Antibodies, which inhibit binding of Dkk-1 to LRP5, are useful in compositions for stimulating bone growth, in particular, compositions for treating bone disorders which result in a loss in bone, for example, osteoporosis.
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TITLE OF THE INVENTION
ANTIBODIES SPECIFIC FOR DKK-1

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to antibodies and immunologically functional fragments thereof that selectively bind Dkk-1 and their use for treating a variety of different diseases including preventing or treating conditions relating to loss of bone mass or to stimulate production of new bone, as well as various non-bone related disorders.

(2) Description of Related Art

The skeletal disorder osteoporosis is the leading cause of morbidity in the elderly. Osteoporosis is characterized by bone loss resulting from an imbalance between bone resorption (destruction) and bone formation. This condition leads to an increased risk of bone fractures, which may occur following low levels of trauma. In the United States, there are currently about 20 million people with detectable fractures of the vertebrae due to osteoporosis. Mortality due to bone fractures is not uncommon among the elderly patient population.

Elderly, post-menopausal women are at the highest risk of developing osteoporosis due to a deficiency of estrogen, which is necessary for proper bone maintenance. Insufficient estrogen levels lead to increased production and longevity of destructive osteoclasts, which, in turn, leads to increased bone resorption. As a result, an average of 5% bone loss is observed in the vertebrae per year. Although less common, osteoporosis also affects elderly men. The existence of osteoporosis in elderly men may also be due, in part, to insufficient estrogen levels caused by a decrease in circulating testosterone.

Therapeutic strategies for overcoming bone loss include both the prevention of bone resorption and the stimulation of bone growth. The majority of therapeutic targets that have led to efficacious osteoporosis treatments fall into the former category. Thus, the first line of treatment/prevention of this condition has historically been the inhibition of bone resorption using compounds such as bisphosphonates, estrogens, selective estrogen receptor modulators (SERMs) and calcitonin. Because inhibition of bone resorption cannot restore bone mass, this approach is an ineffective treatment for patients who have already lost a significant amount of bone. Additionally, the effectiveness of osteoporosis treatments that function by this mechanism is not consistent across the skeletal anatomy because the rate of bone turnover differs from one site to another. For example, the bone turnover rate is higher in the trabecular bone of the vertebrae than in the cortex of the long bones; thus, bone resorption inhibitors are less effective in increasing hip bone mineral density (BMD) and preventing hip fracture. Therefore, osteoanabolic agents, which increase cortical/periosteal bone formation and bone mass at long bones, would address an unmet need in the treatment of osteoporosis, especially for patients with high risk of hip fractures.
One potential therapeutic target for metabolic disorders, including osteoporosis, is the low-density lipoprotein receptor related protein 5 (LRP5). LRP5 belongs to the low density lipoprotein receptor (LDLR) gene family of cell surface receptors, characterized by cysteine-rich, complement-type LDLR ligand binding domains. LRP5 was isolated based on its proximity to the locus of osteoporosis pseudoglioma syndrome (OPPG), an autosomal recessive disorder characterized by severe osteoporosis (Hey, et al Gene 216: 103-111 (1998); U.S. Patent Nos. 6,555,654 and 6,545,137). Additional support for the notion that LRP5 represents a therapeutic target for osteoporosis comes from the observation that loss of function mutations of LRP5 lead to OPPG (Gong et al, Cell 107: 513-523 (2001)).

Interestingly, aberrant expression of LRP5 is also associated with high bone mass trait (HBM), an autosomal dominant human genetic skeletal condition characterized by strikingly increased bone mass. Positional cloning of the HBM mutation demonstrated that HBM results from a G171V mutation of the LRP5 gene which leads to a gain of function (See for example, Little et al, Am. J. Hum. Genet. 70: 11-19 (2002); U.S. Patent Nos. 6,770,461 and 6,780,609; U.S. Published Patent Application Nos. 20040038860 and 20050070699). These findings, together with the fact that null mutation of LRP5 in mice results in severe bone loss (Kato, J. Cell Biol. 157(2): 303-314 (2002)), demonstrated an essential role for LRP5 in bone formation and bone mass in humans.

Despite its specific role in stimulating bone growth, the LRP5 gene was shown to have a nearly ubiquitous expression profile. The mechanism by which activation of LRP5 leads to osteogenesis is not known. At the molecular level, it was recently shown that LRP5 and a closely related LRP6 are involved in Wnt signaling as co-receptors for Wnt. Wnt genes encode secreted proteins implicated in a diverse array of developmental and adult physiological processes, such as mediating cell growth and differentiation in the central nervous system. It was also shown that LRP5 and LRP6 are receptors for the secreted protein dickkopf-1 (Dkk-1) and that their association with Dkk-1 represses Wnt signaling (Mao et al, Nature 411: 321-325 (2001); Semenov et al, Curr. Biol., (2001); Bafico et al, Nat Cell Biol 3: 683-686 (2001)).

Dickkopf-1 (Dkk-1) is a secreted protein that participates in embryonic head induction and antagonizes Wnt (Glinka et al, Nature 391: 357-362 (1998)). The amino acid sequence of human Dkk-1 and nucleotides encoding it have been described (U.S. Patent Nos. 6,344,541; 6844422; 7,057,017; Published Patent Application No. 20050069915; Krupnick et al, Gene 238: 301-313(1999)). Expression of Dkk-1 in human was thought to be restricted to placenta, suggesting a role for Dkk-1 in embryonic development (Krupnick et al, supra). Allen and colleagues (U.S. Published Patent Application No. 20040038860) describe assays relating to the interaction between LRP5, HBM or LRP6 with Dkk-1. Antibodies that bind Dkk-1 have been described in the aforementioned patents and patent applications and in U.S. Published patent Application Nos. 20050079173 and 20060127393.

Human Dkk-1 is a member of a Dickkopf gene family which includes Dkk-1, Dkk-2, Dkk-3, and Dkk-4 (Krupnick et al, supra). Although Dkk-1 and Dkk-4 have been shown to suppress Wnt-induced secondary axis induction in Xenopus embryos, neither block axis induction triggered by
Xenopus Dishevelled or Frizzled, suggesting that their Wnt inhibitory activity is upstream of Frizzled in the Wnt signaling pathway (Krupnick et al., supra). It has been suggested that Dkk-1 might have an inhibitory effect on bone formation, making them potential targets for the prevention or treatment of osteoporosis (Patel and Kareasny, N. Eng. J. Med. 346: 1572-1573 (2002); Boyden et al, N. Eng. J. Med. 346: 1513-1521 (2002)). There is a need for reagents and methods that will selectively inhibit the interaction of Dkk-1 with LRP5/6 and thus stimulate the Wnt signaling pathway in bone with a corresponding increase in bone anabolism without cross reacting other members of the Dickkopf gene family.

BRIEF SUMMARY OF THE INVENTION

The present invention provides antibodies and immunologically functional fragments thereof that selectively bind Dkk-1. The antibodies and immunologically active fragments also block or reduce binding between Dkk-1 and LRP5 and/or LRP6, thereby stimulating at least one activity associated with Wnt signaling. In particular, the antibodies and immunologically functional fragments thereof selectively inhibit the interaction of Dkk-1 with LRP5/6 and thus stimulate the Wnt signaling pathway in bone with a corresponding increase in bone mass without detectable cross reaction with other members of the Dickkopf gene family. The antibodies and fragments include antibodies with a naturally occurring structure, as well as polypeptides that have an antigen binding domain (for example, a domain antibody). The antibodies and fragments can be used to treat a variety of different diseases including preventing or treating conditions relating to loss of bone mass or to stimulate production of new bone, as well as various non-bone related disorders. Nucleic acids molecules, vectors, and host cells useful in the production of the antibodies and selective binding agents are also provided.

Some of the antibodies and immunologically functional fragments that are provided include (a) one or more light chain (LC) complementary determining regions (CDRs) selected from the group consisting of (i) an LC CDR1 with at least 80% sequence identity to SEQ ID NO: 12, (ii) an LC CDR2 with at least 80% sequence identity to SEQ ID NO: 13; and (iii) an LC CDR3 with at least 80% sequence identity to SEQ ID NO: 14; (b) one or more heavy chain (HC) CDRs selected from the group consisting of (i) an HC CDR1 with at least 80% sequence identity to SEQ ID NO: 9; (ii) an HC CDR2 with at least 80% sequence identity to SEQ ID NO: 10; and (iii) a HC CDR3 with at least 80% sequence identity to SEQ ID NO: 11; or (c) one or more LC CDRs of (a) and one or more HC CDRs of (b).

Such antibodies or fragments can specifically bind a Dkk-1 polypeptide. Certain antibodies or fragments include one, two, three, four, five or all six of the foregoing CDRs.

The light chain and heavy chains of other antibodies or fragments are provided as are described above but have at least 90% sequence identity to the foregoing sequences. Still other antibodies or fragments thereof have a light chain in which CDR1 has the amino acid sequence as set forth in SEQ ID NO: 12, CDR2 has the amino acid sequence as set forth in SEQ ID NO: 13 and/or CDR3 has the amino acid sequence as set forth in SEQ ID NO: 14. Some antibodies and fragments may also
have a heavy chain in which CDR1 has the amino acid sequence as set forth in SEQ ID NO:9, CDR2 has the amino acid sequence as set forth in SEQ ID NO: 10 and/or HC CDR3 has the amino acid sequence as set forth in SEQ ID NO: 11. Particular antibodies or fragments include a light chain CDR3 with the amino acid sequence of SEQ ID NO: 14 and/or a heavy chain CDR3 with the amino acid sequence of SEQ E) NO: 11.

Further provided are antibodies and immunologically functional fragments that are include (a) a light chain variable region (VL) having at least 80% sequence identity with SEQ ID NO:4; (b) a heavy chain variable region (VH) having at least 80% sequence identity with SEQ ID NO:8; or (c) a VL θf(a) and a VH of(b).

Further still, provided are antibodies or immunologically functional fragments that are similar in structure but the VL has at least 90% sequence identity with SEQ ID NO:4; and the VH has at least 90% sequence identity with SEQ ID NO:8. In particular antibodies or functional fragments, the VL has at least 95% sequence identity with SEQ ID NO:4; and the VH has at least 95% sequence identity with SEQ ID NO:8. In further still aspects, the antibodies or immunologically functional fragments include a VL that has the amino acid sequence of SEQ ID NO:4, and/or a VH that has the amino acid sequence of SEQ ID NO:8.

