Testing of hematologic parameters may occur as frequently as needed for a given
30 individual, such as, for example, once per day, once per week, once per every two weeks, once per month, once per each 2 month period, once per each 3 month period, once per each 6 month period, or once per year. In addition, the frequency of testing may vary over time.

For example, upon initial dosing of an individual it may be desirable to test for one or more hematologic parameters more frequently, e.g., once per day, once per week, once per every two weeks, or once per month, and then decrease the frequency of testing over time, e.g., after one month, two months, six months, 1 year, two years, or longer, of treatment, the frequency of testing may reduced to, for example, once per month, once per every two months, once per every three months, once per every six months, or once per year. Similarly, it may be desirable to test more frequently when adjusting a patient's dose of an activin-ActRIIa antagonist (e.g., either amount or frequency of administration) and then decrease the frequency of testing over time, for example, once the patient's response to the activin-ActRIIa antagonist has been established.

In various embodiments, patients being treated with an activin-ActRIIa antagonist, or candidate patients for treatment with an activin-ActRIIa antagonist, may be mammals such as rodents and primates, and particularly human patients.

In certain embodiments, patients being treated with an activin-ActRIIa antagonist, or candidate patients to be treated with an activin-ActRIIa antagonist, are patients in need of bone and/or cartilage formation, prevention of bone loss, increased bone mineralization or prevention of bone demineralization, such as patients with low bone density, decreased bone strength, or bone damage due to breakage, loss or demineralization. In exemplary embodiments, the patients or candidate patients may be patients suffering from, or at risk for developing, osteoporosis (including secondary osteoporosis), hyperparathyroidism, Cushing's disease, Paget's disease, thyrotoxicosis, chronic diarrheal state or malabsorption, renal tubular acidosis, or anorexia nervosa. Osteoporosis resulting from drugs or another medical condition is known as secondary osteoporosis. Medications that can cause secondary osteoporosis include, for example, corticosteroids, methotrexate (Rheumatrex, Immunex, Folex PFS), cyclosporine (Sandimmune, Neoral), luteinizing hormone-releasing hormone agonists (Lupron, Zoladex), and heparin (Calciparine, Liquaemin). Bone loss resulting from cancer therapy is widely recognized and termed cancer therapy induced bone loss (CTIBL).

In certain embodiments, patients being treated with an activin-ActRIIa antagonist, or candidate patients to be treated with an activin-ActRIIa antagonist, are patients suffering from or at high risk for developing breast cancer. As every woman is at risk for developing breast cancer, a woman with a high risk for developing breast cancer is a woman whose risk factors confer a greater probability of developing the disease compared to the general

population or the population of women within a certain age group. Exemplary risk factors include age, family history or genetic makeup, lifestyle habits such as exercise and diet, exposure to radiation or other cancer-causing agents, age at the time the first child was born, genetic changes, and weight gain after menopause. Exemplary patients include, for example, patients that have mutations in the BRCA1/2 genes or other genes shown to predispose women to breast and ovarian cancer are also included. Patients also include individuals with solid tumors, metastatic cancer, precancerous lesions of the breast, benign lesions of the breast, or with any abnormal proliferative lesions including typical hyperplasia, atypical hyperplasia, and noninvasive or in situ carcinoma. Patients also include those with both hormone-dependent or hormone-responsive cancers (e.g., estrogen receptor positive cancers) and hormone-independent cancers (e.g., estrogen receptor negative or estrogen receptor mutant cancers). Patients suffering from cancers in which growth factors or oncogenes are activated (e.g., breast cancers in which c-*erbB*-2 (also known as *HER-2/Neu*) tyrosine kinase is expressed) are also contemplated.

In certain embodiments, patients being treated with an activin-ActRIIa antagonist, or candidate patients to be treated with an activin-ActRIIa antagonist, are patients with undesirably low red blood cell or hemoglobin levels, such as patients having an anemia, and those that are at risk for developing undesirably low red blood cell or hemoglobin levels, such as those patients that are about to undergo major surgery or other procedures that may result in substantial blood loss, such as having blood drawn and stored for a later transfusion. Patients and candidate patients may also include those patients in need of an increase in red blood cells and/or hemoglobin levels that do not respond well to Epo. When observing hemoglobin levels in humans, a level of less than normal for the appropriate age and gender category may be indicative of anemia, although individual variations are taken into account. Potential causes of anemia include blood-loss, nutritional deficits, medication reaction, various problems with the bone marrow and many diseases. More particularly, anemia has been associated with a variety of disorders that include, for example, chronic renal failure, myelodysplastic syndrome, rheumatoid arthritis, bone marrow transplantation. Anemia may also be associated with the following conditions: solid tumors (e.g. breast cancer, lung cancer, colon cancer); tumors of the lymphatic system (e.g. chronic lymphocyte leukemia, non-Hodgkins and Hodgkins lymphomas); tumors of the hematopoietic system (eg. leukemia, myelodysplastic syndrome, multiple myeloma); radiation therapy; chemotherapy (e.g.

platinum containing regimens); inflammatory and autoimmune diseases, including, but not limited to, rheumatoid arthritis, other inflammatory arthritides, systemic lupus erythematosus (SLE), acute or chronic skin diseases (e.g. psoriasis), inflammatory bowel disease (e.g. Crohn's disease and ulcerative colitis); acute or chronic renal disease or failure including
5 idiopathic or congenital conditions; acute or chronic liver disease; acute or chronic bleeding; situations where transfusion of red blood cells is not possible due to patient allo- or auto-antibodies and/or for religious reasons (e.g. some Jehovah's Witnesses); infections (e.g. malaria, osteomyelitis); hemoglobinopathies, including, for example, sickle cell disease, thalassemias; drug use or abuse, e.g. alcohol misuse; pediatric patients with anemia from any
10 cause to avoid transfusion; and elderly patients or patients with underlying cardiopulmonary disease with anemia who cannot receive transfusions due to concerns about circulatory overload.

As used herein, a therapeutic that "prevents" a disorder or condition refers to a compound that, in a statistical sample, reduces the occurrence of the disorder or condition in
15 the treated sample relative to an untreated control sample, or delays the onset or reduces the severity of one or more symptoms of the disorder or condition relative to the untreated control sample. The term "treating" as used herein includes prophylaxis of the named condition or amelioration or elimination of the condition once it has been established. In either case, prevention or treatment may be discerned in the diagnosis provided by a
20 physician or other health care provider and the intended result of administration of the therapeutic agent.

6. Pharmaceutical Compositions

In certain embodiments, activin-ActRIIa antagonists (e.g., ActRIIa polypeptides) of
25 the present invention are formulated with a pharmaceutically acceptable carrier. For example, an ActRIIa polypeptide can be administered alone or as a component of a pharmaceutical formulation (therapeutic composition). The subject compounds may be formulated for administration in any convenient way for use in human or veterinary medicine.

30 In certain embodiments, the therapeutic method of the invention includes administering the composition systemically, or locally as an implant or device. When

administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Therapeutically useful agents other than the activin-ActRIIa antagonists which may also optionally be included in the composition as described above, may be administered simultaneously or sequentially with the subject compounds (e.g.,
5 ActRIIa polypeptides) in the methods of the invention.

Typically, activin-ActRIaI antagonists will be administered parenterally. Pharmaceutical compositions suitable for parenteral administration may comprise one or more ActRIIa polypeptides in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or
10 sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as
15 glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

20 Further, the composition may be encapsulated or injected in a form for delivery to a target tissue site (e.g., bone marrow). In certain embodiments, compositions of the present invention may include a matrix capable of delivering one or more therapeutic compounds (e.g., ActRIIa polypeptides) to a target tissue site (e.g., bone marrow), providing a structure for the developing tissue and optimally capable of being resorbed into the body. For
25 example, the matrix may provide slow release of the ActRIIa polypeptides. Such matrices may be formed of materials presently in use for other implanted medical applications.

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
30 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 above mentioned 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.

In certain embodiments, methods of the invention can be administered for orally, e.g., in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of an agent as an active ingredient. An agent may also be administered as a bolus, electuary or paste.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), one or more therapeutic compounds of the present invention may be mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose, and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming, and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol, and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

The compositions of the invention may also contain adjuvants, such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

It is understood that the dosage regimen will be determined by the attending physician considering various factors which modify the action of the subject compounds of the invention (e.g., ActRIIa polypeptides). The various factors include, but are not limited to, the patient's red blood cell count, hemoglobin level or other diagnostic assessments, the desired target red blood cell count, the patient's age, sex, and diet, the severity of any disease that may be contributing to a depressed red blood cell level, time of administration, and other clinical factors. The addition of other known growth factors to the final composition may also affect the dosage. Progress can be monitored by periodic assessment of red blood cell and hemoglobin levels, as well as assessments of reticulocyte levels and other indicators of the hematopoietic process.

Experiments with primates and humans have demonstrated that effects of ActRIIa-Fc on red blood cell levels are detectable when the compound is dosed at intervals and amounts sufficient to achieve serum concentrations of about 100 ng/ml or greater, for a period of at least about 20 to 30 days. Dosing to obtain serum levels of 200 ng/ml, 500 ng/ml, 1000
5 ng/ml or greater for a period of at least 20 to 30 days may also be used. Bone effects can be observed at serum levels of about 200 ng/ml, with substantial effects beginning at about 1000 ng/ml or higher, over a period of at least about 20 to 30 days. Thus, if it is desirable to achieve effects on red blood cells while having little effect on bone, a dosing scheme may be designed to deliver a serum concentration of between about 100 and 1000 ng/ml over a
10 period of about 20 to 30 days. Alternatively, if it is desirable to achieve effects on bone, breast cancer, etc., while having little effect on, or reducing effects on red blood cell levels, a dosing scheme may be designed to deliver a dosing scheme of between about 100 and 1000 ng/ml with a dosing frequency that occurs less than once every 60 days, once every 90 days, or once every 120 days. In humans, serum levels of 200 ng/ml may be achieved with a single
15 dose of 0.1 mg/kg or greater and serum levels of 1000 ng/ml may be achieved with a single dose of 0.3 mg/kg or greater. The observed serum half-life of the molecule is between about 20 and 30 days, substantially longer than most Fc fusion proteins, and thus a sustained effective serum level may be achieved, for example, by dosing with about 0.05 to 0.5 mg/kg on a weekly or biweekly basis, or higher doses may be used with longer intervals between
20 dosings. For example, doses of 0.1 to 1 mg/kg might be used on a monthly or bimonthly basis.

In certain embodiments, the present invention also provides gene therapy for the in vivo production of ActRIIa polypeptides. Such therapy would achieve its therapeutic effect by introduction of the ActRIIa polynucleotide sequences into cells or tissues having the
25 disorders as listed above. Delivery of ActRIIa polynucleotide sequences can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Preferred for therapeutic delivery of ActRIIa polynucleotide sequences is the use of targeted liposomes.

Various viral vectors which can be utilized for gene therapy as taught herein include
30 adenovirus, herpes virus, vaccinia, or an RNA virus such as a retrovirus. The retroviral vector may be a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine

leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

5 Retroviral vectors can be made target-specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody. Those of skill in the art will recognize that specific polynucleotide sequences can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector containing the ActRIIa polynucleotide.

10 Alternatively, tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes gag, pol and env, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for ActRIIa polynucleotides is a colloidal dispersion
15 system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles in vitro and in vivo. RNA, DNA and intact virions can be encapsulated within the aqueous
20 interior and be delivered to cells in a biologically active form (see e.g., Fraley, et al., Trends Biochem. Sci., 6:77, 1981). Methods for efficient gene transfer using a liposome vehicle, are known in the art, see e.g., Mannino, et al., Biotechniques, 6:682, 1988. The composition of the liposome is usually a combination of phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical
25 characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebroside, and gangliosides. Illustrative
30 phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine. The targeting of liposomes is also possible based on, for example, organ-specificity, cell-specificity, and organelle-specificity and is known in the art.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain embodiments and embodiments of the present invention, and are not intended to limit the invention.

Example 1: ActRIIa-Fc Fusion Proteins

Applicants constructed a soluble ActRIIa fusion protein that has the extracellular domain of human ActRIIa fused to a human or mouse Fc domain with a minimal linker in between. The constructs are referred to as ActRIIa-hFc and ActRIIa-mFc, respectively.