Some antibodies or fragments have a light chain that comprises or consists of the amino acid sequence of SEQ ID NO:2 or 3 and/or a heavy chain that comprises or consists of the amino acid sequence of SEQ ID NO:6 or 7.

Also included are antibodies or an immunologically functional fragments that specifically bind a mature human Dkk-1 protein consisting of amino acids 32-266 of SEQ ID NO:35 and having a tertiary structure established by a disulfide bond between cysteine residues 220 and 245, wherein the antibody binds to an epitope comprising in part a loop consisting of the amino acids between cysteine residues 201 and 210 of SEQ ID NO:35.

Further provided are antibodies or fragments that compete with an antibody such as those described above for specific binding to a Dkk-1 polypeptide. For example, some antibodies and fragments compete with an antibody that consists of two identical heavy chains and two identical light chains, wherein the heavy chains consist of the amino acid sequence set forth in SEQ ID NO:3 and the light chains consist of amino acid sequence set forth in SEQ ID NO:7.

The various antibodies and fragments that are provided can include a single light and/or heavy chain or a single variable light domain and/or a single variable heavy domain. Other antibodies and fragments include two light and/or two heavy chains. In those instances in which the antibody or fragment includes two light and/or heavy chains, the two light chains in some instances are identical to one another; likewise, the two heavy chains in some instances are identical. The antibodies that are provided may include, for example, monoclonal antibodies, a human antibody, a chimeric antibody, or a humanized antibody. The immunologically functional fragments may include, but are not limited to, a
scFv, a Fab, a Fab', a (Fab')2, or a domain antibody. In some instances, the antibody or fragment dissociates from a Dkk-1 polypeptide with a Kd of about 269 pM or less.

Further provided are pharmaceutical compositions that include any of the foregoing antibodies and immunologically active fragments. Such compositions typically also include a buffer, a pharmaceutically acceptable diluent, a carrier, a solubilizer, an emulsifier, or a preservative. The use of the foregoing antibodies and immunologically active fragments in the preparation of a pharmaceutical composition or medicament is also described.

A variety of nucleic acids encoding the foregoing antibodies are also provided. Some nucleic acids, for instance, encode (a) a light chain CDR with the amino acid sequence as set forth in SEQ ID NO:14; and/or (b) a heavy chain CDR with the amino acid sequence as set forth in SEQ ID NO:1, such that the encoded CDR(s) encode an antibody or an immunologically functional fragment thereof that can specifically bind a Dkk-1 polypeptide. In particular aspects, the nucleic acids comprise or consist of a sequence that encodes a variable light region (VL) and/or a variable heavy region (VH) of an antibody or immunologically active fragment, wherein the VL has at least 80%, 90% or 95% sequence identity with SEQ ID NO:4 and the VH has at least 80%, 90%, or 95% sequence identity with SEQ ID NO:8. Some of the nucleic acids include a sequence that encodes a VL that comprises or consists of SEQ ID NO:4 and/or a sequence that encodes a VH that comprises or consists of SEQ ID NO:8. Still other nucleic acids include sequences that encode both a VL or VH with the foregoing sequence characteristics. Expression vectors comprising the foregoing nucleic acids are also disclosed herein, as are cells (for example, lower eukaryotic cells such as yeast cells or higher eukaryote cells such as mammalian cells such as CHO cells or insect cells) that comprise such expression vectors. Methods of producing an antibody or an immunologically active fragment thereof by culturing cells that contain such expression vectors are also described.

In another aspect, the use of the foregoing antibodies or immunologically functional fragments in the treatment of a variety of diseases is disclosed. In particular methods, an effective amount of an antibody or immunologically active fragment as described herein is administered to an individual in need thereof to treat osteoporosis, arthritis, multiple myeloma, metastatic bone disease, periodontal disease, diseases responsive to stem cell renewal, inflammatory diseases, neurological diseases, ocular diseases, renal diseases, pulmonary diseases, and skin diseases. Some treatment methods involve treating rheumatoid arthritis, psoriatic arthritis or osteoarthritis.

Further provided herein are methods of treating or preventing loss of bone mass comprising administering to an individual in need thereof a therapeutically effective amount of an antibody or immunologically functional fragment thereof as described herein. In a particular aspect, the individual is one that suffers from osteoporosis or other bone loss disease or disorder, for example, osteopenia, Paget's disease, periodontitis, rheumatoid arthritis, and bone loss due to immobilization. In a further aspect of this embodiment, the individual is one who suffers from cancer that metastasizes to bone, and in another aspect, the patient is one who suffers from multiple myeloma.
Methods of inducing or stimulating increased bone mass are also disclosed. Such methods involve administering to an individual a therapeutically effective amount of an antibody or immunologically functional fragment thereof as disclosed herein. In one aspect, the individual suffers from cancer that metastasizes to bone, and in another aspect, the patient suffers from multiple myeloma. In yet another aspect, the individual is selected from those who have osteoporosis, osteopenia, Paget's disease, periodontitis, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and bone loss due to immobilization. In an additional aspect of this method, the individual is a bone graft recipient or one who suffers from a bone fracture.

The Dkk-1 antibodies and immunologically functional fragments thereof disclosed herein may provide a therapeutic treatment for alleviating the bone-destructive effects of cancer cells (for example, multiple myeloma, breast cancer, prostate cancer, and the like) invading the bone micro-environment.

In light of the above, further provided is a method of inducing Wnt activity in an individual comprising administering to the individual a therapeutically effective amount of an antibody or immunologically functional fragment thereof as described herein.

Definitions

As used herein, the terms "antibody," "immunoglobulin," "immunoglobulins" and "immunoglobulin molecule" are used interchangeably. Each immunoglobulin molecule has a unique structure that allows it to bind its specific antigen, but all immunoglobulins have the same overall structure as described herein. The basic immunoglobulin structural unit is known to comprise a tetramer of subunits. Each tetramer has two identical pairs of polypeptide chains, each pair having one "light" chain (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively.

The light and heavy chains are subdivided into variable regions and constant regions (See generally, Fundamental Immunology (Paul, W., ed., 2nd ed. Raven Press, N.Y., 1989), Ch. 7. The variable regions of each light/heavy chain pair form the antibody binding site. Thus, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are the same. The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. The terms include naturally occurring forms, as well as fragments and derivatives. Included within the scope of the term are classes of immunoglobulins (Igs), namely, IgG, IgA, IgE, IgM, and IgD.
Also included within the scope of the terms are the subtypes of IgGs, namely, IgGi, IgG2, IgG3 and IgG4. The term is used in the broadest sense and includes single monoclonal antibodies (including agonist and antagonist antibodies) as well as antibody compositions which will bind to multiple epitopes or antigens. The terms specifically cover monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (for example, bispecific antibodies), and antibody fragments so long as they contain or are modified to contain at least the portion of the CH2 domain of the heavy chain immunoglobulin constant region which comprises an N-linked glycosylation site of the CH2 domain, or a variant thereof. In addition, these terms can refer to an antibody fragment of at least the Fab region that at least contains an N-linked glycosylation site.

The term "Fc" fragment refers to the 'fragment crystallized' C-terminal region of the antibody containing the CH2 and CH3 domains (Figure 1). The term "Fab" fragment refers to the 'fragment antigen binding' region of the antibody containing the VH, CH1, VL and CL domains (See Figure 1).

The term "monoclonal antibody" (mAb) as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each mAb is directed against a single determinant on the antigen. In addition to their specificity, monoclonal antibodies are advantageous in that they can be synthesized by hybridoma culture, uncontaminated by other immunoglobulins. The term "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies herein can be made by the hybridoma method first described by Kohler et al., (1975) Nature, 256:495, or may be made by recombinant DNA methods (See, for example, U.S. Patent No. 4,816,567).

The term "fragments" within the scope of the terms "antibody" or "immunoglobulin" include those produced by digestion with various proteases, those produced by chemical cleavage and/or chemical dissociation and those produced recombinantly, so long as the fragment remains capable of specific binding to a target molecule. Among such fragments are Fc, Fab, Fab', Fv, F(ab')2, and single chain Fv (scFv) fragments. Hereinafter, the term "immunoglobulin" also includes the term "fragments" as well.

Immunoglobulins further include immunoglobulins or fragments that have been modified in sequence but remain capable of specific binding to a target molecule, including: interspecies chimeric and humanized antibodies; antibody fusions; heteromeric antibody complexes and antibody fusions, such as diabodies (bispecific antibodies), single-chain diabodies, and intrabodies (See, for example,

The term "epitope" refers to a site on an antigen to which B and/or T cells respond or a site on a molecule against which an antibody will be produced and/or to which an antibody will bind. For example, an epitope can be recognized by an antibody defining the epitope. A linear epitope is an epitope wherein an amino acid primary sequence comprises the epitope recognized. A linear epitope typically includes at least 3, and more usually, at least 5, for example, about 8 to about 10 amino acids in a unique sequence. A conformational epitope, in contrast to a linear epitope, is an epitope wherein the primary sequence of the amino acids comprising the epitope is not the sole defining component of the epitope recognized (e.g., an epitope wherein the primary sequence of amino acids is not necessarily recognized by the antibody defining the epitope). Typically a conformational epitope encompasses an increased number of amino acids relative to a linear epitope. With regard to recognition of conformational epitopes, the antibody recognizes a three-dimensional structure of the peptide or protein. For example, when a protein molecule folds to form a three-dimensional structure, certain amino acids and/or the polypeptide backbone forming the conformational epitope become juxtaposed enabling the antibody to recognize the epitope.

Methods of determining conformation of epitopes include but are not limited to, for example, x-ray crystallography, two-dimensional nuclear magnetic resonance spectroscopy and site-directed spin labeling and electron paramagnetic resonance spectroscopy. See, for example, Epitope Mapping Protocols in Methods in Molecular Biology (1996) Vol. 66, Morris (Ed.).

As used herein, the term "Dkk-1" includes, for example, rhesus monkey, murine, and human forms of Dkk-1. The amino acid sequences for the human and Rhesus monkey Dkk-1 proteins are shown, respectively, in SEQ ED NOS:35 and 38. The human Dkk-1 protein (SEQ ID NO:35) has a leader sequence consisting of amino acids 1-31 of SEQ ID NO:35. The murine Dkk-1 protein sequence has been disclosed in Glinka, *et al.*, Nature 391: 357-362 (1998). The Rhesus monkey Dkk-1 has been disclosed in International Publication No. WO2005049640. The term "Dkk-1" also includes variants of such native sequences that are immunologically cross-reactive with these native proteins. These Dkk-1 proteins can inhibit the interaction between LRP5 or LRP6 proteins with Wnt. An exemplary amino acid sequence for the human LRP5 is given in SEQ ID NO:39. An exemplary amino acid sequence encoding human LRP6 is given in SEQ ID NO:40. The term can also refer to a fragment of a native or variant form of Dkk-1 that contains an epitope to which the antibody disclosed herein can specifically bind.