ActRIIa-hFc is shown below as purified from CHO cell lines (SEQ ID NO: 7):

ILGRSETQECLFFNANWEKDRTNQTGVEPCYGDKDKRRHCFATWKNISGSIEIVKQG
CWLDDINCYDRTDCVEKKDSPEVYFCCCEGNMCNEKFSYFPEMEVTQPTSNPVT
PKPPTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
VPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTTPVLDSGDSFLYSLKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK

The ActRIIa-hFc and ActRIIa-mFc proteins were expressed in CHO cell lines. Three different leader sequences were considered:

- (i) Honey bee mellitin (HBML): MKFLVNVALVFMVVYISYIYA (SEQ ID NO: 8)
- (ii) Tissue Plasminogen Activator (TPA): MDAMKRGLCCVLLLCGAVFVSP (SEQ ID NO: 9)
- (iii) Native: MGAAAKLAFAVFLISCSSGA (SEQ ID NO: 10).

The selected form employs the TPA leader and has the following unprocessed amino acid sequence:

MDAMKRGLCCVLLLCGAVFVSPGAAILGRSETQECLFFNANWEKDRTNQTGVEPCY
GDKDKRRHCFATWKNISGSIEIVKQGCWLDDINCYDRTDCVEKKDSPEVYFCCCEG
NMCNEKFSYFPEMEVTQPTSNPVT
PKPPTGGGTHTCPPCPAPELLGGPSVFLFPPKPK

DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
 VLTVLHQDWLNGKEYKCKVSNKALPVPIEKTISKAKGQPREPQVYTLPPSREEMTKN
 QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQ
 QGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:13)

5 This polypeptide is encoded by the following nucleic acid sequence:

ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCT
 TCGTTTCGCCCCGGCGCCGCTATACTTGGTAGATCAGAACTCAGGAGTGTCTTTT
 TTTAATGCTAATTGGGAAAAAGACAGAACCAATCAAACCTGGTGTGTAACCGTGT
 ATGGTGACAAAGATAAACGGCGGCATTGTTTTGCTACCTGGAAGAATATTTCTGG
 10 TTCCATTGAATAGTGAAACAAGGTTGTTGGCTGGATGATATCAACTGCTATGACA
 GGACTGATTGTGTAGAAAAAAAAGACAGCCCTGAAGTATATTTCTGTTGCTGTGA
 GGGCAATATGTGTAATGAAAAGTTTTCTTATTTTCCGGAGATGGAAGTCACACAG
 CCCACTTCAAATCCAGTTACACCTAAGCCACCCACCGGTGGTGGAACCTCACACAT
 GCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCC
 15 CCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTG
 GTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC
 GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAG
 CACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGC
 AAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGTCCCCATCGAGAAA
 20 ACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCC
 CCATCCCGGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTCAAA
 GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAG
 AACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCT
 ATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCAT
 25 GCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCT
 GTCTCCGGGTAAATGAGAATTC (SEQ ID NO:14)

Both ActRIIa-hFc and ActRIIa-mFc were remarkably amenable to recombinant expression. As shown in figure 1, the protein was purified as a single, well-defined peak of protein. N-terminal sequencing revealed a single sequence of -ILGRSTQE (SEQ ID NO: 30 11). Purification could be achieved by a series of column chromatography steps, including, for example, three or more of the following, in any order: protein A chromatography, Q sepharose chromatography, phenylsepharose chromatography, size exclusion

chromatography, and cation exchange chromatography. The purification could be completed with viral filtration and buffer exchange. The ActRIIa-hFc protein was purified to a purity of >98% as determined by size exclusion chromatography and >95% as determined by SDS PAGE.

5 ActRIIa-hFc and ActRIIa-mFc showed a high affinity for ligands, particularly activin A. GDF-11 or Activin A ("ActA") were immobilized on a Biacore CM5 chip using standard amine coupling procedure. ActRIIa-hFc and ActRIIa-mFc proteins were loaded onto the system, and binding was measured. ActRIIa-hFc bound to activin with a dissociation constant (K_D) of 5×10^{-12} , and the protein bound to GDF11 with a K_D of 9.96×10^{-9} . See figure 10 2. ActRIIa-mFc behaved similarly.

The ActRIIa-hFc was very stable in pharmacokinetic studies. Rats were dosed with 1 mg/kg, 3 mg/kg or 10 mg/kg of ActRIIa-hFc protein and plasma levels of the protein were measured at 24, 48, 72, 144 and 168 hours. In a separate study, rats were dosed at 1 mg/kg, 10 mg/kg or 30 mg/kg. In rats, ActRIIa-hFc had an 11-14 day serum half life and circulating 15 levels of the drug were quite high after two weeks (11 μ g/ml, 110 μ g/ml or 304 μ g/ml for initial administrations of 1 mg/kg, 10 mg/kg or 30 mg/kg, respectively.) In cynomolgus monkeys, the plasma half life was substantially greater than 14 days and circulating levels of the drug were 25 μ g/ml, 304 μ g/ml or 1440 μ g/ml for initial administrations of 1 mg/kg, 10 mg/kg or 30 mg/kg, respectively. Preliminary results in humans suggests that the serum half 20 life is between about 20 and 30 days.

Example 2: Characterization of an ActRIIa-hFc Protein

ActRIIa-hFc fusion protein was expressed in stably transfected CHO-DUKX B11 cells from a pAID4 vector (SV40 ori/enhancer, CMV promoter), using a tissue plasminogen 25 leader sequence of SEQ ID NO:9. The protein, purified as described above in Example 1, had a sequence of SEQ ID NO:7. The Fc portion is a human IgG1 Fc sequence, as shown in SEQ ID NO:7. Sialic acid analysis showed that the protein contained, on average, between about 1.5 and 2.5 moles of sialic acid per molecule of ActRIIa-hFc fusion protein.

This purified protein showed a remarkably long serum half-life in all animals tested, 30 including a half-life of 25-32 days in human patients (see Example 6, below). Additionally, the CHO cell expressed material has a higher affinity for activin B ligand than that reported

for an ActRIIa-hFc fusion protein expressed in human 293 cells (del Re et al., J Biol Chem. 2004 Dec 17;279(51):53126-35.) Additionally, the use of the tPa leader sequence provided greater production than other leader sequences and, unlike ActRIIa-Fc expressed with a native leader, provided a highly pure N-terminal sequence. Use of the native leader sequence resulted in two major species of ActRIIa-Fc, each having a different N-terminal sequence.

Example 3. ActRIIa-hFc Increases Red Blood Cell Levels in Non-Human Primates

The study employed four groups of five male and five female cynomolgus monkeys each, with three per sex per group scheduled for termination on Day 29, and two per sex per group scheduled for termination on Day 57. Each animal was administered the vehicle (Group 1) or ActRIIa-Fc at doses of 1, 10, or 30 mg/kg (Groups 2, 3 and 4, respectively) via intravenous (IV) injection on Days 1, 8, 15 and 22. The dose volume was maintained at 3 mL/kg. Various measures of red blood cell levels were assessed two days prior to the first administration and at days 15, 29 and 57 (for the remaining two animals) after the first administration.

The ActRIIa-hFc caused statistically significant increases in mean red blood cell parameters (red blood cell count [RBC], hemoglobin [HGB], and hematocrit [HCT]) for males and females, at all dose levels and time points throughout the study, with accompanying elevations in absolute and relative reticulocyte counts (ARTC; RTC). See Figures 3 – 6.

Statistical significance was calculated for each treatment group relative to the mean for the treatment group at baseline.

Notably, the increases in red blood cell counts and hemoglobin levels are roughly equivalent in magnitude to effects reported with erythropoietin. The onset of these effects is more rapid with ActRIIa-Fc than with erythropoietin.

Similar results were observed with rats and mice.

Example 4. ActRIIa-hFc Increases Red Blood Cell Levels and Markers of Bone Formation in Human Patients

The ActRIIa-hFc fusion protein described in Example 1 was administered to human patients in a randomized, double-blind, placebo-controlled study that was conducted to evaluate, primarily, the safety of the protein in healthy, postmenopausal women. Forty-eight subjects were randomized in cohorts of 6 to receive either a single dose of ActRIIa-hFc or placebo (5 active:1 placebo). Dose levels ranged from 0.01 to 3.0 mg/kg intravenously (IV) and 0.03 to 0.1 mg/kg subcutaneously (SC). All subjects were followed for 120 days. In addition to pharmacokinetic (PK) analyses, the biologic activity of ActRIIa-hFc was also assessed by measurement of biochemical markers of bone formation and resorption, and FSH levels.

To look for potential changes, hemoglobin and RBC numbers were examined in detail for all subjects over the course of the study and compared to the baseline levels. Platelet counts were compared over the same time as the control. There were no clinically significant changes from the baseline values over time for the platelet counts.

PK analysis of ActRIIa-hFc displayed a linear profile with dose, and a mean half-life of approximately 25-32 days. The area-under-curve (AUC) for ActRIIa-hFc was linearly related to dose, and the absorption after SC dosing was essentially complete (see Figures 7 and 8). These data indicate that SC is a desirable approach to dosing because it provides equivalent bioavailability and serum-half life for the drug while avoiding the spike in serum concentrations of drug associated with the first few days of IV dosing (see Figure 8). ActRIIa-hFc caused a rapid, sustained dose-dependent increase in serum levels of bone-specific alkaline phosphatase (BAP), which is a marker for anabolic bone growth, and a dose-dependent decrease in C-terminal type 1 collagen telopeptide and tartrate-resistant acid phosphatase 5b levels, which are markers for bone resorption. Other markers, such as PINP showed inconclusive results. BAP levels showed near saturating effects at the highest dosage of drug, indicating that half-maximal effects on this anabolic bone biomarker could be achieved at a dosage of 0.3 mg/kg, with increases ranging up to 3 mg/kg. Calculated as a relationship of pharmacodynamic effect to AUC for drug, the EC₅₀ is 51,465 (day*ng/ml). See Figure 9. These bone biomarker changes were sustained for approximately 120 days at the highest dose levels tested. There was also a dose-dependent decrease in serum FSH levels consistent with inhibition of activin.

Overall, there was a very small non-drug related reduction in hemoglobin over the first week of the study probably related to study phlebotomy in the 0.01 and 0.03 mg/kg groups whether given IV or SC. The 0.1 mg/kg SC and IV hemoglobin results were stable or showed modest increases by Day 8-15. At the 0.3 mg/kg IV dose level there was a clear
5 increase in HGB levels seen as early as Day 2 and often peaking at Day 15-29 that was not seen in the placebo subjects. At the 1.0 mg/kg IV dose and the 3.0 mg/kg IV dose, mean increases in hemoglobin of greater than 1 g/dl were observed in response to the single dose, with corresponding increases in RBC counts and hematocrit. These hematologic parameters peaked at about 60 days after the dose and substantial decrease by day 120. This indicates
10 that dosing for the purpose of increasing red blood cell levels may be more effective if done at intervals less than 120 days (i.e., prior to return to baseline), with dosing intervals of 90 days or less or 60 days or less may be desirable. For a summary of hematological changes, see Figures 10-13.

Overall, ActRIIa-hFc showed a dose-dependent effect on red blood cell counts and
15 reticulocyte counts, and a dose-dependent effect on markers of bone formation.

Example 5. Treatment of an Anemic Patient with ActRIIa-hFc

A clinical study was designed to treat patients with multiple doses of ActRIIa-hFc, at dose levels of 0.1 mg/kg, 0.3 mg/kg and 1.0 mg/kg, with dosing every thirty days. Normal
20 healthy patients in the trial exhibited an increase in hemoglobin and hematocrit that is consistent with the increases seen in the Phase I clinical trial reported in Example 4, except that, in some instances, the hemoglobin and hematocrit were elevated beyond the normal range. An anemic patient with hemoglobin of approximately 7.5 also received two doses at the 1 mg/kg level, resulting in a hemoglobin level of approximately 10.5 after two months.
25 The patient's anemia was a microcytic anemia, thought to be caused by chronic iron deficiency.