The term "osteopenia" refers to a patient with bone loss of at least one standard deviation compared with a standard patient considered to have normal bone mineral density (BMD). For present purposes, the measurement is determined by Dual Energy X-ray Absorptiometry (DEXA) and the patient's BMD is compared with an age and gender-matched standard (Z score), hi determining osteopenia, BMD measurements may be taken of one or more bones.
The term "therapeutically effective amount" refers to the amount of an anti-Dkk-1 antibody determined to produce a therapeutic response in a mammal. Such therapeutically effective amounts are readily ascertained by one of ordinary skill in the art.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows a diagram of the plasmid encoding the RH2-18 light chain. OriP is the Epstein Barr virus origin of replication for expression in eukaryotic cells. HCMV intron A promoter is the human cytomegalovirus promoter and first intron. LC Lambda encodes the light chain lambda constant region. Leader encodes a leader or signal sequence for secretion of the light chain polypeptide into the culture medium. BGH pA is the bovine growth hormone polyadenylation signal sequence. SV40 promoter is the SV40 virus promoter. GS is Glutamine synthase. SV40 is the SV40 polyadenylation signal sequence. Kan is the kanamycin gene for selection of the vector in *E. coli*.

Figure 1B shows a diagram of the plasmid encoding the RH2-18 heavy chain. OriP is the Epstein Barr virus origin of replication for expression in eukaryotic cells. HCMV intron A promoter is the human cytomegalovirus promoter and first intron. IgG2M4 encodes the heavy chain IgG2M4 constant region. Leader encodes a leader or signal sequence for secretion of the light chain polypeptide into the culture medium. BGH pA is the bovine growth hormone polyadenylation signal sequence. Kan is the kanamycin gene for selection of the vector in *E. coli*.

Figure 1C shows the amino acid sequences of the RH2-18 light chain and heavy chain amino acid sequences (SEQ ID NO:3 and SEQ ID NO:7, respectively). The leader sequences for the light and heavy chain sequences are not shown. The variable regions are shown in italics.

Figure 1D shows the amino acid sequence of the light chain variable region (SEQ ID NO:4) aligned with the sequence for the region in the germline (SEQ ID NO:16). The three light chain (LC) complementary determining regions (CDRs) are underlined and the amino acid sequence differences in the frameworks between the variable region sequence in RH2-18 and the germline sequence are shown in bold-faced type.

Figure 1E shows the amino acid sequence of the heavy chain variable region (SEQ ID NO:8) aligned with the sequence for the region in the germline (SEQ ID NO:15). The three heavy chain (HC) CDRs are underlined and the amino acid sequence differences in the frameworks between the variable region sequence in RH2-18 and the germline sequence are shown in bold-faced type.

Figure 1F shows the results of a LABCHIP 90 capillary electrophoresis of 12 converted anti-Dkk-1 antibodies purified for *in vitro* analysis. Lane 2 is the RH2-18 anti-Dkk-1 antibody.

Figure 2A shows Eu-Dkk-1 binding to HEK293hLrp5 cells and inhibitory activity of anti-Dkk-1 antibodies RH2-10, RH2-18, RH2-31, RH2-59, and RH2-80 at various concentrations. 8B4 is a control antibody that is non-specific for Dkk-1.
Figure 2B shows the result for RH2-18 antibodies retitrated using an extended dose range. The results show that for this assay format the RH2-18 antibodies had an effective dose of about 5 nM.

Figure 3 shows the neutralizing activities of anti-Dkk-1 antibodies RH1-10, RH2-18, RH2-31, RH2-59, and RH2-80 antibodies on Dkk-1 function in Wnt3A induced signaling. Treatment with Wnt3A significantly stimulated the signaling pathway (black bar) compared to control treatment (open bar). Anti-Dkk-1 antibodies were added at indicated concentrations.

Figure 4 shows the effect of the RH2-18, Rh2-59, and Rh2-80 antibodies on osteoblastic cell differentiation. Differentiation of C3H10T1/2 cells towards the osteoblastic phenotype was determined by increased endogenous ALP activities.

Figure 5A shows a dot-blot binding analysis using antibody RH2-18 showing its specificity for the C-terminal region of Dkk-1. Rhesus monkey Dkk-1 proteins were fused to a green fluorescent protein (GFP) tag (loading control). Full-length rhesus Dkk-1 protein, C-terminal region (ΔN-Dkk-1, encoding residues 159 to 266) or N-terminal region (ΔC-Dkk-1, encoding residues 1-158) were expressed and analyzed by dot-immunoblotting using RH2-18 antibody.

Figure 5B shows a dot-blot binding analysis that shows that RH2-18 antibody binding is lost when various amino acid substitutions are made in the Dkk-1 C-terminal domain. Rhesus Dkk-1 proteins were fused to a GFP tag (loading control). Full-length rhesus Dkk-1 protein, C-terminal region (ΔN-Dkk-1, encoding residues 159 to 266) or N-terminal region (ΔC-Dkk-1) were expressed and analyzed by dot-immunoblotting using RH2-18 antibody. Alanine-substitutions were introduced in ΔN-Dkk-1 and the position numbers of the amino acid residues substituted are indicated.

Figure 5C shows a structural-homology model of Dkk-1 C-terminal domain (amino acids 187 to 266) showing the amino acid residues necessary for binding of RH2-18 antibody. Amino acid numbers given have been substituted by Alanine-scanning. Substitutions of amino acid residues found to result in diminished antigen-antibody interaction in immunoblotting experiments using non-denatured protein are amino acids Si87 to Vi88, R203 to K208, E241, aL243. Amino acid residues (Ri71 to Li74) contributing to the RH2-18 antibody binding epitope outside the Dkk-1-homology model are listed. Substitution of Amino acid C22O causes a loss of RH2-18 antibody binding to Dkk-1. Substitution of the remaining amino acid residues did not appear to affect binding of RH2-18 antibody to Dkk-1.

Figure 6A shows an amino acid sequence alignment of human Dkk-1, Dkk-2, Dkk-4, and rhesus monkey Dkk-1. The conserved amino acids are shown in red and non-conserved amino acids are shown in green. Amino acid residues in Dkk-1 that are necessary for RH2-18 binding are enclosed within blue boxes. Note the lack of sequence conservation between Dkk-1 and Dkk-2 and Dkk-4 for amino acid residues Ri71 to Li74, Si87 to Vi88, S207, and E241.

Figure 6B shows a dot-blot analysis using different Dkk isoforms that shows specificity of the RH2-18 antibodies to Dkk-1. Native recombinant rhesus monkey Dkk-1, Dkk-2, and cynomolgus
monkey Dkk-4 proteins (0.1 ng to 100 ng) were used. A non-related recombinant protein was loaded as a control for non-specific assay signal (HIS protein) and probed with the RH2-18 antibodies.

Figure 7 shows that distal femur bone mineral density (BMD) was increased 5.2 to 8.7% in a dose effect fashion by RH2-18 antibodies in the dose range 0.5 to 5 mg/kg. Error bars = SEM. N=1 I/group.

Figure 8 shows that whole femur BMD was increased 4.7 to 4.8% by RH2-18 antibodies in the dose range 1.5 to 5 mg/kg. Error bars = SEM. N=1 I/group.

Figure 9 shows that central femur BMD was increased 3.2 to 3.5% in a dose effect fashion by RH2-18 antibodies in the dose range 1.5 to 5 mg/kg. Error bars = SEM. N=1 I/group.

Figure 10A shows the transcriptional effects of RH2-80 antibodies (Dkk-IAB) on cultured cancer cells in a TOPflash transcription assay performed in HCT1 16 cells in complete medium. 8B4 was the control non-specific antibody. Con was vehicle control.

Figure 10B shows the transcriptional effects of RH2-80 antibodies (Dkk-IAB) on cultured cancer cells in a cell proliferation assay performed in HCT1 16 cells in complete medium. 8B4 was the control non-specific antibody. Con was vehicle control.

Figure 11 shows a xenograph model for tumor growth and the effect of RH2-59 antibody on tumor growth. Subcutaneous injection of 1x10? HCT1 16 cells in 100 µL PBS into the right flank of six week old NOD.CB 11-Prkdcscid/J (SCID) mice. Treatments were followed on the second day after injection and continued twice week for a total of seven treatments. Phosphate buffered saline (PBS) and a non-specific antibody (NS AB) served as negative controls. Tumors were isolated at about 3.5 weeks. Tumor mass was obtained by excising the tumor post necropsy and weighing. Statistics were performed using one-way ANOVA. No statistical difference was observed among all samples. "Tumor cells were un-intentionally injected into dermis. 1/5 mice in each group had tumor rupture.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions comprising an antibody or immunologically functional fragments thereof, that selectively inhibits binding of Dkk-1 to LRP5 by targeting a multi-dimensional conformational epitope in the C-terminal region of Dkk-1. The antibody further includes modifications to the Fc domain of the antibody, which renders the antibody unable to bind, to a physiologically relevant degree, any Fc receptors or Clq, but without substantial modification of the binding to FcRn or modification of the half-life. In other words, provided is a composition comprising an antibody recognizes a multi-dimensional conformation epitope of the Dkk-1 while neither provoking either antibody-dependent cellular cytotoxicity (ADCC) or complement-mediated cytotoxicity (CMC), nor forming immune complexes. In a currently preferred embodiment, the antibody is a fully human monoclonal antibody, which preferably does not provoke either antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC), or form immune complexes to any extent, while retaining its normal pharmacokinetic (PK) properties. Compositions comprising
the antibody in a growing mouse model for stimulating bone anabolism has shown that compositions comprising the antibody are useful for treatment of osteoporosis.