Example 6. ActRIIa-mFc Increases Red Blood Cell Levels in Mice by Stimulation of Splenic Red Blood Cell Release

30 In this study the effects of the in vivo administration of ActRIIa-mFc on the frequency of hematopoietic progenitors in bone marrow and spleen was analyzed. One group of mice

was injected with PBS as a control and a second group of mice administered two doses of ActRIIa-mFc at 10 mg/kg and both groups sacrificed after 8 days. Peripheral blood was used to perform complete blood counts and femurs and spleens were used to perform in vitro clonogenic assays to assess the lymphoid, erythroid and myeloid progenitor cell content in
5 each organ. In the peripheral blood a significant increase in the red blood cell and hemoglobin content was seen in compound treated mice. In the femurs there was no difference in the nucleated cell numbers or progenitor content between the control and treated groups. In the spleens, the compound treated group experienced a statistically significant increase in the nucleated cell number before red blood cell lysis and in the mature erythroid
10 progenitor (CFU-E) colony number per dish, frequency and total progenitor number per spleen. In addition, an increase was seen in the number of myeloid (CFU-GM), immature erythroid (BFU-E) and total progenitor number per spleen.

Animals:

Sixteen BDF1 female mice 6 -8 weeks of age were used in the study. Eight mice were
15 injected subcutaneously with test compound ActRIIa-mFc at days 1 and 3 at a dose of 10 mg/kg and eight mice were injected subcutaneously with vehicle control, phosphate buffered saline (PBS), at a volume of 100 μ L per mouse. All mice were sacrificed 8 days after first injection in accordance with the relevant Animal Care Guidelines. Peripheral blood (PB) samples from individual animals were collected by cardiac puncture and used for complete
20 blood counts and differential (CBC/Diff). Femurs and spleens were harvested from each mouse.

Tests performed:

CBC/Diff Counts

PB from each mouse was collected via cardiac puncture and placed into the
25 appropriate microtainer tubes. Samples were sent to CLV for analysis on a CellDyn 3500 counter.

Clonogenic Assays

Clonogenic progenitors of the myeloid, erythroid and lymphoid lineages were assessed using the in vitro methylcellulose-based media systems described below.

Mature Erythroid Progenitors:

Clonogenic progenitors of the mature erythroid (CFU-E) lineages were cultured in MethoCult™ 3334, a methylcellulose-based medium containing recombinant human (rh) Erythropoietin (3 U/mL).

5 Lymphoid Progenitors:

Clonogenic progenitors of the lymphoid (CFU-pre-B) lineage were cultured in MethoCult® 3630, a methylcellulose-based medium containing rh Interleukin 7 (10 ng/mL).

Myeloid and Immature Erythroid Progenitors:

10 Clonogenic progenitors of the granulocyte-monocyte (CFU-GM), erythroid (BFU-E) and multipotential (CFU-GEMM) lineages were cultured in MethoCult™ 3434, a methylcellulose-based medium containing recombinant murine (rm) Stem Cell Factor (50 ng/mL), rh Interleukin 6 (10 ng/mL), rm Interleukin 3 (10 ng/mL) and rh Erythropoietin (3 U/mL).

Methods:

15 Mouse femurs and spleens were processed by standard protocols. Briefly, bone marrow was obtained by flushing the femoral cavity with Iscove's Modified Dulbecco's Media containing 2% fetal bovine serum (IMDM 2% FBS) using a 21 gauge needle and 1 cc syringe. Spleen cells were obtained by crushing spleens through a 70 µm filter and rinsing the filter with IMDM 2% FBS. Nucleated cell counts in 3% glacial acetic acid were then
20 performed on the single cells suspensions using a Neubauer counting chamber so that the total cells per organ could be calculated. To remove contaminating red blood cells, total spleen cells were then diluted with 3 times the volume of ammonium chloride lysis buffer and incubated on ice 10 minutes. The cells were then washed and resuspended in

IMDM 2% FBS and a second cell count were performed to determine the cell
25 concentration of cells after lysis.

Cell stocks were made and added to each methylcellulose-based media formulation to obtain the optimal plating concentrations for each tissue in each media formulation. Bone marrow cells were plated at 1×10^5 cells per dish in MethoCult™ 3334 to assess mature erythroid progenitors, 2×10^5 cells per dish in MethoCult™ 3630 to assess lymphoid
30 progenitors and 3×10^4 cells per dish in MethoCult™ 3434 to assess immature erythroid and

myeloid progenitors. Spleen cells were plated at 4×10^5 cells per dish in MethoCultTM 3334 to assess mature erythroid progenitors, 4×10^5 cells per dish in MethoCultTM 3630 to assess lymphoid progenitors and 2×10^5 cells per dish in MethoCultTM 3434 to assess immature erythroid and myeloid progenitors. Cultures plated in triplicate dishes were incubated at 37°C, 5% CO₂ until colony enumeration and evaluation was performed by trained personnel. Mature erythroid progenitors were cultured for 2 days, lymphoid progenitors were cultured for 7 days and mature erythroid and myeloid progenitors were cultured for 12 days.

Analysis:

The mean $\pm$ 1 standard deviation was calculated for the triplicate cultures of the clonogenic assays and for the control and treatment groups for all data sets.

Frequency of colony forming cells (CFC) in each tissue was calculated as follows:

Cells plated per dish

Mean CFC scored per dish

Total CFC per femur or spleen was calculated as follows:

Total CFC scored $\times$ nucleated cell count per femur or spleen (following RBC lysis)

Number of nucleated cells cultured

Standard t-tests were performed to assess if there was a differences in the mean number of cells or hematopoietic progenitors between the PBS control mice and compound treated mice. Due to the potential subjectivity of colony enumeration, a p value of less than 0.01 is deemed significant. Mean values ($\pm$ SD) for each group are shown in the tables below.

Table: Hematologic Parameters

Treatment Group	White Blood Cells ($\times 10^9/L$)	Red Blood Cells ($\times 10^9/L$)	Hemoglobin (g/L)	Hematocrit (L/L)
PBS (n=8)	6.37 $\pm$ 2.83	10.9 $\pm$ 0.7	154.5 $\pm$ 5.9	0.506 $\pm$ 0.029
ActRIIa-mFc (n=8)	8.92 $\pm$ 3.69	11.8 $\pm$ 0.3*	168.3 $\pm$ 4.3**	0.532 $\pm$ 0.014

* = $p < 0.01$

** = $p < 0.0005$

Table: CFC From Femur and Spleen

Treatment Group	Total CFC per Femur	Total CFC per Spleen	Total CFU-E per Femur	Total CFU-E per Spleen
PBS (n=8)	33437 +/- 7118	4212 +/- 1148	27185 +/- 12893	6743 +/- 1591
ActRIIa-mFc (n=8)	31068 +/- 8024	6816 +/- 1516*	18118 +/- 6672	27313 +/- 11790

* = $p < 0.005$ **= $p < 0.0001$

5

Treatment of mice with ActRIIa-mFc resulted in significant increases in a number of hematopoietic parameters. In the peripheral blood a significant increase in the red blood cell and hemoglobin content was seen in compound treated mice. In the femurs there was no difference in the nucleated cell numbers or progenitor content between the control and treated groups. In the spleens, the compound treated group experienced a statistically significant increase in the nucleated cell number before red blood cell lysis and in the mature erythroid progenitor (CFU-E) colony number per dish, frequency and total progenitor number per spleen. In addition, an increase was seen in the number of myeloid (CFU-GM), immature erythroid (BFU-E) and total progenitor number per spleen.

15

Example 7. Alternative ActRIIa-Fc Proteins

A variety of ActRIIa variants that may be used according to the methods described herein are described in the International Patent Application published as WO2006/012627 (see e.g., pp. 55-58), incorporated herein by reference in its entirety. An alternative construct may have a deletion of the C-terminal tail (the final 15 amino acids of the extracellular domain of ActRIIa. The sequence for such a construct is presented below (Fc portion underlined)(SEQ ID NO: 12):

20

25

ILGRSETQECLFFNANWEKDRTNQTGVEPCYGDKDKRRHCFATWKNISGSIEIVKQG
 CWLDDINCYDRTDCVEKKDSPEVYFCCCEGNMCNEKFSYFPEMTGGGTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPVPPIEKTISKAKGQPRE

PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFCVMHEALHNHYTQKSLSLSPGK

Example 8: Effect of ActRIIA-mFc on Chemotherapy-Induced Anemia in Mice

5 Applicants investigated the effect of ActRIIA-mFc on chemotherapy-induced anemia in mice. In the first of two studies, 6-week-old female C57BL/6 mice were treated with a single dose of ActRIIA-mFc (10 mg/kg, s.c.) or vehicle (phosphate-buffered saline) 3 days before a single dose of the chemotherapeutic paclitaxel (20 mg/kg, i.p.). Blood samples were collected before chemotherapy and then 3, 7, and 14 days (n = 6 per cohort per time point) after paclitaxel. ActRIIA-mFc prevented the decline in hematocrit level otherwise observed after paclitaxel (Figure 15), and similar effects were observed for hemoglobin concentration and RBC count. In a second study, 6-week-old female C57BL/6 mice were given a varying number of ActRIIA-mFc doses (10 mg/kg, s.c.), or vehicle (PBS), beginning before paclitaxel (20 mg/kg single dose, i.p.) and continuing at intervals of 3 or 4 days. Blood samples were collected 3, 7, and 14 days (n = 8 per cohort per time point) after paclitaxel. At 14 days, ActRIIA-mFc treatment increased hematocrit level progressively as a function of dose number (Figure 16). Thus, ActRIIA-mFc can stimulate erythropoiesis sufficiently to attenuate or prevent chemotherapy-induced anemia.

20 Example 9: Effect of ActRIIA-mFc on Anemia in a Mouse Model of Chronic Kidney Disease

Applicants investigated the effect of ActRIIA-mFc on nephrectomy-induced anemia in mice as a model of chronic kidney disease. In the first of two studies, female C57BL/6 mice underwent a partial surgical nephrectomy, with removal of approximately five-sixths of total kidney volume, to reduce production of erythropoietin. Mice were given a 4-week recovery period with a high-fat diet to further promote renal deficiency and were then treated twice-weekly with ActRIIA-mFc (10 mg/kg, s.c.) or vehicle (PBS) for a total of 8 weeks. Blood samples were collected before the onset of dosing, after 4 weeks of treatment, and after 8 weeks of treatment (n = 8 per cohort per time point). Control mice exhibited a decline in hematocrit level over the 8-week treatment period, whereas ActRIIA-mFc treatment prevented the decline at 4 weeks and also produced a beneficial trend at 8 weeks (Figure 17). Similar benefits of ActRIIA-mFc treatment over control were observed in a second study that

differed mainly in the use of a longer recovery period (2 months) and a standard diet. Thus, ActRIIA-mFc can stimulate erythropoiesis sufficiently to prevent or attenuate anemia in a model of chronic kidney disease.

Taken together, these findings indicate that soluble ActRIIA-Fc fusion proteins can be used as antagonists of signaling by TGF- family ligands to increase circulating levels of red blood cells, and thereby, to treat hypoproliferative anemias resulting from chronic diseases such as cancer and renal disease, and potentially other inflammatory or infectious diseases as well. Note that effects of ACE-011 on anemia in human patients are typically robust compared to the more modest effects in rodents.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

While specific embodiments of the subject matter have been discussed, the above specification is illustrative and not restrictive. Many variations will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

We Claim:

1. A method for managing a patient that has been treated with, or is a candidate to be treated with, an activin-ActRIIa antagonist, the method comprising monitoring in the patient one or more hematologic parameters that correlate with increased red blood cell levels.
2. The method of claim 1, wherein the hematologic parameters are one or more of the following: red blood cell levels, blood pressure, or iron stores.
3. The method of claim 1, the method further comprising administering to the patient the activin-ActRIIa antagonist in an amount that is appropriate to the observed level of said hematologic parameter in the patient.
4. The method of claim 1, wherein, if the patient has blood pressure elevated above baseline or is hypertensive, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.
5. The method of claim 1, wherein, if the patient has blood pressure elevated above baseline or is hypertensive, the patient is treated with a blood pressure lowering agent prior to administration of the activin-ActRIIa antagonist.
6. The method of claim 1, wherein if the patient has uncontrolled hypertension, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.
7. The method of claim 1, wherein if the patient has a red blood cell level greater than the normal range for patients of similar age and sex, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.
8. The method of claim 1, wherein if the patient has a hemoglobin level of greater than 15g/dl, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.
9. The method of claim 1, wherein if the patient has a hemoglobin level greater than 10, 11 or 12 g/dl, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.
10. The method of claim 1, wherein if the patient has iron stores that are lower than the normal range for patients of similar age and sex, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.
11. The method of claim 1, wherein if the patient has a transferrin saturation of less than 20%, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.
12. The method of claim 1, wherein if the patient has a ferritin level of less than 100 ng/ml, dosing with the activin-ActRIIa antagonist is reduced, delayed or terminated.