Of the various osteoanabolic targets, the canonical Wnt signaling pathway in bone offers the best opportunity to elicit an effective and safe anabolic response to therapy. Canonical Wnts signal through two co-receptors, frizzled and the LDL receptor-related proteins (LRPs) 5 and 6. Hypermorphic mutations of LRP5 cause significant increases in BMD in comparison to age-matched normal controls (about 5 SD above the mean in clinical populations). The responsible mutations (e.g., G171V) have been tested in transgenic mice leading to increased bone mass and bone formation rates. Canonical Wnt signaling is blocked in the presence of the inhibitory protein Dickkopf-1 (Dkk1), which is highly expressed in bone. Dkk-1 is a 266 amino acid protein with a 26kDa molecular mass. The protein has two cysteine rich domains-amino acids 97 to 138 and 183 to 245, a motif that is highly conserved among species. Dkk-1 shares a high percentage of sequence identity/similarity between species (human: rhesus 97/99, human: mouse 80/87 and rhesus: mouse 79/87). Interestingly, Dkk-1 loses its ability to inhibit hypermorphic G171 mutants of LRP5, a key signaling defect of the mutated receptor. Further, heterozygous knockout mice lacking Dkk-1 similarly show an increase in bone mass (as do G171V-LRP5 mice), which is accompanied by a four-fold rise on bone formation rates. The composite data surrounding LRP5 and its inhibition by Dkk-1 suggest that an osteoanabolic response could be generated through selective activation of the receptor or by interfering therapies that prevent Dkk-1 inhibition of LRP5 signaling in the bone microenvironment. Indeed, as shown in the Examples, the neutralizing anti-Dkk-1 antibodies disclosed herein (at 0.5 to 5.0 mg/kg, s.c. twice weekly) increased bone mass in growing mice with PTH-like effects on the distal and whole femur. Other anti-Dkk-1 antibodies have also been shown to increase bone mass in growing mice, for example, see for example, U.S. Published Application No. 20060127393.

Therefore, fore, a variety of anti-Dkk-1 antibodies and immunologically functional fragments thereof, including single chain antibodies, domain antibodies, and polypeptides with an antigen binding region, useful for regulating the activity of Dkk-1 are provided. These anti-Dkk-1 antibodies and immunologically functional fragments thereof specifically bind to the human Dkk-1 polypeptide, relieve Dkk-1 inhibition of the Wnt signaling pathway, and induce bone formation in bone tissue.

In certain embodiments of the invention, the anti-Dkk-1 antibody is of the IgG1, IgG2 or IgG4 subtype. In preferred embodiments, the antibody is a fully human monoclonal antibody, which preferably does not provoke either antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC), or form immune complexes to any extent, while retaining its normal pharmacokinetic (PK) properties. In a currently preferred embodiment, the antibody has an IgG2m4 isotype (See U.S. Application No. 11/581,931 filed October 17, 2006 and U.S. Application 11/256,332 filed October 21, 2005).
The variable regions of each light/heavy immunoglobulin chain pair comprising an antibody typically forms the antigen binding site. Variable regions of immunoglobulin chains generally exhibit the same overall structure consisting of relatively conserved framework regions (FR) joined by three hypervariable regions or "complementarily determining regions" (CDRs). The CDRs from the two chains of each heavy chain/light chain pair typically are aligned by the framework regions to form a structure that binds specifically with a specific epitope on the target protein. From the N-terminal to C-terminal of the immunological chain, naturally-occurring light and heavy chain variable regions both typically conform with the following order of these elements: FRl, CDRl, FR2, CDR2, FR3, CDR3 and FR4. A numbering system has been devised for assigning numbers to amino acids that occupy positions in each of these domains. This numbering system has been described in Chothia and Lesk, J. Mol. Biol. 196: 901-917 (1987); Chothia et al, Nature 342: 878-883 (1989).

Specific examples of some of the full-length light and heavy immunoglobulin chains of the anti-Dkk-1 antibodies that are provided and their corresponding nucleotide and amino acid sequences are summarized in Table 1.

| Antibody Name | Chain Name | Chain Type | Nucleotide Sequence (SEQ ID NO: | Amino Acid Sequence (SEQ ED NO: | |
|---------------|------------|------------|--------------------------------|-------------------------------|
| RH1-10        | L1         | Light      | 17                             | 18                           |
| RH2-18        | L2         | Light      | 2                              | 2                             |
| RH2-59        | L3         | Light      | 21                             | 22                           |
| RH2-80        | L4         | Light      | 25                             | 26                           |
| RH1-10        | H1         | Heavy      | 19                             | 20                           |
| RH2-18        | H2         | Heavy      | 5                              | 6                             |
| RH2-59        | H3         | Heavy      | 23                             | 24                           |
| RH2-80        | H4         | Heavy      | 27                             | 28                           |

An anti-Dkk-1 antibody can be formed by combining any one of the light chains listed in Table 1 with any of the heavy chains listed in Table 1. In some instances, the antibody include at least one heavy chain and one light chain from those listed in Table 1 and in other instances, the antibody contains two identical light chains and two identical heavy chains. As an example, an antibody or immunologically functional fragment can include two L2 light chains and two H1 heavy chains, or two L2 light chains and two H3 heavy chains, or two L2 light chains and two H4 heavy chains and other similar combinations of pairs of light chains and pairs of heavy chains as listed in Table 1.

Exemplary anti-Dkk-1 antibodies capable of binding to the aforementioned multidimensional conformational epitope in the C-terminal region of Dkk-1 are the monoclonal antibodies.
RHI-IO, RH2-18, RH2-59, and RH2-80 (see, examples below), each of which comprises a light chain and a heavy chain.

The complete light chain of RHI-IO is encoded by the nucleotide sequence shown in SEQ ID NO:17, and the complete heavy chain of RHI-IO by the nucleotide sequence shown in SEQ ID NO:19. The corresponding light and heavy chain amino acid sequences of RHI-10 are shown, respectively, in SEQ ID NOS: 18 and 20. Amino acid residues 1 to 20 of SEQ ID NO:18 and residues 1 to 19 of SEQ ID NO:20 correspond to the signal sequences of these the light and heavy chains of RHI-10, respectively. The amino acid sequence of the light chain without the signal sequence is shown in SEQ ID NO:42, the amino acid sequence of the heavy chain lacking the signal sequence is shown in SEQ ID NO:41. Thus, in one aspect of the foregoing embodiment, the heavy chain may consist of amino acids 20 to 457 of SEQ ID NO:20 (H1 corresponding to SEQ ID NO:41), and in another aspect of this embodiment, the light chain may consist of amino acids 21 to 237 of SEQ ID NO:18 (L1 corresponding to SEQ ID NO:42). In yet another aspect of this embodiment, the antibody comprises both a heavy chain consisting of amino acids 20 to 457 of SEQ ID NO:20 and a light chain consisting of amino acids 21 to 237 of SEQ ID NO:18. In some instances, the antibody consists of two identical heavy chains each consisting of amino acids 20-457 of SEQ ID NO:20 and two identical light chains each consisting of amino acids 21 to 237 of SEQ ID NO:18.

The complete light chain of RH2-18 is encoded by the nucleotide sequence shown in SEQ ID NO:1, and the complete heavy chain of RH2-18 by the nucleotide sequence shown in SEQ ID NO:5. The corresponding light and heavy chain amino acid sequences of RH2-18 are shown, respectively, in SEQ ID NOS:2 and 6. Amino acid residues 1 to 20 of SEQ ID NO:2 and residues 1 to 19 of SEQ ID NO:6 correspond to the signal sequences of these the light and heavy chains of RH2-18, respectively. The amino acid sequence of the light chain without the signal sequence is shown in SEQ ID NO:3, the amino acid sequence of the heavy chain lacking the signal sequence is shown in SEQ ID NO:7. Thus, in one aspect of the foregoing embodiment, the heavy chain may consist of amino acids 20 to 457 of SEQ ID NO:6 (H2 corresponding to SEQ ID NO:7), and in another aspect of this embodiment, the light chain may consist of amino acids 21 to 237 of SEQ ID NO:2 (L2 corresponding to SEQ ID NO:3). In yet another aspect of this embodiment, the antibody comprises both a heavy chain consisting of amino acids 20 to 457 of SEQ ID NO:6 and a light chain consisting of amino acids 21 to 237 of SEQ ID NO:2. In some instances, the antibody consists of two identical heavy chains each consisting of amino acids 20-457 of SEQ ID NO:6 and two identical light chains each consisting of amino acids 21 to 237 of SEQ ID NO:2.

The complete light chain of RH2-59 is encoded by the nucleotide sequence shown in SEQ ID NO:21, and the complete heavy chain of RH2-59 by the nucleotide sequence shown in SEQ ID NO:23. The corresponding light and heavy chain amino acid sequences of RH2-59 are shown, respectively, in SEQ ID NOS:22 and 24. Amino acid residues 1 to 20 of SEQ ID NO:22 and residues 1 to 19 of SEQ ID NO:24 correspond to the signal sequences of these the light and heavy chains of RH2-
59, respectively. The amino acid sequence of the light chain without the signal sequence is shown in SEQ ID NO:44, the amino acid sequence of the heavy chain lacking the signal sequence is shown in SEQ ID NO:43. Thus, in one aspect of the foregoing embodiment, the heavy chain may consist of amino acids 20 to 457 of SEQ ID NO:24 (H3 corresponding to SEQ ID NO:43), and in another aspect of this embodiment, the light chain may consist of amino acids 21 to 237 of SEQ ID NO:22 (L3 corresponding to SEQ ID NO:44). In yet another aspect of this embodiment, the antibody comprises both a heavy chain consisting of amino acids 20 to 457 of SEQ ID NO:24 and a light chain consisting of amino acids 21-237 of SEQ ID NO:22. In some instances, the antibody consists of two identical heavy chains each consisting of amino acids 20 to 457 of SEQ ID NO:24 and two identical light chains each consisting of amino acids 21 to 237 of SEQ ID NO:22.

The complete light chain of RH2-80 is encoded by the nucleotide sequence shown in SEQ ID NO:25, and the complete heavy chain of RH2-80 by the nucleotide sequence shown in SEQ ID NO:27. The corresponding light and heavy chain amino acid sequences of RH2-80 are shown, respectively, in SEQ ID NOS:26 and 28. Amino acid residues 1 to 20 of SEQ ID NO:26 and residues 1 to 19 of SEQ ID NO:28 correspond to the signal sequences of these the light and heavy chains of RH2-80, respectively. The amino acid sequence of the light chain without the signal sequence is shown in SEQ ID NO:46, the amino acid sequence of the heavy chain lacking the signal sequence is shown in SEQ ID NO:45. Thus, in one aspect of the foregoing embodiment, the heavy chain may consist of amino acids 20 to 457 of SEQ ID NO:28 (H4 corresponding to SEQ ID NO:45), and in another aspect of this embodiment, the light chain may consist of amino acids 21 to 237 of SEQ ID NO:26 (L4 corresponding to SEQ ID NO:46). In yet another aspect of this embodiment, the antibody comprises both a heavy chain consisting of amino acids 20 to 457 of SEQ ID NO:28 and a light chain consisting of amino acids 21 to 237 of SEQ ID NO:26. In some instances, the antibody consists of two identical heavy chains each consisting of amino acids 20 to 457 of SEQ ID NO:28 and two identical light chains each consisting of amino acids 21 to 237 of SEQ ID NO:26.