13. The method of claim 1, wherein if the patient has iron stores that are lower than the normal range for patients of similar age and sex, the patient is treated with an iron supplement prior to dosing with the activin-ActRIIa antagonist.
14. The method of claim 1, wherein if the patient has a transferrin saturation of less than 20%, the patient is treated with an iron supplement prior to dosing with the activin-ActRIIa antagonist.
15. The method of claim 1, wherein if the patient has a ferritin level of less than 100 ng/ml, the patient is treated with an iron supplement prior to dosing with the activin-ActRIIa antagonist.
16. The method of claim 1, wherein monitoring red blood cell levels comprises monitoring one or more of the following: hemoglobin levels and hematocrit levels.
17. The method of claim 1, wherein monitoring blood pressure comprises monitoring one or more of the following: systolic blood pressure, diastolic blood pressure or mean arterial blood pressure.
18. The method of claim 1, wherein monitoring iron stores comprises monitoring one or more of the following: transferrin saturation, ferritin levels or total iron binding capacity.
19. A method for dosing a patient with an activin-ActRIIa antagonist, the method comprising dosing the patient in amounts and at intervals selected so as to reduce the risk of causing a rise in hemoglobin levels greater than 1 g/dl in two weeks.
20. The method of any one of claims 1-19, wherein the patient is suffering from anemia.
21. The method of any one of claims 1-19, wherein the patient is in need of bone growth, increased bone density or increased bone strength.
22. The method of any one of claims 1-19, wherein the patient is suffering from a bone-related disorder.
23. The method of any one of claims 1-19, wherein the patient has cancer.
24. The method of claim 23, wherein the patient has breast cancer.
25. The method of any of claims 1-19, wherein the activin-ActRIIa antagonist is an antibody that binds to a target protein selected from the group consisting of: an activin and ActRIIA.
26. The method of any of claims 1-19, wherein the activin-ActRIIa antagonist is inhibin or a conservative variant of inhibin.

27. The method of any of claims 1-19, wherein the activin-ActRIIa antagonist is a protein comprising a follistatin domain that binds to and antagonizes activin.
28. The method of any of claims 1-19, wherein the activin-ActRIIa antagonist is a protein selected from the group consisting of: follistatin, follistatin-related gene (FLRG) and a conservative variant of the foregoing.
29. The method of any one of claims 1-19, wherein the activin-ActRIIa antagonist is an ActRIIa polypeptide selected from the group consisting of:
- a) a polypeptide comprising an amino acid sequence at least 90% identical to SEQ ID NO: 2;
 - b) a polypeptide comprising an amino acid sequence at least 90% identical to SEQ ID NO: 3; and
 - c) a polypeptide comprising at least 50 consecutive amino acids selected from SEQ ID NO: 2.
 - d) a polypeptide comprising an amino acid sequence at least 90% identical to SEQ ID NO: 7;
 - e) a polypeptide comprising an amino acid sequence at least 90% identical to SEQ ID NO: 12; and
 - f) a polypeptide comprising at least 50 consecutive amino acids selected from SEQ ID NO: 7.
30. The method of claim 29, wherein the polypeptide has one or more of the following characteristics:
- i) binds to an ActRIIa ligand with a K_D of at least 10^{-7} M; and
 - ii) inhibits ActRIIa signaling in a cell.
31. The method of claim 29, wherein said polypeptide is a fusion protein including, in addition to an ActRIIa polypeptide domain, one or more polypeptide portions that enhance one or more of in vivo stability, in vivo half life, uptake/administration, tissue localization or distribution, formation of protein complexes, and/or purification.
32. The method of claim 29, wherein said fusion protein includes a polypeptide portion selected from the group consisting of: an immunoglobulin Fc domain and a serum albumin.
33. The method of claim 29, wherein said polypeptide includes one or more modified amino acid residues selected from: a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid,

an amino acid conjugated to a lipid moiety, and an amino acid conjugated to an organic derivatizing agent.

34. The method of claim 29, wherein the activin-ActRIIa antagonist is an ActRIIa-Fc fusion protein comprising an amino acid sequence selected from the group consisting of:

- a) the amino acid sequence of SEQ ID NO: 3,
- b) the amino acid sequence of SEQ ID NO: 2,
- c) the amino acid sequence of SEQ ID NO: 7,
- d) an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 7,
- e) an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 7,
- f) the amino acid sequence of SEQ ID NO: 12, and
- g) the amino acid sequence of SEQ ID NO: 13.

35. A method for administering an ActRIIa-Fc fusion protein to a patient, the method comprising administering the ActRIIa-Fc fusion protein no more frequently than once per 60 days.

36. The method of claim 35, wherein the activin antagonist is administered to the patient no more frequently than once per 90 days.

37. The method of claim 35, wherein the activin antagonist is administered to the patient no more frequently than once per 120 days.

38. A method for administering an ActRIIa-Fc fusion protein to a patient in need thereof, the method comprising administering the ActRIIa-Fc fusion protein to a patient having a hemoglobin level of less than 10 g/dL.

39. The method of claim 38, wherein the patient has a hemoglobin level of less than 11 g/dL.

40. The method of claim 38, wherein the patient has a hemoglobin level of less than 12 g/dL.

41. The method of any of claims 38-40, wherein the patient is in need of bone growth, increased bone density or increased bone strength.

42. The method of any of claims 38-40, wherein the patient is suffering from a bone-related disorder.

43. The method of any of claims 38-40, wherein the patient has cancer.

44. The method any of claims 38-40, wherein the patient has breast cancer.

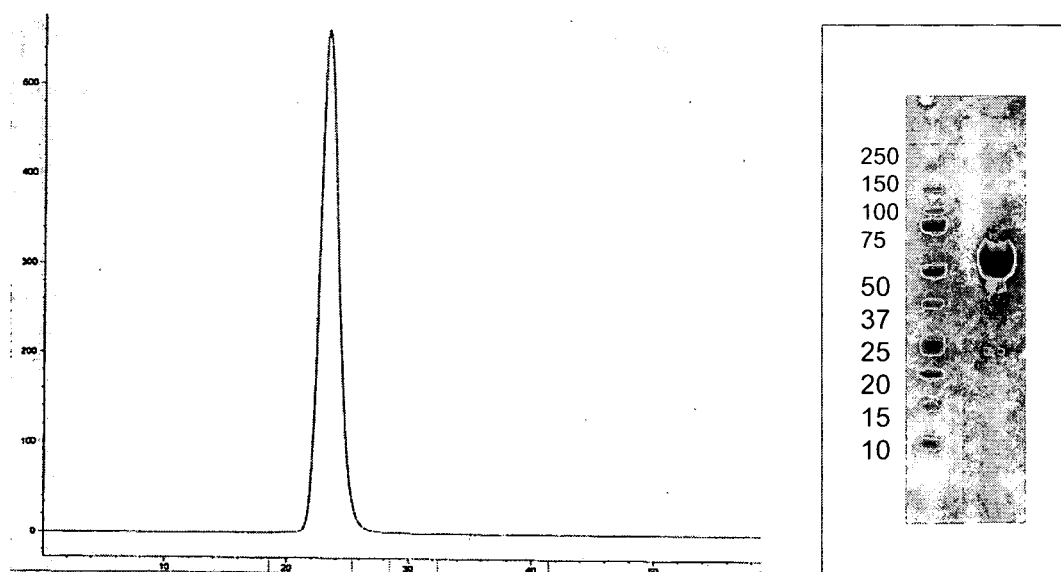


Figure 1

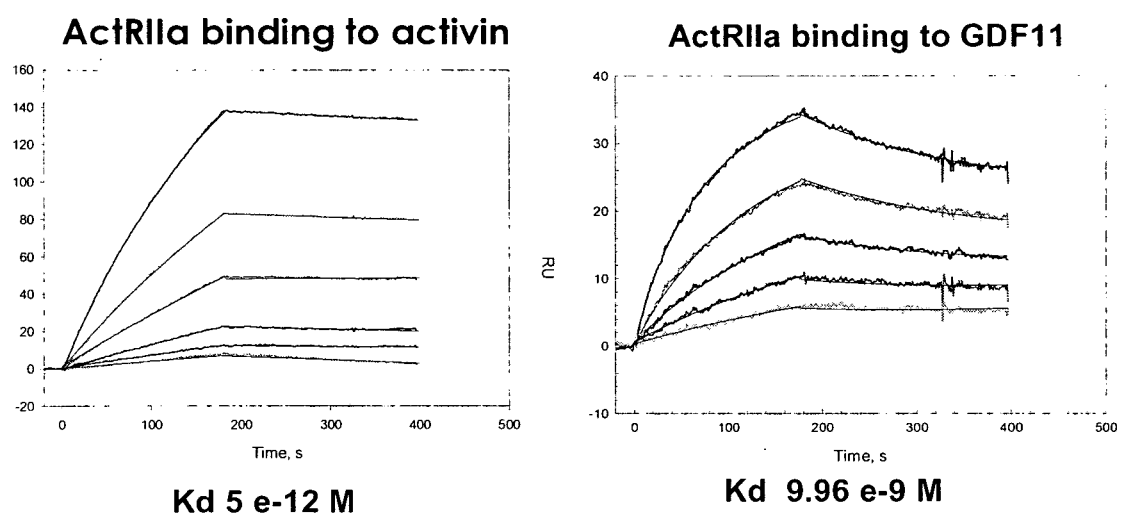


Figure 2

Figure 3A

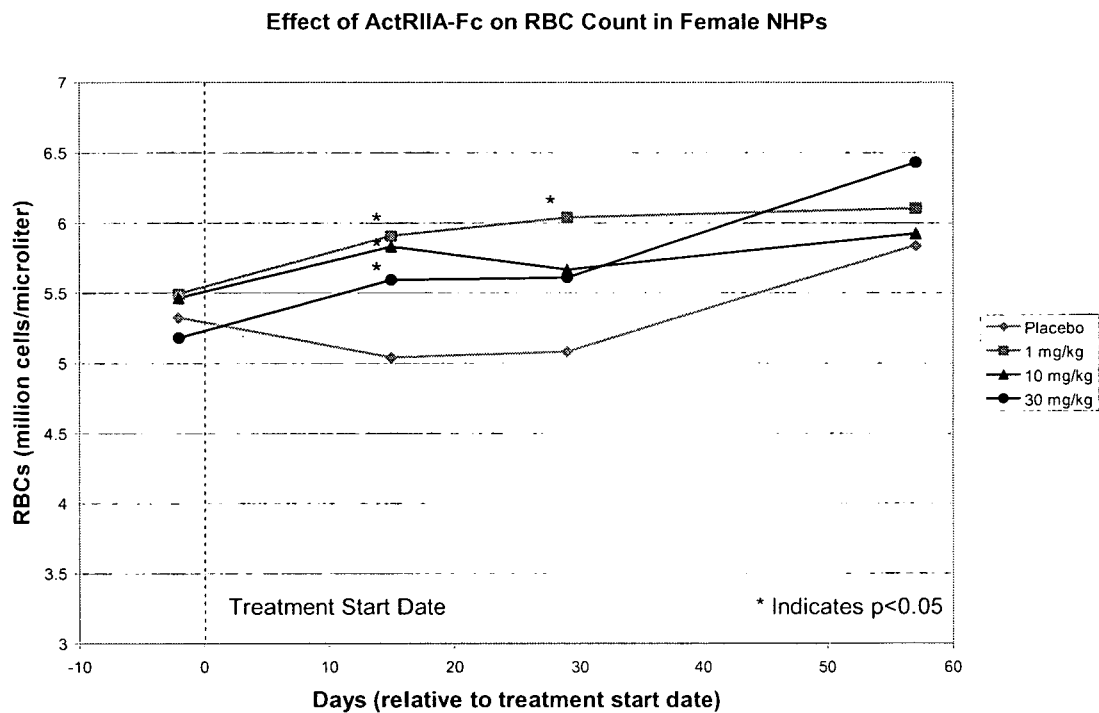


Figure 3B

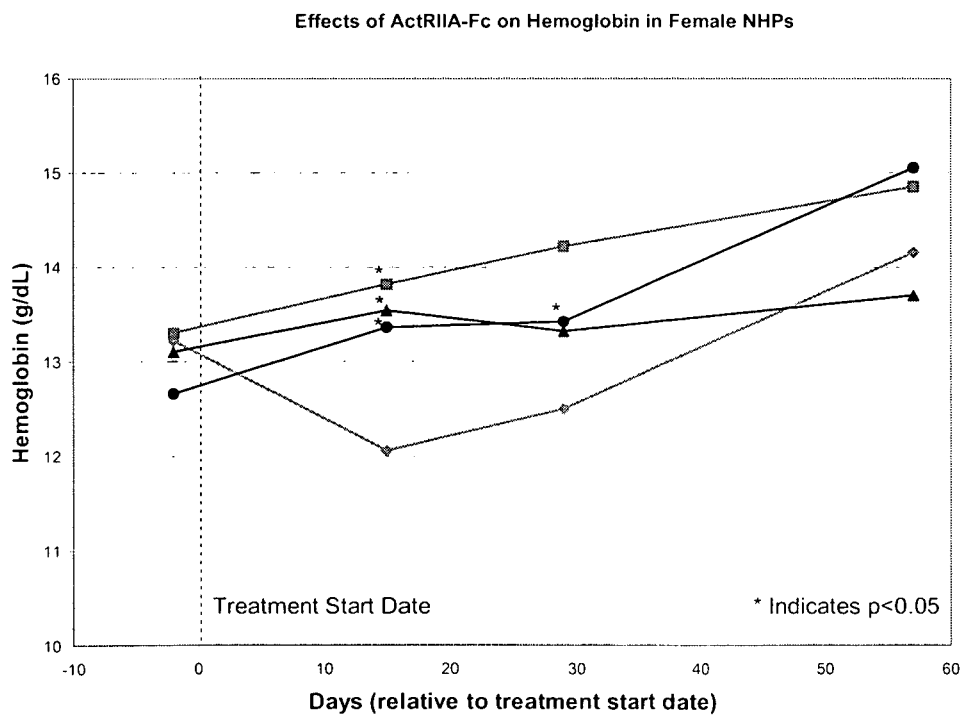


Figure 3