Other anti-Dkk-1 antibodies that are provided are variants of antibodies formed by any combination of the heavy and light chains disclosed above and comprise light and/or heavy chains that each have at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99% identity to the amino acid sequences of these light and heavy chains. In some instances, such antibodies include at least one heavy chain and one light chain, whereas in other instances such variant forms contain two identical light chains and two identical heavy chains.

Also provided are anti-Dkk-1 antibodies that comprise a light chain variable region selected from the group consisting of VL1, VL2, VL3, and VL4 and/or a heavy chain variable region selected from the group consisting of VH1, VH2, VH3, and VH4 as shown in Table 2 below, and immunologically functional fragments, derivatives, muteins and variants of these light chain and heavy chain variable regions.
Table 2

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Chain Name</th>
<th>Chain Type</th>
<th>Nucleotide Sequence (SEQ ID NO:)</th>
<th>Amino Acid Sequence (SEQ ID NO:)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH1-10</td>
<td>VL1</td>
<td>Light</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>RH2-18</td>
<td>VL2</td>
<td>Light</td>
<td>51</td>
<td>4</td>
</tr>
<tr>
<td>RH2-59</td>
<td>VL3</td>
<td>Light</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>RH2-80</td>
<td>VL4</td>
<td>Light</td>
<td>53</td>
<td>49</td>
</tr>
<tr>
<td>RH1-10</td>
<td>VH1</td>
<td>Heavy</td>
<td>54</td>
<td>58</td>
</tr>
<tr>
<td>RH2-18</td>
<td>VH2</td>
<td>Heavy</td>
<td>55</td>
<td>8</td>
</tr>
<tr>
<td>RH2-59</td>
<td>VH3</td>
<td>Heavy</td>
<td>56</td>
<td>59</td>
</tr>
<tr>
<td>RH2-80</td>
<td>VH4</td>
<td>Heavy</td>
<td>57</td>
<td>60</td>
</tr>
</tbody>
</table>

Thus, the anti-Dkk-1 antibodies that are provided thus include, but are not limited to, those having the following form: VL1VH1, VL1VH2, VL1VH3, VL1VH4, VL2VH1, VL2VH2, VL2VH3, VL2VH4, VL3VH1, VL3VH2, VL3VH3, VL3VH4, VL4VH1, VL4VH2, VL4VH3, and VL4VH3. In some instances, the foregoing antibodies include two light chain variable region domains and two heavy chain variable region domains wherein each light chain is the same and each heavy chain is the same. In other instances, the foregoing antibodies include two light chain variable region domains and two heavy chain variable region domains wherein each light chain is different and each heavy chain is different.

As a specific example of such anti-Dkk-1 antibodies, particular antibodies or immunologically functional fragments thereof can comprise the variable region of the light chain or the variable region of the heavy chain of RH2-18, wherein the light chain variable region consists of amino acids 21 to 132 of SEQ ID NO: 2 (VL1 corresponding to SEQ ID NO:4) and the heavy chain variable region consists of amino acids 20 to 131 of SEQ ID NO:6 (VH1 corresponding to SEQ ID NO:8). In one aspect of this embodiment, the antibody consists of two identical heavy chains and two identical light chains. Also provided, for instance, is an antibody comprising a light chain variable region that consists of amino acids 21 to 132 of SEQ ID NO:2 or an antigen-binding or an immunologically functional fragment thereof and further comprising a heavy chain variable region that consists of amino acids 20 to 131 of SEQ ID NO:6.

Particular anti-Dkk-1 antibodies can comprise a light chain variable domain comprising a sequence of amino acids that differs from the sequence of a light chain variable domain selected from VL1, VL2, VL3, or VL4 from 1 up to about 20 amino acid residues, wherein each such sequence difference is independently either a deletion, insertion, or substitution of one amino acid. The light chain variable region in some antibodies comprises a sequence of amino acids that has at least 70%, 75%, 80%,
85%, 90%, 95%, 97%, or 99% sequence identity to the amino acid sequences of the light chain variable region of VL1, VL2, VL3, or VL4.

Particular anti-Dkk-1 antibodies can comprise a heavy chain variable domain comprising a sequence of amino acids that differs from the sequence of a heavy chain variable domain selected from VH1, VH2, VH3, or VH4 from 1 up to about 20 amino acid residues, wherein each such sequence difference is independently either a deletion, insertion, or substitution of one amino acid. The heavy chain variable region in some antibodies comprises a sequence of amino acids that has at least 70%, 75%, 80%, 85%, 90%, 95%, 97% or 99% sequence identity to the amino acid sequences of the heavy chain variable region of VH1, VH2, VH3, or VH4.

Particular anti-Dkk-1 antibodies that are disclosed herein can comprise one or more amino acid sequences that are identical or have substantial sequence identity to the amino acid sequences of one or more of the CDRs as summarized in Table 3.
Table 3
CDRs

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Chain</th>
<th>CDR</th>
<th>Amino Acid Sequence</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH2-18</td>
<td>Light</td>
<td>LC CDR1</td>
<td>TGSSSNIGAGYDVH</td>
<td>12</td>
</tr>
<tr>
<td>RH2-18</td>
<td>Light</td>
<td>LC CDR2</td>
<td>GYSNRPS</td>
<td>13</td>
</tr>
<tr>
<td>RH2-18</td>
<td>Light</td>
<td>LC CDR3</td>
<td>QSYDNSLSSY</td>
<td>14</td>
</tr>
<tr>
<td>RHI-10</td>
<td>Light</td>
<td>LC CDR1</td>
<td>TGSSSNIGAGYDVH</td>
<td>12</td>
</tr>
<tr>
<td>RHI-10</td>
<td>Light</td>
<td>LC CDR2</td>
<td>GNSNRPS</td>
<td>13</td>
</tr>
<tr>
<td>RHI-10</td>
<td>Light</td>
<td>LC CDR3</td>
<td>QSYDNSLSGY</td>
<td>61</td>
</tr>
<tr>
<td>RH2-59</td>
<td>Light</td>
<td>LC CDR1</td>
<td>TGSSSNIGAGYDVH</td>
<td>12</td>
</tr>
<tr>
<td>RH2-59</td>
<td>Light</td>
<td>LC CDR2</td>
<td>ANTRNPS</td>
<td>62</td>
</tr>
<tr>
<td>RH2-59</td>
<td>Light</td>
<td>LC CDR3</td>
<td>QSYDTSPASAYV</td>
<td>63</td>
</tr>
<tr>
<td>RH2-80</td>
<td>Light</td>
<td>LC CDR1</td>
<td>TGSSSNIGAAYDVH</td>
<td>64</td>
</tr>
<tr>
<td>RH2-80</td>
<td>Light</td>
<td>LC CDR2</td>
<td>VNNNRPS</td>
<td>65</td>
</tr>
<tr>
<td>RH2-80</td>
<td>Light</td>
<td>LC CDR3</td>
<td>QSYDNSLNNAYV</td>
<td>66</td>
</tr>
<tr>
<td>RH2-18</td>
<td>Heavy</td>
<td>HC CDR1</td>
<td>DYYIH</td>
<td>9</td>
</tr>
<tr>
<td>RH2-18</td>
<td>Heavy</td>
<td>HC CDR2</td>
<td>WISHNSGATTYAQKFQA</td>
<td>10</td>
</tr>
<tr>
<td>RH2-18</td>
<td>Heavy</td>
<td>HC CDR3</td>
<td>EDY</td>
<td>11</td>
</tr>
<tr>
<td>RHI-10</td>
<td>Heavy</td>
<td>HC CDR1</td>
<td>GYYLH</td>
<td>67</td>
</tr>
<tr>
<td>RHI-10</td>
<td>Heavy</td>
<td>HC CDR2</td>
<td>WISHNSGATNYAQNFGQ</td>
<td>68</td>
</tr>
<tr>
<td>RH2-59</td>
<td>Heavy</td>
<td>HC CDR1</td>
<td>DYYIH</td>
<td>9</td>
</tr>
<tr>
<td>RH2-59</td>
<td>Heavy</td>
<td>HC CDR2</td>
<td>WISHNSGATTYAQKFQA</td>
<td>10</td>
</tr>
<tr>
<td>RH2-59</td>
<td>Heavy</td>
<td>HC CDR3</td>
<td>EDY</td>
<td>11</td>
</tr>
<tr>
<td>RH2-80</td>
<td>Heavy</td>
<td>HC CDR1</td>
<td>DYYIH</td>
<td>9</td>
</tr>
<tr>
<td>RH2-80</td>
<td>Heavy</td>
<td>HC CDR2</td>
<td>WISHNSGATTYAQKFQA</td>
<td>10</td>
</tr>
<tr>
<td>RH2-80</td>
<td>Heavy</td>
<td>HC CDR3</td>
<td>EDY</td>
<td>11</td>
</tr>
</tbody>
</table>

The anti-Dkk-1 antibodies and immunological functional fragments that are provided can include one or more of the CDRs listed above and can include any combination of the CDRs. For example, some antibodies or fragments can include both the light chain CDR3 and the heavy chain CDR3. Certain antibodies have variant forms of the CDRs listed in Table 3, with one or more of the CDRs each having at least 80%, 85%, 90% or 95% sequence identity to a CDR sequence listed in Table 3. For example, the antibody or fragment can include both a light chain CDR3 and a heavy chain CDR3 that each have at least 80%, 85%, 90% or 95% sequence identity to the light chain CDR3 sequence and the heavy chain CDR3, respectively, listed in Table 3. Differences from the listed sequences usually are
conservative substitutions. Polypeptides comprising one or more of the light or heavy chain CDRs may be produced by using a suitable vector to express the polypeptides in a suitable host cell as described in greater detail below.

The heavy and light chain variable regions and the CDRs that are disclosed in Table 2 and 3 can be used to prepare any of the various types of immunologically functional fragments that are known in the art including, but not limited to, domain antibodies, Fab fragments, Fab' fragments, F(ab')2 fragments, Fv: fragments, single-chain antibodies and scFvs.

Anti-Dkk-1 Antibody Epitope

The anti-Dkk-1 antibodies bind to a complex multi-dimensional conformational epitope in the C-terminus region of the Dkk-1. The C-terminal domain of Dkk-1 is predicted to form a globular tertiary structure by homology model with colipase (as described below). The results shown in Example 5 indicate that the epitope recognized by the anti-Dkk-1 antibodies as exemplified by the RH2-18 antibody is a complex epitope affected by both sequence and tertiary structure of Dkk-1 in its cysteine-rich-domain-2 in the C-terminus. Figure 5C shows a structural-homology model of Dkk-1 C-terminal domain (amino acid residues 187 to 266) indicating the amino acid residues necessary for binding of Dkk-1 to the RH2-18 antibody as determined by alanine-scanning (See Figure 5C). Substitutions of amino acid residues S187 to V88, R203 through K208, E24, and L243 were found to result in diminished antigen-antibody interaction in immunoblotting experiments using non-denatured protein. Thus, these amino acid residues appear to play an important role in the formation of the complex epitope. Amino acid residues R171 to L174, which are outside the amino acid sequence shown in the C-terminal homology model, were also found to contribute to the complex epitope, in addition, substituting amino acid C22O with alanine also resulted in a loss of RH2-18 binding to Dkk-1. However, specific substitutions of the amino acid residues as indicated in Fig. 5c mapping to the second and third fingers (loops) of the Dkk-1 C-terminal domain did not appear to adversely affect binding of RH2-18 to Dkk-1 and, therefore, are not considered necessary for the binding of RH2-18 to Dkk-1.

Thus, the RH2-18 antibody binds a complex epitope comprising amino acid residues from different discrete regions of the second cysteine-rich domain were identified as being necessary for antibody binding: amino acid residues S187 and V88, both in a region preceding the first finger domain, and amino acids R203, H204, F205, W206, S207, and K208, all of which comprise the first finger of the domain. Further towards the C-terminus of the domain, and preceding the second finger, are E241 and L243, which were also required for RH2-18 binding to Dkk-1. Finally, Cys220 is necessary for RH2-18 binding to Dkk-1. Cys220 is predicted to play an important role in establishing a proper tertiary structure, again an indication of the complex nature of this epitope. Together, the data suggest that RH2-18 epitope is defined by a topographical surface of Dkk-1 C-terminal region that includes but is not limited to the first finger domain A202-I209 of Dkk-1. Thus, the anti-Dkk-1 antibodies bind to a complex epitope of mature human Dkk-1 protein consisting of amino acids 32-266 of SEQ ED NO:35 and
having a tertiary structure established by a disulfide bond between cysteine residues 220 and 245, wherein the antibody binds to an epitope comprising a loop consisting of the amino acids between cysteine residues 201 and 210 of SEQ ID NO:35.

The Dkk-1 amino acid sequence is closely related to that of Dkk-2 and Dkk-4 with 50% and 45% identity at the amino acid level, respectively. A comparison of the amino acid sequences of human Dkk-1, -2, -4, and Rhesus monkey Dkk-1 (Figure 6A) shows a lack of conservation of amino acid residues located within Dkk-1 that were identified as important for RH2-18 binding to Dkk-1 as described above. The amino acid residues in Dkk-1 that are necessary for RH2-18 binding are enclosed within the blue boxes. As can be seen, there is a complete lack of sequence conservation within the corresponding regions in Dkk-2 and Dkk-4. Based on the epitope mapping of amino acid residues in Dkk-1, which showed that amino acid residues Arg171 to Leul74, Serl87, Vall88, Ser207, and Glu241 are necessary for binding of RH2-18 to Dkk-1 and based on the lack of sequence conservation between Dkk-1 and Dkk-2 and Dkk-4 at amino acid residues Arg171 to Leul74, Serl87, Vall88, Ser207, and Glu241, RH2-18 and other anti-Dkk-1 antibodies sharing a similar epitope are predicted to have a high degree of selectivity for Dkk-1. As shown in Example 5, there was little or no detectable specific binding of RH2-18 to Dkk-2 or Dkk-4. Selectivity of the antibody towards Dkk-1 was minimally 100-fold and the detection limit for binding of RH2-18 to rhesus Dkk-1 was found to be about 1 ng. These data indicate RH2-18 antibody and similar anti-Dkk-1 antibodies described herein recognize a novel, complex, three-dimensional epitope covering several discrete regions of Dkk-1, which indicates that RH2-18 and the similar antibodies are unique.

Production of Anti-Dkk-1 Antibodies

The anti-Dkk-1 antibodies herein can be made by a hybridoma method first described by Kohler et al., (1975) Nature, 256:495, or may be made by recombinant DNA methods (See, for example, Example 1; U.S. Patent No. 4,816,567). In a currently preferred aspect, the anti-Dkk-1 antibodies herein are made by recombinant DNA methods.

Recombinant DNA constructs encoding the anti-Dkk-1 antibodies herein can be used to transform lower eukaryote host cells such as yeast or filamentous fungi, plant host cells, mammalian host cells, insect host cells, or microbial host cells. Transformation can be performed using any known method for introducing nucleic acids into a host cell. The optimal transformation procedure used will depend upon the type of host cell being transformed.

Typical recombinant DNA expression constructs comprise a nucleic acid molecule encoding a polypeptide encoding the one or more of the following: a heavy chain constant region (for example, Cgamma-I, CH2 and/or CH3); a heavy chain variable region; a light chain constant region; a light chain variable region; and, one or more CDRs of the light or heavy chain of the anti-Dkk-1 antibody. These nucleic acid sequences are inserted into an appropriate expression vector using standard ligation techniques. For example, in one embodiment, a nucleic acid encoding the RH2-18 heavy chain and a
nucleic acid encoding the RH2-18 light chain are each ligated into an expression vector and each chain is separately expressed. Alternatively, a single nucleic acid encoding both the heavy and light chains joined by a peptide cleavage site such that after expression of the light and heavy chains as a single polypeptide, the peptide cleavage site is cleaved to produce separate light and heavy chains.

The expression vector is typically selected to be functional in the particular host cell employed. Suitable expression vectors can be purchased, for example, from Invitrogen Life Technologies or BD Biosciences. Other useful vectors for cloning and expressing the antibodies and fragments of the invention include those described in Bianchi and McGrew, Biotech. Biotechnol. Bioeng. 84(4):439-44 (2003). Additional suitable expression vectors are discussed, for example, in Methods Enzymol. 185 (D.V. Goeddel, ed.), 1990, New York: Academic Press.

Typically, expression vectors further include nucleic acid sequences for plasmid or virus maintenance and for cloning and expression of exogenous nucleotide sequences. These nucleic acid sequences typically include one or more of the following operatively-linked nucleotide expression sequences: a promoter, one or more enhancer sequences, an origin of replication, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a sequence encoding a leader sequence for polypeptide secretion, a ribosome binding site, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element.

Optionally, the vector may contain a tag-encoding sequence located at the 5’ or 3’ end of the coding sequence and encoding a polyHis tag (such as hexaHis), or another tag for which commercially available antibodies exist, such as FLAG, HA (hemaglutinin from influenza virus), or myc. The tag can serve as a means for affinity purification of the antibody from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified antibody polypeptide by various means such as using certain peptidases for cleavage.

The nucleotide expression sequences in the expression vector may be homologous (from the same species or strain as the host cell), heterologous (from a species other than the host cell species or strain), hybrid (a combination of sequences from more than one source), synthetic, or native. As such, the source of an expression sequence may be any prokaryotic or eukaryotic organism. Expression sequences useful in the vectors may be obtained by any of several methods well known in the art. An origin of replication is typically a part of prokaryotic expression vectors, particularly those purchased commercially, and the origin aids in the amplification of the vector in a host cell. If the vector does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and ligated into the vector.

The expression vectors will typically contain a promoter that is recognized by the host organism and is operably linked to nucleic acid encoding the anti-Dkk-1 antibody thereof to produce the anti-Dkk-1 antibody. Promoters may be inducible promoters or constitutive promoters. Inducible
promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. Constitutive promoters, on the other hand, initiate continuous gene product production; that is, there is little or no experimental control over gene expression. A large number of promoters, recognized by a variety of potential host cells, are well known. A suitable promoter is operably linked to the DNA encoding anti-Dkk-1 antibody by removing the promoter from the source DNA by restriction enzyme digestion or amplifying the promoter by polymerase chain reaction and inserting the desired promoter sequence into the vector. Suitable promoters for use with yeast hosts are also well known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include, but are not limited to, those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, retroviruses, hepatitis-B virus and most preferably Simian Virus40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, for example, heat-shock promoters and the actin promoter. An enhancer sequence may be inserted into the vector to increase transcription in higher eukaryotes of a nucleic acid encoding an anti-Dkk-1 antibody. Enhancers are cis-acting elements of DNA, usually about 10 to 300 bp in length, that act on promoters to increase transcription. Enhancers are relatively orientation and position independent.

In expression vectors, a transcription termination sequence is typically located 3’ of the end of a polypeptide-coding region and serves to terminate transcription. A transcription termination sequence used for expression in prokaryotic cells typically is a G-C rich fragment followed by a poly-T sequence.

A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes used in expression vectors encode proteins that (a) confer resistance to antibiotics or other toxins, for example, ampicillin, tetracycline, or kanamycin for prokaryotic host cells; (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Examples of selectable markers include the kanamycin resistance gene, the ampicillin resistance gene and the tetracycline resistance gene. A bacterial neomycin resistance gene can also be used for selection in both prokaryotic and eukaryotic host cells.

A ribosome-binding site is usually necessary for translation initiation of mRNA and is characterized by a Shine-Dalgarno sequence (prokaryotes) or a Kozak sequence (eukaryotes). The element is typically located 3’ to the promoter and 5’ to the coding sequence of the polypeptide to be expressed.