Figure 4A

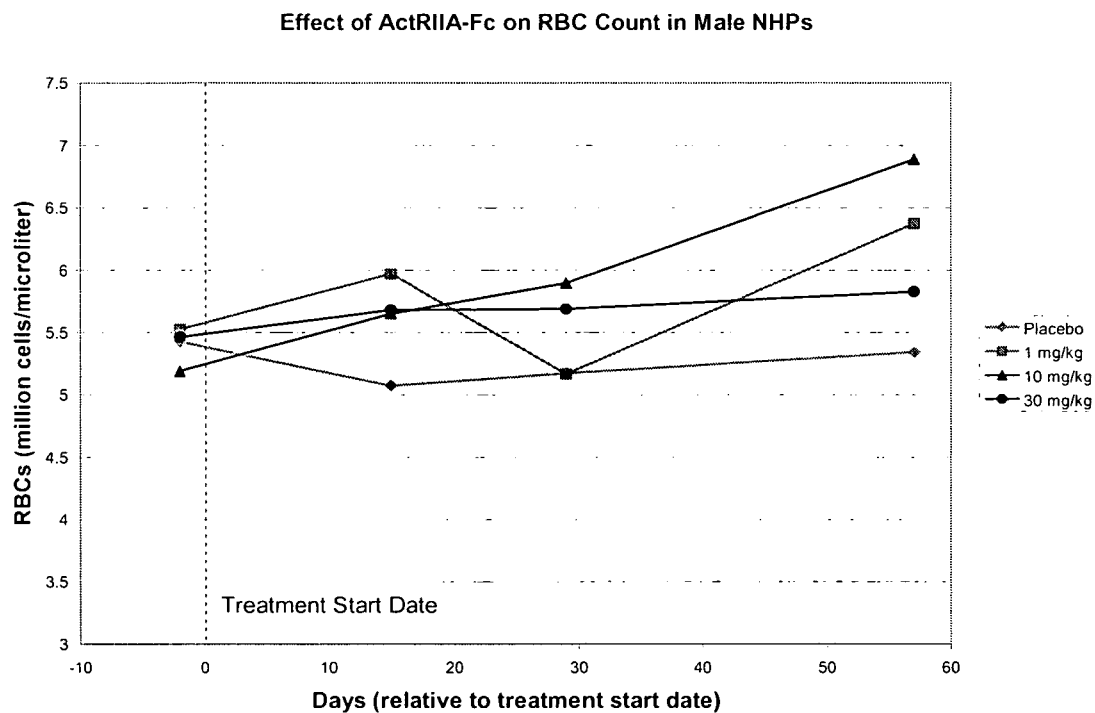


Figure 4B

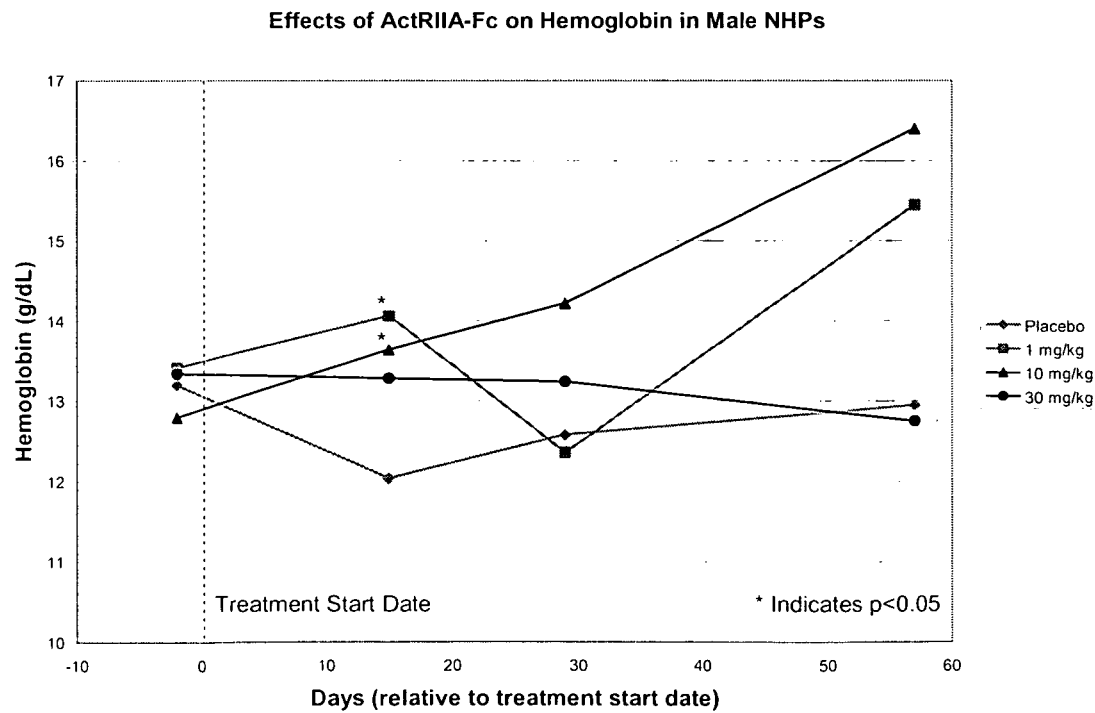


Figure 4

Figure 5A

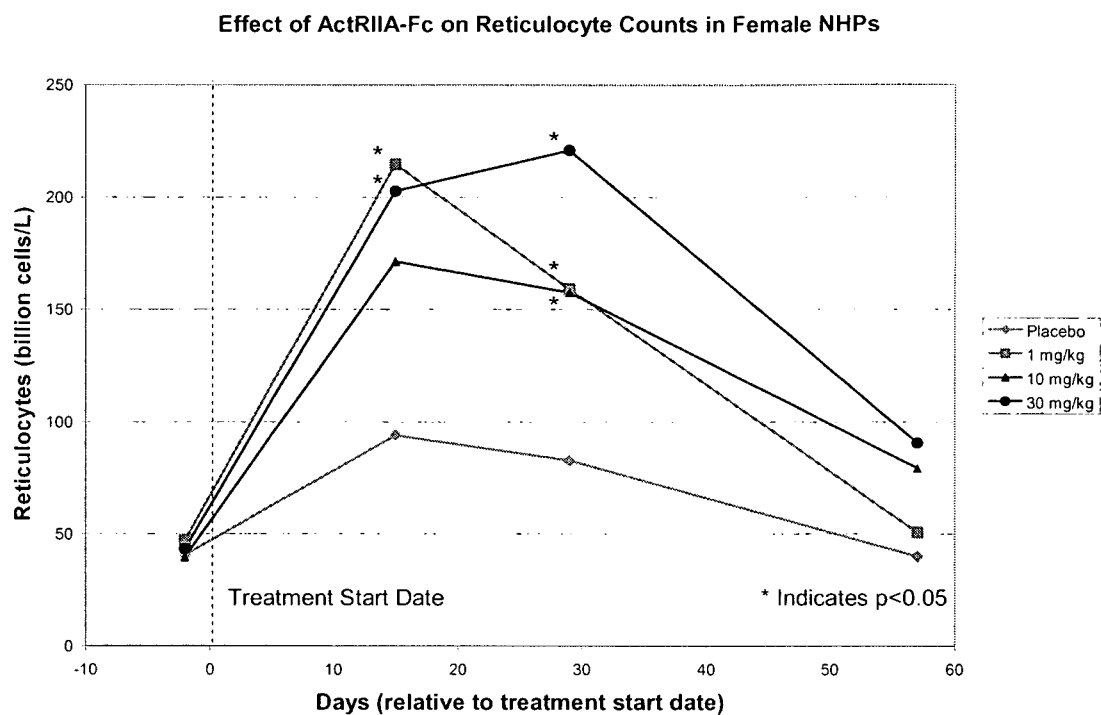


Figure 5B

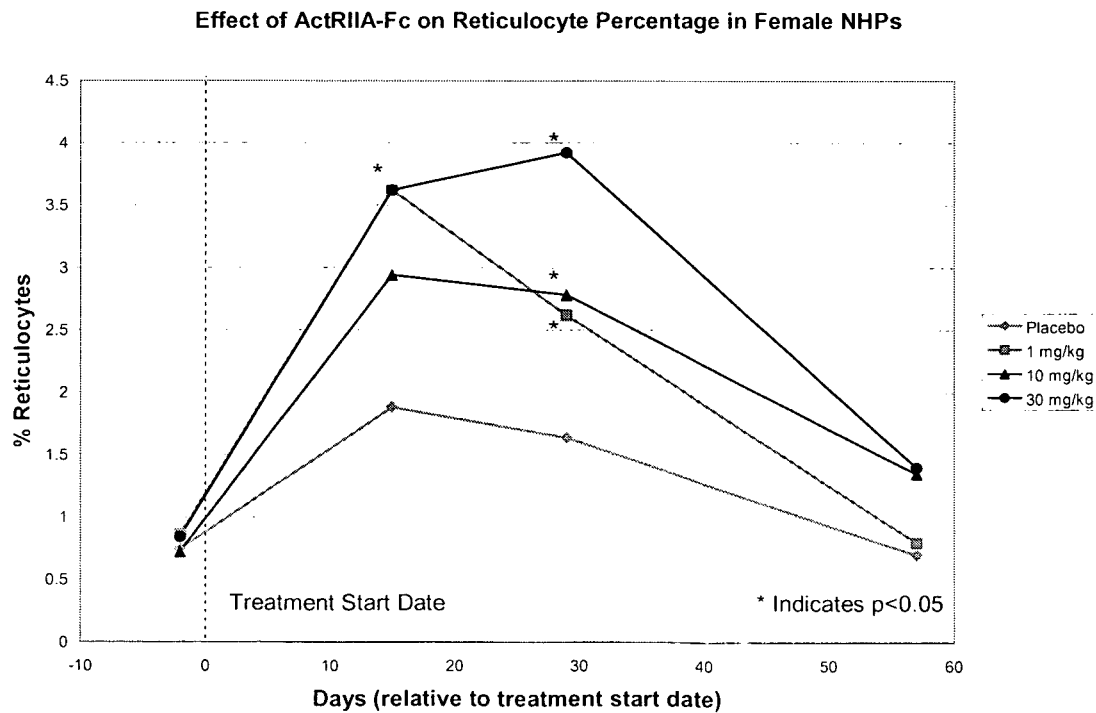


Figure 5

Figure 6A

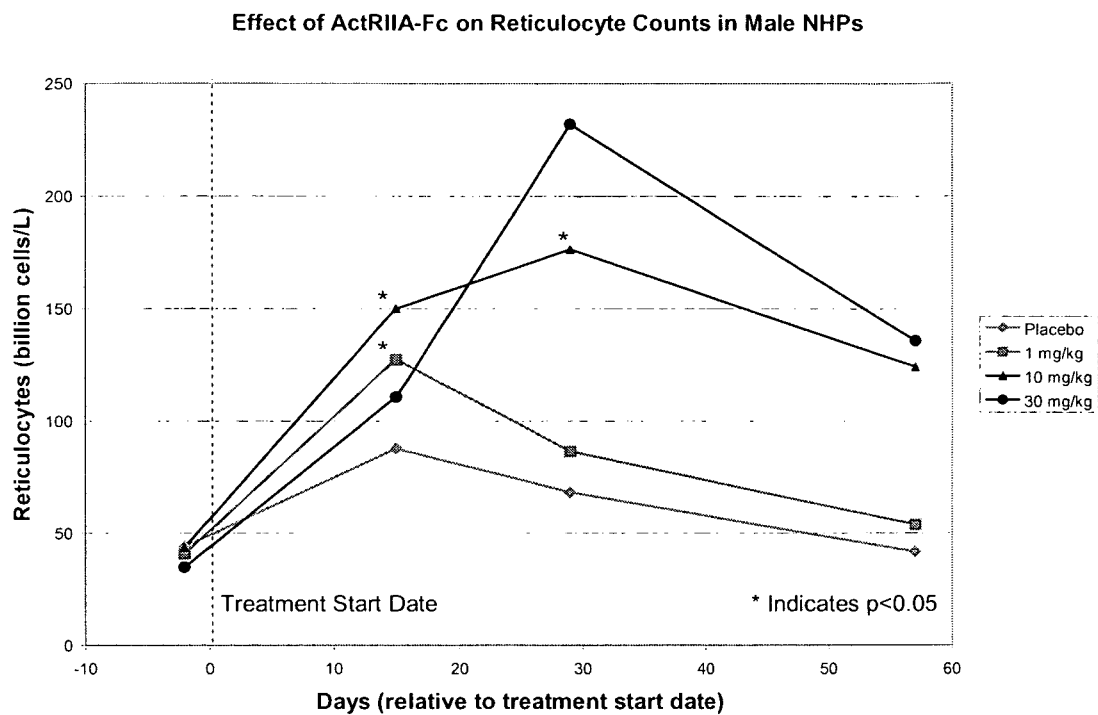


Figure 6B

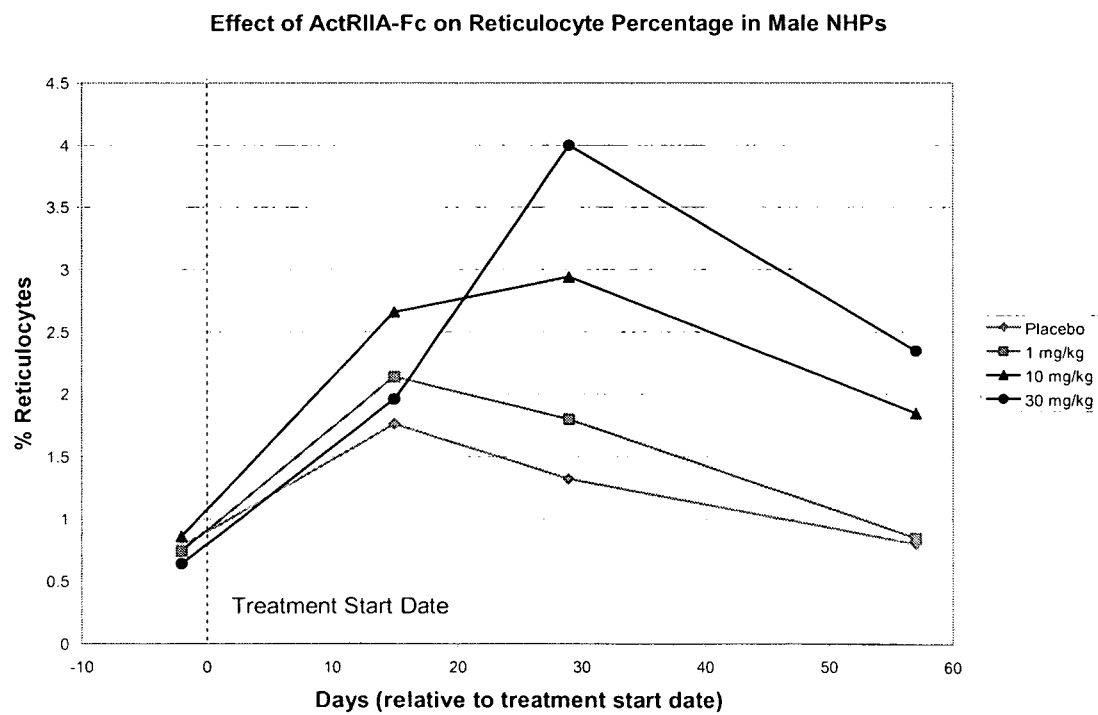


Figure 6

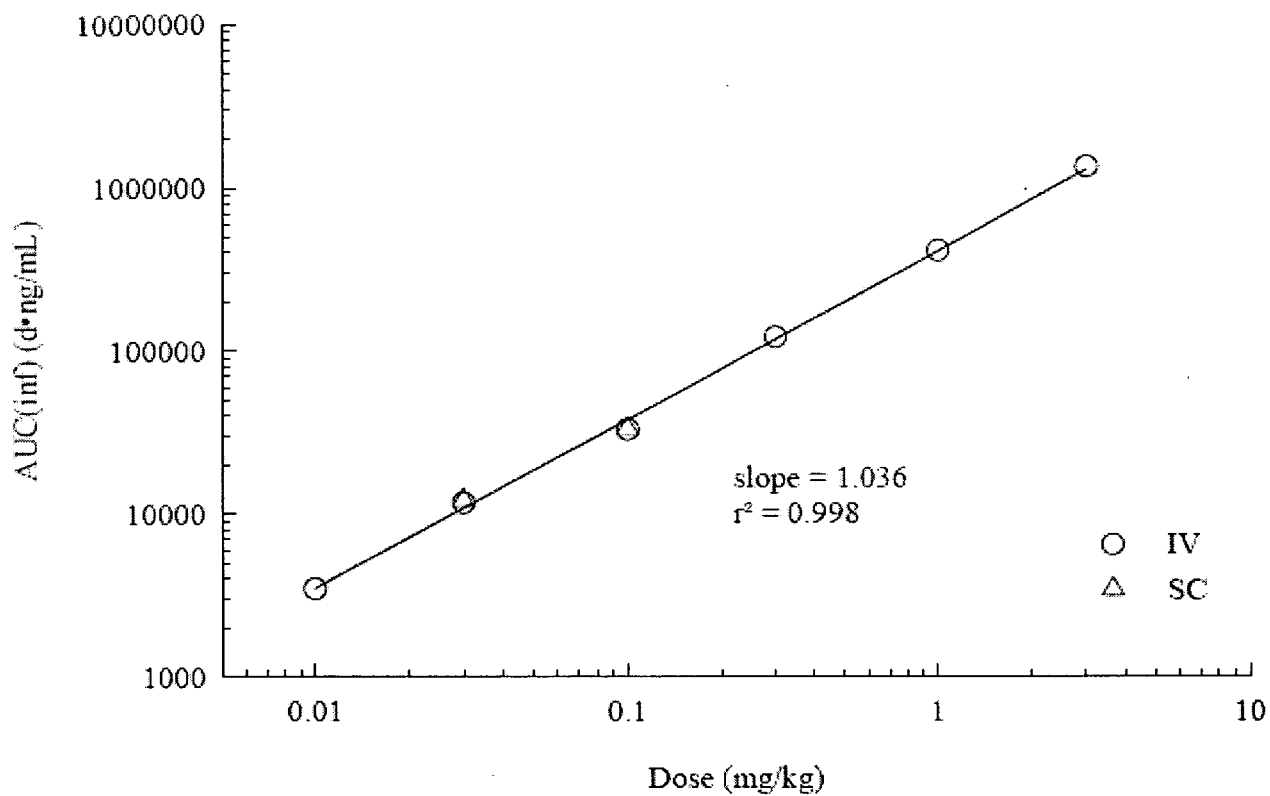


Figure 7

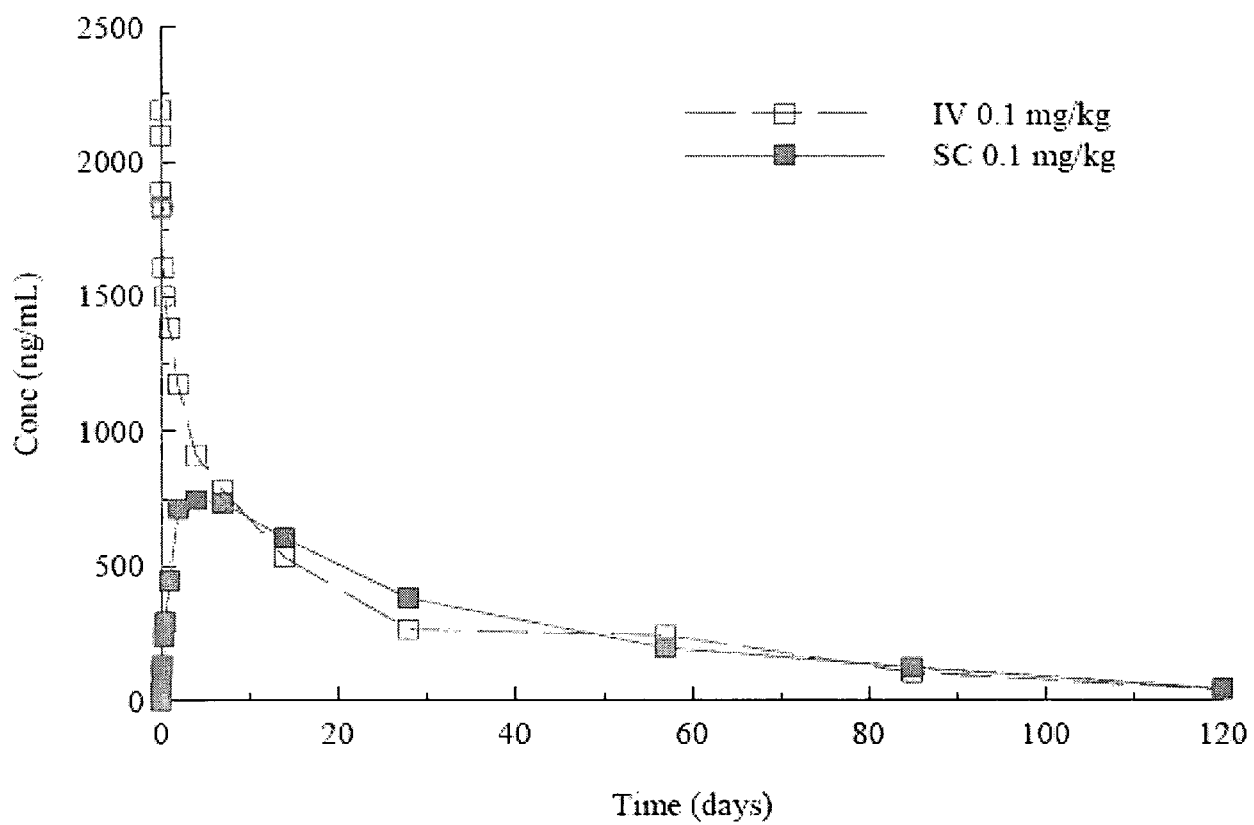


Figure 8