In some cases, anti-Dkk-1 antibodies having particular glycosylation structures or patterns are desired. For example, many mammalian and plant cells will produce proteins with particular N-glycans that render the protein immunogenic when introduced into an individual. In the case of the
anti-Dkk-1 antibodies where invoking an immune response against the anti-Dkk-1 antibodies is undesirable, it is preferred that the glycosylation pathway of the mammalian host cells be modified to produce anti-Dkk-1 antibodies without the undesirable N-glycans. Methods for modifying the glycosylation pathway in mammalian cells to produce antibodies with particular N-glycans has been described in, for example, International Patent Application No. WO0061739, and U.S. Published Patent Application Nos. 20040093621, 20040259150, 20030157108, 20040191256, 20040136986, and U.S. Patent No. 6,946,292. A method for modifying the glycosylation pathway in plants has been described by Cox et al, Nature Biotechnology, doi:10.1038/nbtl260, published on-line 26 November 2006. Many lower eukaryote cells also produce proteins with particular N-glycans that render the protein immunogenic when introduced into an individual. In the case of the anti-Dkk-1 antibodies, it is preferred that the glycosylation pathway of the lower eukaryote host cells be modified to produce anti-Dkk-1 antibodies without the undesirable N-glycans. Methods for modifying the glycosylation pathway in lower eukaryotes, including yeast, to produce antibodies with particular N-glycans and glycosylation patterns have been described in for example, U.S. Patent No. 7,029,872 and in Published U.S. Patent Application Nos. 20060034829, 20060024304, 20060034828, 20060034830, 20060029604, and 20060024292.

The transformed host cell, when cultured under appropriate conditions, synthesizes an anti-Dkk-1 antibody that can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). The selection of an appropriate host cell will depend upon various factors, such as desired expression levels, polypeptide modifications that are desirable or necessary for activity (such as glycosylation or phosphorylation) and ease of folding into a biologically active molecule.

Mammalian cell lines available as hosts for expression are well known in the art and include, but are not limited to, many immortalized cell lines available from the American Type Culture Collection (ATCC), such as Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (for example, Hep G2), and a number of other cell lines. In certain embodiments, the best cell line for expressing a particular DNA construct may be selected by testing various cell lines to determine which ones have the highest levels of expression levels and produce antibodies with constitutive Dkk-1 binding properties.

In particular embodiments, it is preferable that the antibodies be produced in a lower eukaryote cell genetically engineered to produce glycoproteins having humanlike N-glycan structures. U.S. Patent No. 7,029,872 and in Published U.S. Patent Application Nos. 20060034829, 20060024304, 20060034828, 20060034830, 20060029604, and 20060024292 disclose producing antibodies in lower eukaryote cells such as yeast and filamentous fungi that have predominantly particular N-glycan structures. Genetically engineered lower eukaryotes that can be used to produce the antibodies include those selected from the group consisting of Pichia pastoris, Pichia fermentans, Pichia trehalophila, Pichia kochamiae, Pichia membraefaciens, Pichia opuntiae, Pichia thermotolerans, Pichia salictaria,

Anti-Dkk-1 Antibody Compositions

Further provided are compositions comprising an effective amount of the anti-Dkk-1 antibodies or immunologically functional fragments thereof together with one or more of the following: a pharmaceutically acceptable diluent, a carrier, a solubilizer, an emulsifier, a preservative, and/or an adjuvant. Thus, the use of the antibodies and immunologically active fragments that are provided herein in the preparation of a pharmaceutical composition or medicament is also included. Such compositions can be used in the treatment of a variety of bone disorders such as osteoporosis. Acceptable formulation components for pharmaceutical preparations are nontoxic to recipients at the dosages and concentrations employed.

In addition to the anti-Dkk-1 antibodies and immunologically functional fragments that are provided, the compositions may also contain components for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. Suitable materials for formulating pharmaceutical compositions include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulfite or sodium hydrogen-sulfite); buffers (such as acetate, borate, bicarbonate, Tris-HCl, citrates, phosphates, or other organic acids); bulking agents (such as mannitol or glycerin); chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-t cyclodextrin or hydroxypropyl-beta-cyclodextrin); fillers; monosaccharides; disaccharides; and other carbohydrates (such as glucose, mannose or dextrose); proteins (such as serum albumin, gelatin or immunoglobulins); coloring, flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol; sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate 80, triton, tromethamine, lecithin, cholesterol, tyloxapal); stability enhancing agents (such as sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides, preferably sodium or potassium chloride, mannitol sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants.
The primary vehicle or carrier in a pharmaceutical composition may be either aqueous or nonaqueous in nature. Suitable vehicles or carriers for such compositions include water for injection, physiological saline solution or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. Compositions comprising anti-Dkk-1 antibodies or immunologically functional fragments thereof may be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents in the form of a lyophilized cake or an aqueous solution. Further, the anti-Dkk-1 antibodies or immunologically functional fragments thereof may be formulated as a lyophilizate using appropriate excipients such as sucrose. The formulation components are present in concentrations that are acceptable to the site of administration. Buffers are advantageously used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 4.0 to about 8.5, or alternatively, between about 5.0 to 8.0. Pharmaceutical compositions can comprise TRIS buffer of about pH 6.5 to 8.5, or acetate buffer of about pH 4.0 to 5.5, which may further include sorbitol or a suitable substitute therefor.

The effective amount of a pharmaceutical composition comprising anti-Dkk-1 antibodies or immunologically functional fragments thereof will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment, according to certain embodiments, will thus vary depending, in part, upon the molecule delivered, the indication for which the anti-Dkk-1 antibody is being used, the route of administration, and the size (body weight, body surface or organ size) and/or condition (the age and general health) of the patient. A clinician may adjust the dosage and modify the route of administration to obtain the optimal therapeutic effect. Typical dosages range from about 0.1 µg/kg to up to about 100 mg/kg or more, depending on the factors mentioned above. In certain embodiments, the dosage may range from: 0.1 µg/kg up to about 150 mg/kg; or 1 µg/kg up to about 100 mg/kg, or 5 µg/kg up to about 50 mg/kg. In general, it is currently expected that the anti-Dkk-1 antibodies or immunological fragments thereof will be formulated as a sterile, clear liquid at a concentration of at least 10 mg/mL in isotonic buffered saline (20mM histidine, 150 mM sodium chloride, 0.05% polysorbate 80, pH 6.4). A typical antibody formulation is filled as a single dose, 0.6 mL glass vials filled with 3.3 mL of solution per vial and each vial is stopped with a West Fluorotec Teflon-coated stopper and sealed with an aluminum cap.

Although rV administration of antibody therapy for osteoporosis indications is considered acceptable; the optimal profile for the antibodies herein is subcutaneous or intraperitoneal dosing, once biweekly or monthly.

The anti-Dkk-1 antibodies or fragments have therapeutic use in stimulating osteoblast activity and increasing bone mineral density or bone mass. These antibodies and fragments are thus useful for treating patients suffering from various medical disorders that involve excessive bone loss or patients who require the formation of new bone even where there may not necessarily be excessive osteoclast activity. Blocking Dkk-1 activity results in heightened osteoblast activation via signaling
transmitted by Wnt proteins. Excessive osteoclast activity is associated with numerous osteogenic disorders that can be treated with the anti-Dkk-1 antibodies and immunologically functional fragments that are provided, including ostopenia, osteoporosis, periodontitis, Paget's disease, bone loss due to immobilization, lytic bone metastases and arthritis, including rheumatoid arthritis, psoriatic-arthritis, ankylosing spondylitis and other conditions that involve bone erosion.

Various low bone mass conditions can also be treated including a variety of forms of osteoporosis, including but not limited to, glucocorticoid induced osteoporosis, osteoporosis induced after transplantation, osteoporosis associated with chemotherapy, immobilization induced osteoporosis, osteoporosis due to mechanical unloading, and osteoporosis associated with anticonvulsant use.

Additional bone diseases that can be treated with some of the antibodies or fragments include bone disease associated with renal failure and nutritional, gastrointestinal and/or hepatic associated bone diseases.

Different forms of arthritis can also be treated, examples including osteoarthritis and rheumatoid arthritis. The antibodies and fragments can also be used to treat systemic bone loss associated with arthritis (for example, rheumatoid arthritis). In treating arthritis, patients may benefit by perilesional or intralesional injections of the subject antibodies or fragments thereof. For example, the antibody or fragment thereof can be injected adjacent to or directly into an inflamed joint, thus stimulating repair of damaged bone at the site.

Some cancers are known to increase osteoclast activity and induce bone resorption, such as breast and prostate cancer. Multiple myeloma, which arises in bone marrow, also is associated with bone loss, in part likely due to the increased expression of Dkk-1 by plasma cells, which then suppresses the bone building activity of osteoblasts in the vicinity. Reducing Dkk-1 activity by administering the subject antibodies or immunologically functional fragments thereof can result in an increase in osteoblast activity that serves to counteract the excessive osteoclast activity, thereby reducing the severity of the aforementioned disorders, reducing bone erosion and inducing new bone formation in the patient.

Treatment with certain of the anti-Dkk-1-specific antibodies or immunologically functional fragments can induce a significant increase in bone mineral density in a patient suffering from an osteopenic disorder. Inhibiting Dkk-1 with the antibodies or immunologically functional fragments described herein can also be used in various bone repair applications. For example, certain antibodies and fragments can be useful in retarding wear debris osteolysis associated with artificial joints, accelerating the repair of bone fractures, and enhancing the incorporation of bone grafts into the surrounding living bone into which they have been engrafted.

Anti-Dkk-1 antibodies or immunologically functional fragments thereof can be administered alone or in combination with other therapeutic agents, for example, in combination with cancer therapy agents, with agents that inhibit osteoclast activity or with other agents that enhance osteoblast activity. For example, the inventive antibodies can be administered to cancer patients undergoing radiation therapy or chemotherapy.
Anti-Dkk-1 antibodies and immunologically functional fragments thereof may be used alone for the treatment of the above referenced conditions resulting in loss of bone mass or in combination with a therapeutically effective amount of a bone growth promoting (anabolic) agent or a bone anti-resorptive agent including but not limited to: bone morphogenic factors designated BMP-I to BMP-12; transforming growth factor-β and TGF-β family members; fibroblast growth factors FGF-I to FGF-IO; interleukin-1 inhibitors, INFα inhibitors; RANK ligand inhibitors, parathyroid hormone (PTH), E series prostaglandins, bisphosphonates, and bone-enhancing minerals such as fluoride and: calcium. Anabolic agents that can be used in combination with the inventive antibodies and functional fragments thereof include parathyroid hormone and insulin-like growth factor (IGF), wherein the latter agent is preferably complexed with an IGF binding protein.