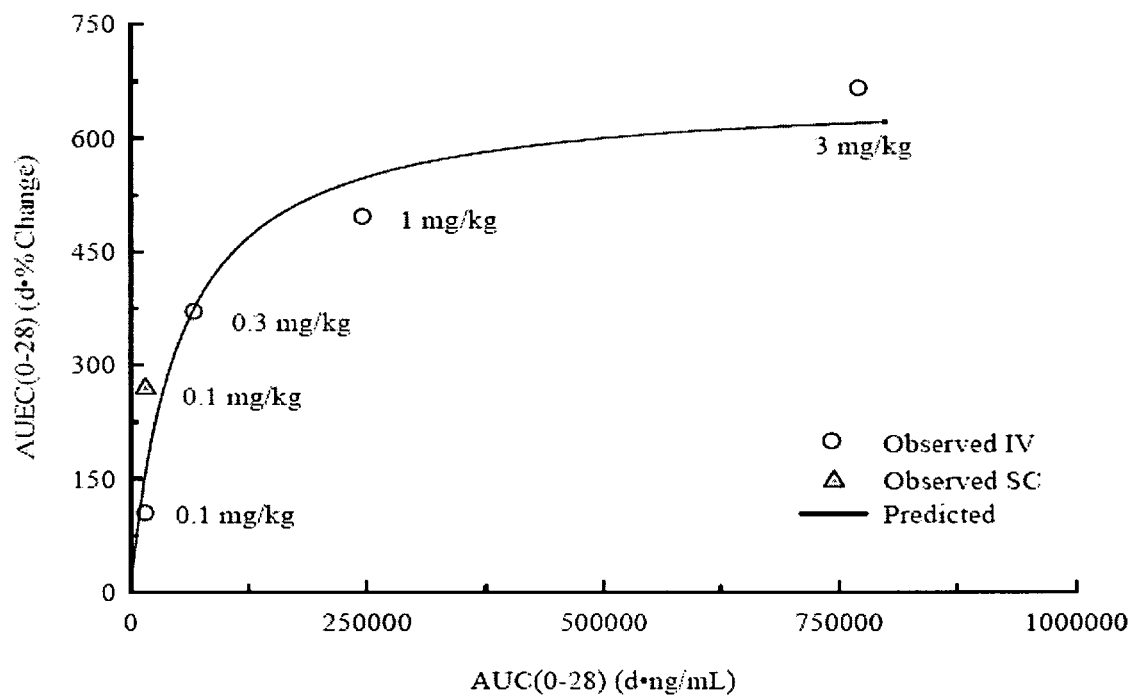


Figure 9

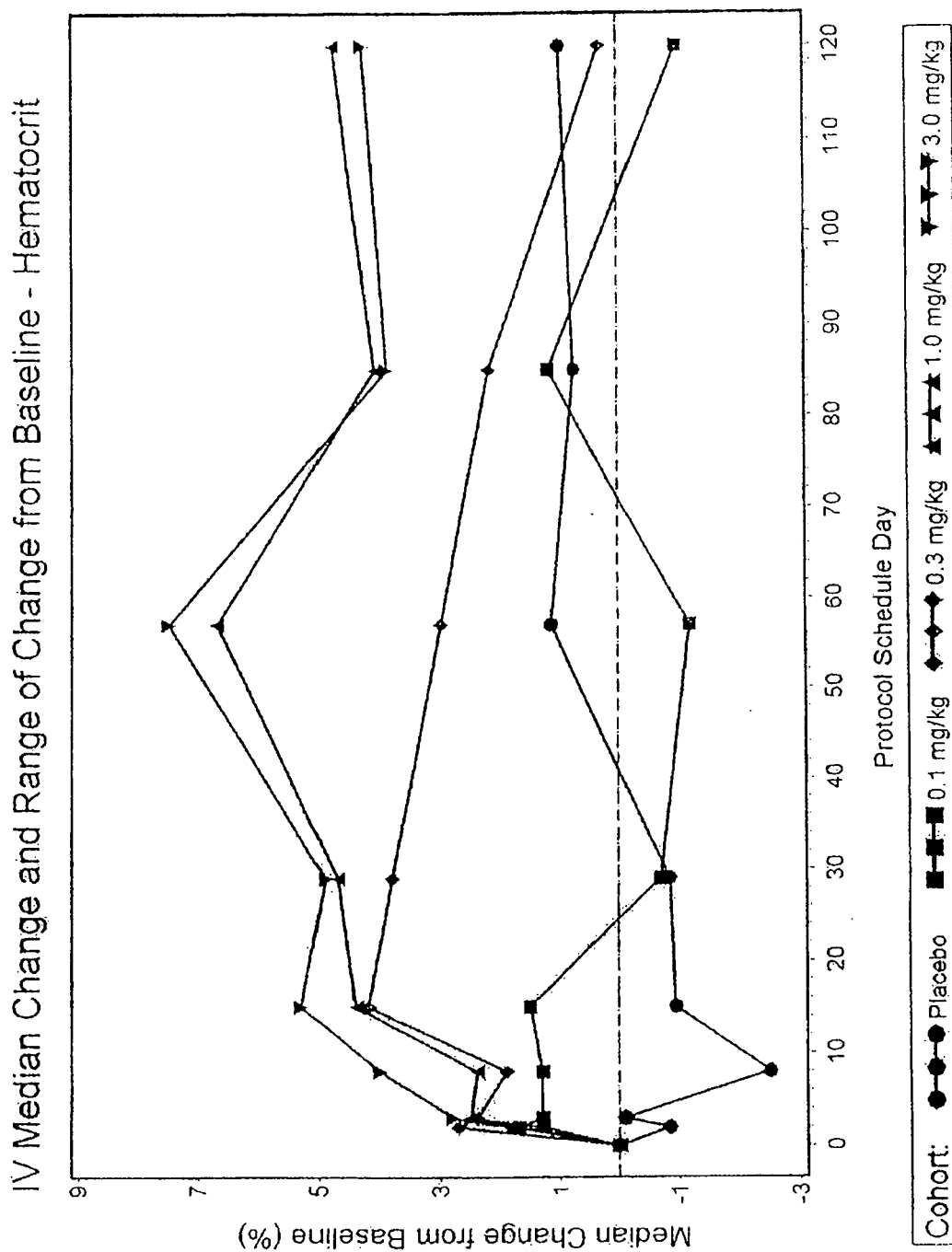


Figure 10

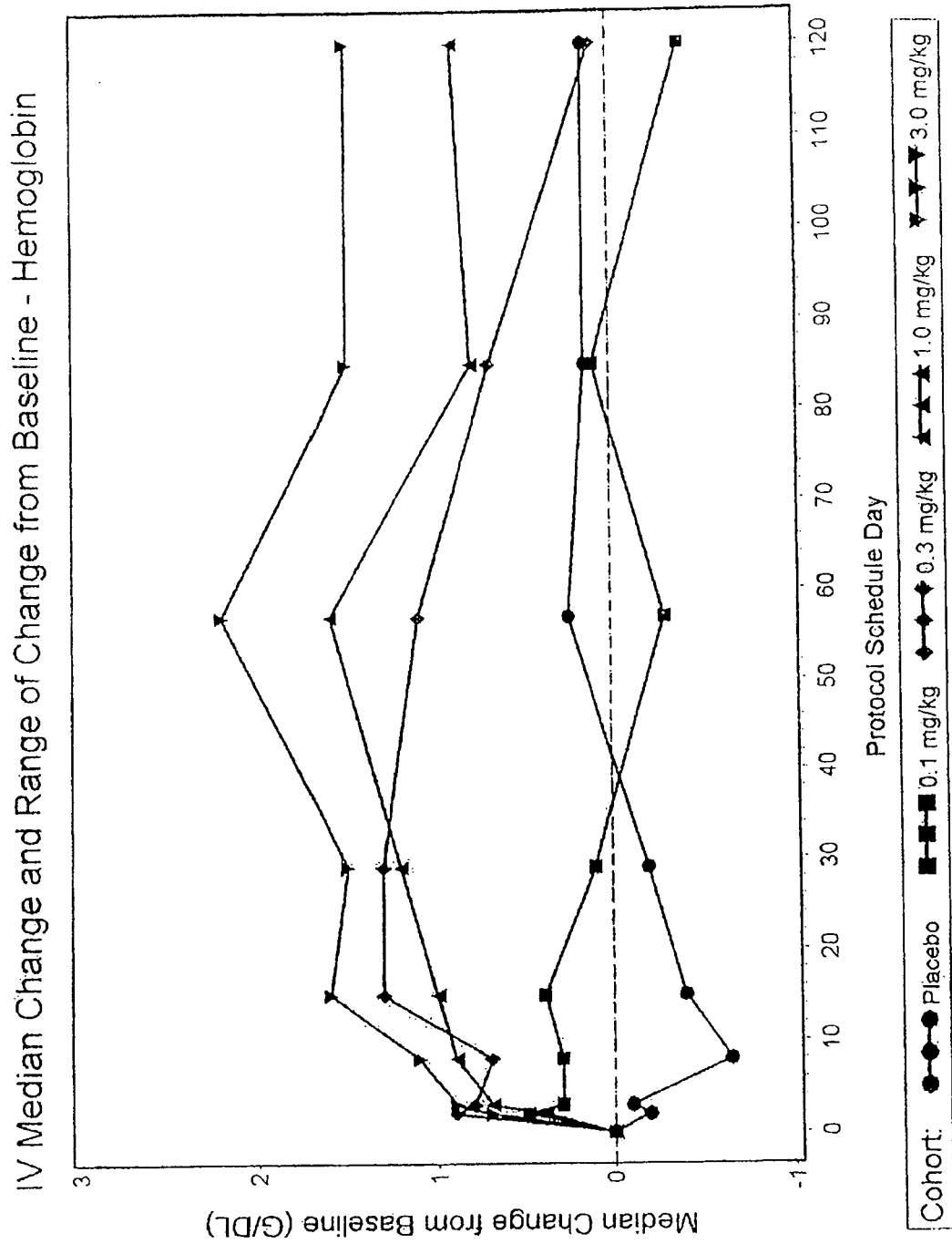


Figure 11

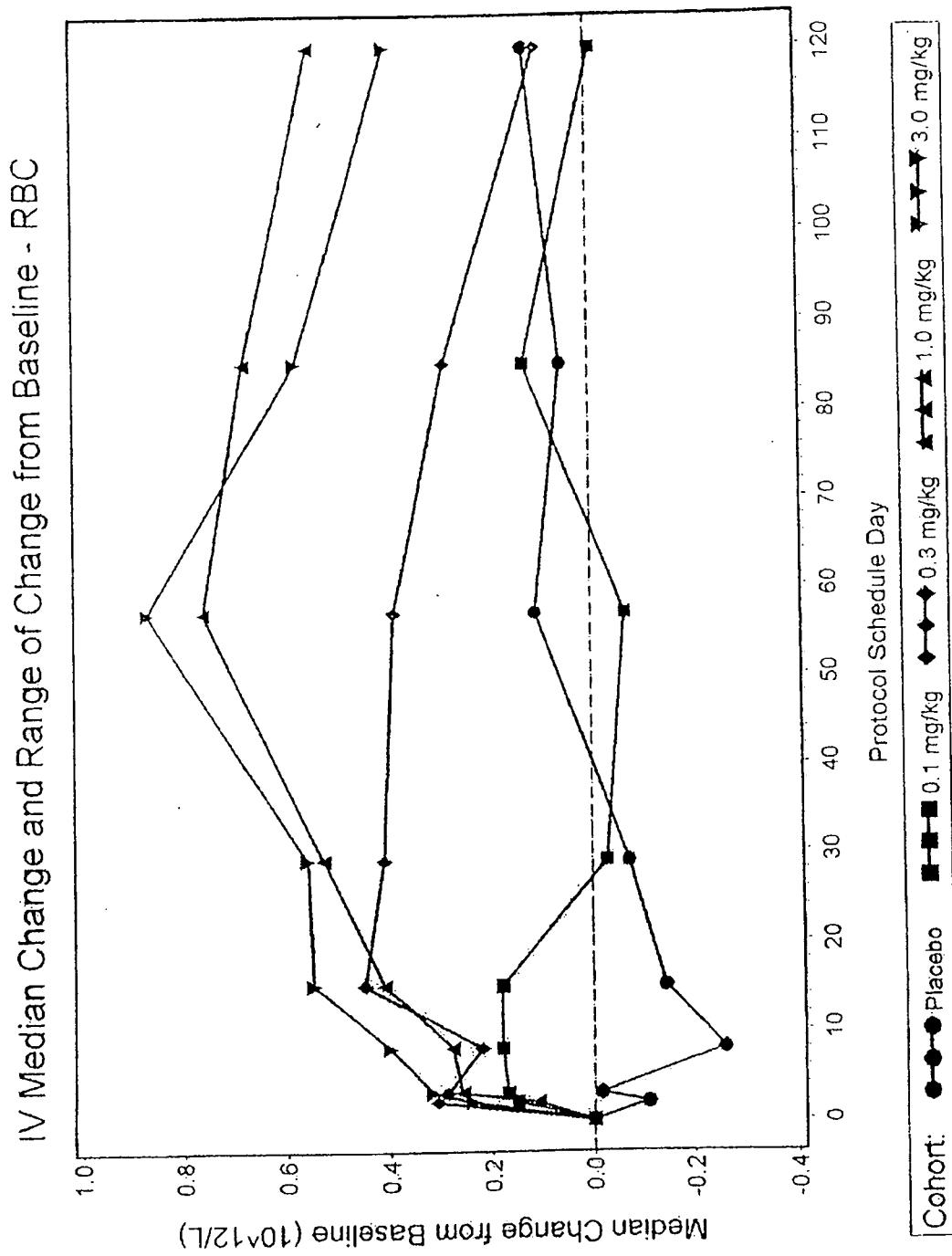


Figure 12

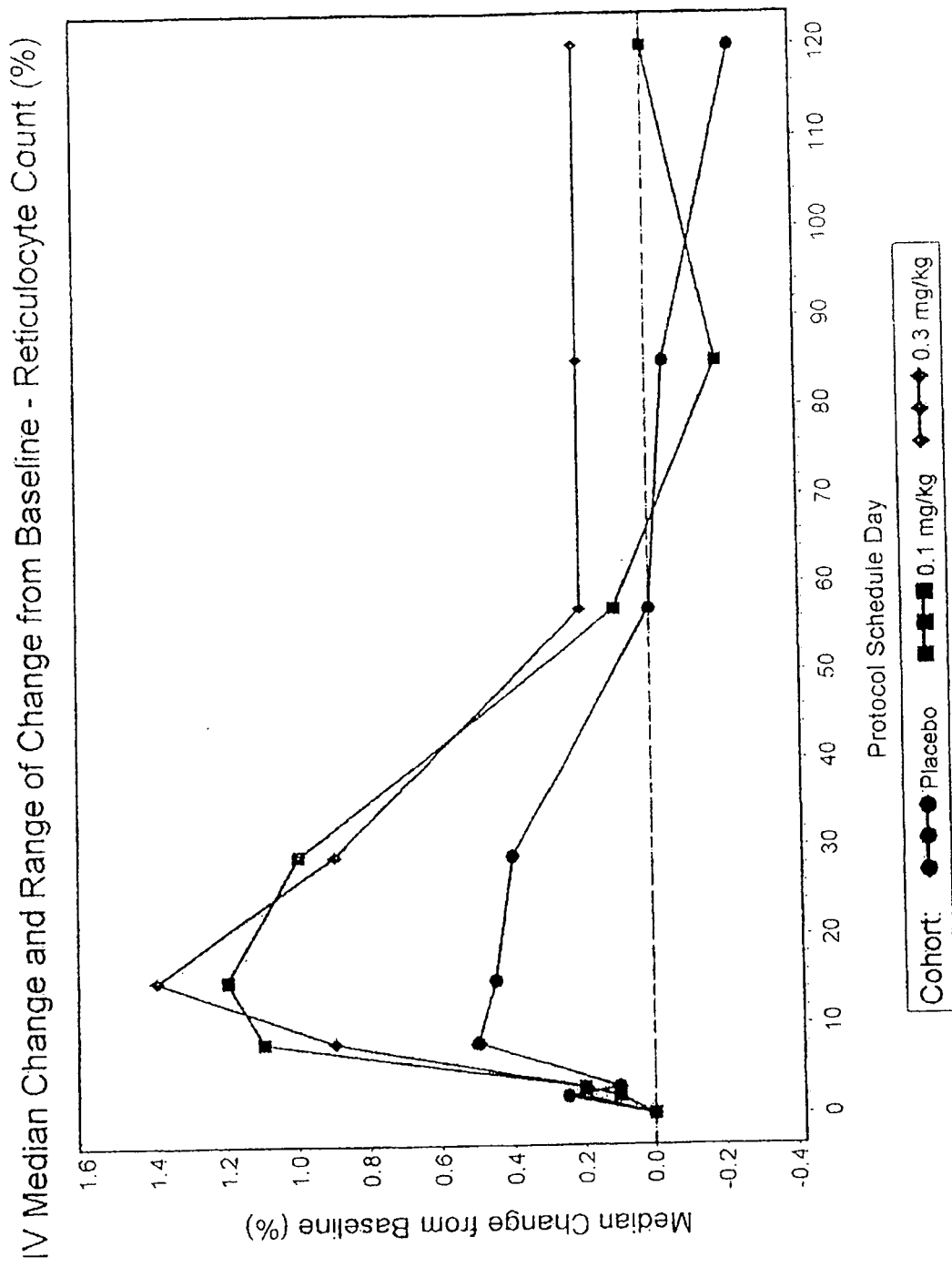


Figure 13

ActRIIa ILGRSETQEC IFFNANWEKD RTNQTGVEPC YGDKDKRRHC FATWKNISGS
ActRIIb GRGEAETREC IYYNANWELE RTNQSGLERC EGEQDKRLHC YASWRNSSGT

IEIVKQGCWL DDINCYDRTD CVEKKDSPEV YFCCCEGNMC NEKFSYFPDM
IELVKKGCWL DDFNCYDRQE CVATEENPQV YFCCCEGNFC NERFTHLPEA

EVTQPTSNPV TPKPPT
GGPEVTYPEPP PTAPT

FIGURE 14

FIGURE 15. Effect of ActRIIA-mFc on chemotherapy-induced anemia in mice

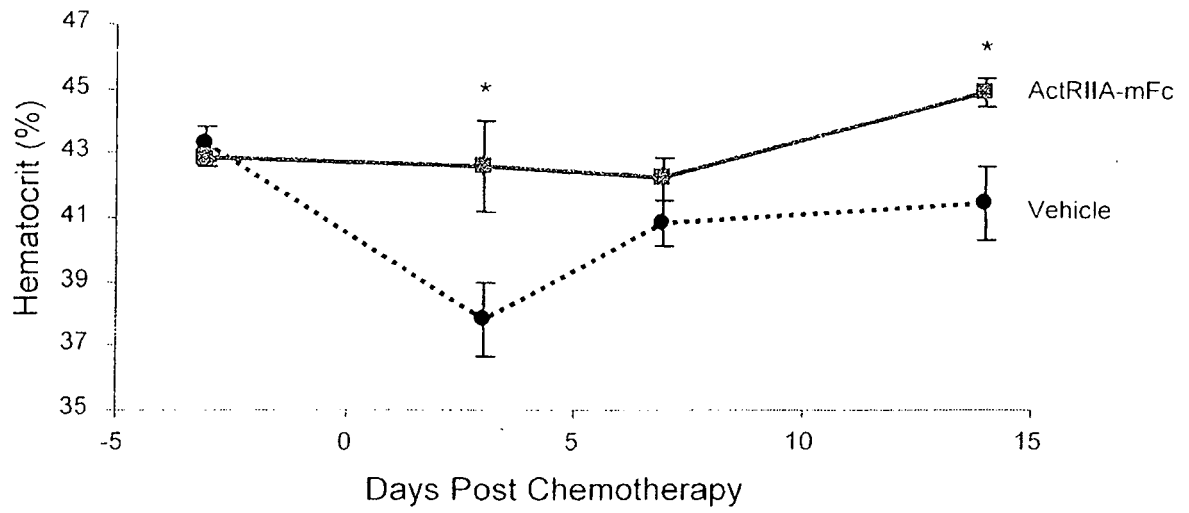


FIGURE 15

FIGURE 16. Dose-dependent effect of ActRIIA-mFc on chemotherapy-induced anemia in mice

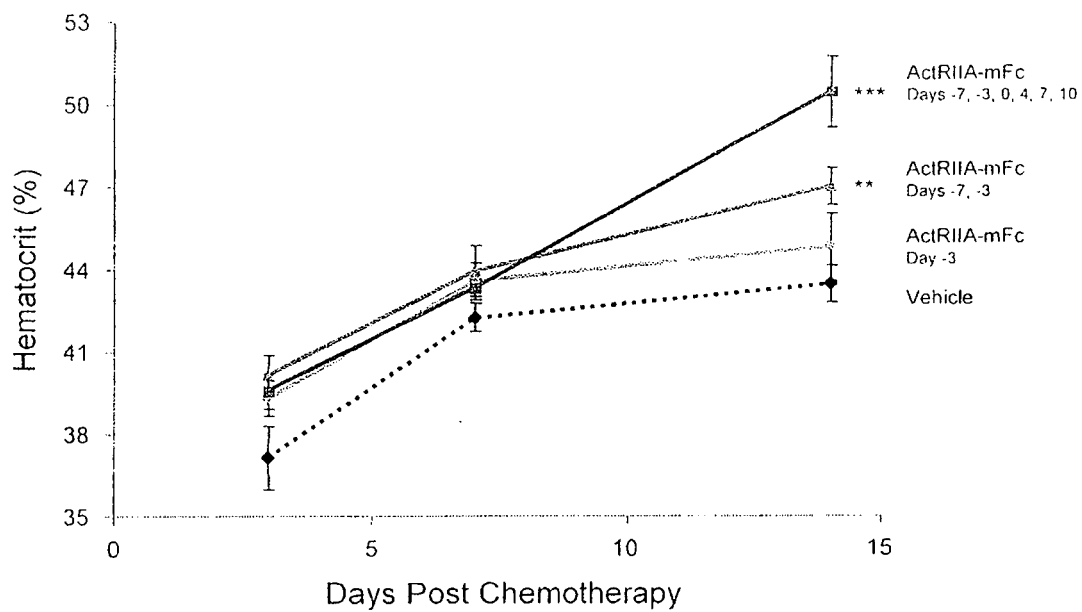


FIGURE 16

FIGURE 17. Effect of ActRIIA-mFc on anemia in mouse model of chronic kidney disease

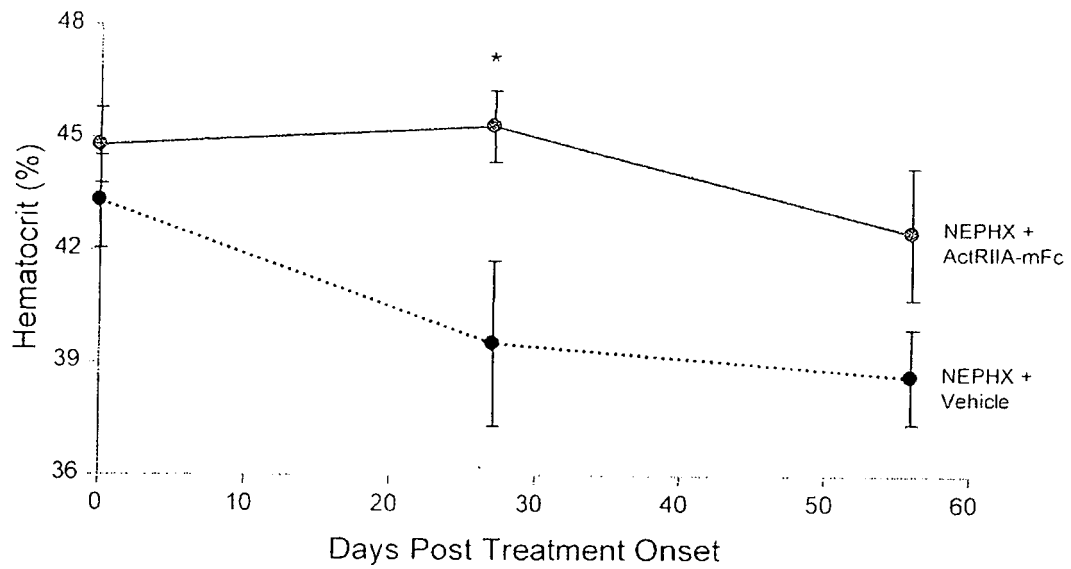


FIGURE 17