In addition, the anti-Dkk-1 antibodies can be administered to patients in combination with antibodies that bind to tumor cells and induce a cytotoxic and/or cytosstatic effect on tumor growth. Examples of such antibodies include those that bind to cell surface proteins Her2, CDC20, CDC33, mucin-like glycoprotein I and epidermal growth factor receptor (EGFR) present on tumor cells and induce a cytostatic and/or cytotoxic effect on tumor cells displaying these proteins. Also, combination therapy can include as cancer therapy agents polypeptides that selectively induce apoptosis in tumor cells, such as the TNF-related polypeptide TRAIL.

The anti-Dkk-1 antibodies or immunologically functional fragments thereof can be administered concurrently with other treatments and therapeutic agents being administered for the same condition. Anti-Dkk-1 antibodies or immunologically functional fragments thereof can be administered prophylactically to prevent or mitigate the onset of loss of bone mass by early stage cancer (stages I or II), or can be given to ameliorate an existing condition of loss of bone mass due to metastasis to the bone. Anti-Dkk-1 antibodies of the invention may be used to prevent and/or treat the growth of tumor cells in bone. Cancer that metastasizes to bone can spread readily as tumor cells stimulate osteoclasts to resorb the internal bone matrix. Treatment with an anti-Dkk-1 antibody or immunologically functional fragment thereof will help maintain bone mineral density at the site of such metastases by stimulating increased osteoblast activity. Any cancer that has potential to metastasize to bone may be prevented or treated with an anti-Dkk-1 antibody administered before or after metastasis has occurred.

It is expected that the antibodies herein will be effective as monotherapy. However, it is also expected that the antibodies herein could be administered with existing treatments for osteoporosis, such as (but not limited to) alendronate, risendronate, ibandronate, zoledronic acid, calcitonin, estrogen(s), PTH, and conjugated estrogens, raloxifene and other selective estrogen receptor modulators, teriparatide, vitamin D and its metabolites, among others. In addition to these approved treatments, it is also expected that the antibodies herein may provide synergistic/additive benefit for any of several approaches currently in development for the treatment of osteoporosis, which include without limitation, cathepsin K inhibitors, ATP6 inhibitors, chloride channel-7 inhibitors, denosumab, or other anti-RANK antibodies or inhibitors, osteoprotegerin-Fc, αvβ3 integrin antagonists, and calcilytics, among others.
Dkk-1 has also been implicated in the pathogenesis of myeloma bone disease through the suppression of osteoblast differentiation. Tian et al. (N. Engl. J. Med. 349: 2483-2494 (2003) found overexpression of the Dkk-1 gene and Dkk-1 protein in multiple myeloma (MM) patients with focal bone lesions. In vitro, recombinant human Dkk-1 or bone marrow plasma with high Dkk-1 levels inhibited osteoblast function. This effect was neutralized by treatment with a polyclonal anti-Dkk-1 antibody. It was also suggested that the reduction of Dkk-1 levels after treatment (autologous stem cell transfer) may be correlated with the normalization of osteoblast function, which could provide a basis for developing agents that block Dkk-1 activity such as the antibodies disclosed herein, thus restoring osteoblast function and counteracting the increased osteoclastogenesis observed in myeloma (See, Politou et al. In J Cancer 119:1728 (2006)).

Mice engrafted with primary multiple myeloma cells expressing varying levels of Dkk-1 when treated with control or Dkk-1 neutralizing antibodies for four to six weeks show reduced BMD in controls but increased BMD from pre-treatment levels (p<0.001) in the anti-Dkk1 antibody group. The bone anabolic effect of anti-DKK1 antibodies was associated with reduced multiple myeloma burden (p<0.04). The authors concluded that Dkk-1 is a key player in multiple myeloma bone disease and that blocking Dkk-1 activity in myelomatous bones reduces osteolytic bone resorption, increases bone formation, and helps control multiple myeloma growth (See, Yaccoby et al., Blood. 2006 Oct 26; [Epub ahead of print]). In addition, PC-3 prostate cancer cells express the Wnt inhibitor Dkk-1. Decreasing Dkk-1 levels enabled the PC-3 cells to induce osteoblastic activity, including alkaline phosphatase production and mineralization, in murine bone marrow stromal cells indicating that Dkk-1 blocked Wnt-mediated osteoblastic activity in PC-3 cells (Hall et al., Cancer Res 65:7554 (2005)). Together, the above results suggest the involvement of Wnt-signaling and Dkk-1 in cancer cells known to invade bone environment. Therefore, the Dkk-1 antibodies and immunologically functional fragments thereof disclosed herein may provide a therapeutic treatment for alleviating the bone-destructive effects of cancér cells (for example, multiple myeloma, breast cancer, prostate cancer, and the like) invading the bone micro-environment.

The following examples are intended to promote a further understanding of the present invention.

EXAMPLE 1

The human anti-Dkk-1 antibodies were prepared using the Cambridge Antibody Technology (CAT) human single chain Fv phage display library (Cambridgeshire, United Kingdom). The library was panned against both Rhesus monkey and mouse Dkk-1 (RhDkk-1 and MsDkk-1, respectively). Each library was subjected to three rounds of solution-based panning against biotin labeled Dkk-1 (100 nM). Though the percentage of sequence identity and similarity is high between mouse and rhesus Dkk-1, six different panning strategies were employed to ensure library selections.
would cross react to Dkk-1 from both species (Table 4). It is from scheme (E) that all subsequent data originates.

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Round 1</th>
<th>Round 2</th>
<th>Round 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MsDkk-1</td>
<td>MsDkk-1</td>
<td>MsDkk-1</td>
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<tr>
<td>B</td>
<td>MsDkk-1</td>
<td>RhDkk-1</td>
<td>MsDkk-1</td>
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<td>C</td>
<td>MsDkk-1</td>
<td>MsDkk-1</td>
<td>RhDkk-1</td>
</tr>
<tr>
<td>D</td>
<td>RhDkk-1</td>
<td>RhDkk-1</td>
<td>RhDkk-1</td>
</tr>
<tr>
<td>E</td>
<td>RhDkk-1</td>
<td>RhDkk-1</td>
<td>MsDkk-1</td>
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<td>F</td>
<td>RhDkk-1</td>
<td>MsDkk-1</td>
<td>RhDkk-1</td>
</tr>
</tbody>
</table>

To validate the antigen specificity of the selected scFv-phage clones, 176 phage clones from each library in round 2 and 88 clones from each third round library were tested in a time-resolved fluorescence (TRF) ELISA assay using HEK293 cells overexpressing LRP5 or LRP6 and rhesus or mouse Dkk-1 protein labeled with fluorescent Europium-chelate (Eu). Eu-Dkk-1 protein binding to human embryonic kidney, HEK293 cells overexpressing human LRP5 or LRP6 was monitored by measuring the time-resolved fluorescence of bound ligand. When Dkk-1 protein bound to LRP5 (or 6)-expressing cells, a strong signal was detected. When the Dkk-1 was tested in the presence of an anti-Dkk-1 antibody, a reduction in fluorescent signal indicated interference with the Dkk-1/LRP5 (or 6) interaction. 264 Dkk-1 scFVs were tested in this primary assay for the ability to inhibit binding of Dkk-1 protein to the cell surface. Based on the above assay, 20 scFVs among the group identified to inhibit binding of Dkk-1 to the cell surface (Table 5) were chosen for conversion to fully human IgGs.

<table>
<thead>
<tr>
<th>SEQ ID</th>
<th>Library</th>
<th>Rhesus</th>
<th>Mouse</th>
<th>Phage ELISA (+)</th>
<th>Phage ELISA (+)</th>
<th>% inhibition</th>
<th>Comments</th>
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<td>RH1-10</td>
<td>BMV</td>
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<td>X</td>
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<td>RH1-12</td>
<td>BMV</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>63%</td>
<td></td>
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<tr>
<td>RH1-25</td>
<td>BMV</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>RH1-26</td>
<td>BMV</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>64%</td>
<td></td>
</tr>
<tr>
<td>RH1-28</td>
<td>BMV</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>80%</td>
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<tr>
<td>RH1-30</td>
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<td></td>
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<td></td>
<td></td>
<td>66%</td>
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<tr>
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<td>X</td>
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<td>X</td>
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<td></td>
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<td></td>
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</table>
DNA encoding the heavy chain variable regions were fused in-frame with DNA encoding the IgG2M4 constant region whereas DNA encoding the light chain variable regions were fused in-frame with DNA encoding either lambda or kappa light chain constant region in alignment with the corresponding variable regions. The resulting antibody expression vectors are shown in the plasmid maps (Figures IA and IB) by using the expression vector encoding antibody RH2-18 as an example. The cloning procedure is described below. The light chain lambda vector that was used was built in-house and comprises cloning sites flanked by a human CMV (HCMV) promoter and leader sequence on the 5′ end of one cloning site and the light chain lambda sequences and bovine growth hormone (BGH) polyadenylation signal on the 3′ side of the other cloning site. The heavy chain IgG2M4 constant region vector that was used was built in-house and comprises cloning sites flanked by an HCMV promoter and leader sequence on the 5′ end of one cloning site and heavy chain IgG2M4 sequences and BGH polyadenylation signal on the 3′ side of the other cloning site. The expression vectors carry oriP from Epstein Barr virus (EBV) viral genome for prolonged expression in 293EBNA cells and the bacterial sequences for kanamycin selection marker and replication origin in E. coli. The leader sequence at the amino termini of the antibodies mediated the secretion of the expressed antibodies into the culture medium. The leader sequence for heavy chain is MEWSWVFLFFLSVTGVHS (SEQ ID NO:29) and light chain: MSVPTQVLGLLLLWLTDARC (SEQ ID NO:30). The rest of the 19 scFv leads were converted to IgG in the same manner.

<table>
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<th>X</th>
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<td>RH3-9</td>
<td>DP47</td>
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<td>X</td>
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<td>DP47</td>
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<td>X</td>
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<tr>
<td>RH3-54</td>
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<td>X</td>
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<td>DP47</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
<td>RH3-84</td>
<td>DP47</td>
<td>X</td>
<td>X</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>RH3-94</td>
<td>DP47</td>
<td>X</td>
<td>X</td>
<td>68%</td>
<td></td>
</tr>
</tbody>
</table>

To make the vectors shown in Figures IA and IB, the respective variable regions were PCR amplified in a volume of 25 μL containing high fidelity PCR master mix, template volume 1 μL and forward and reverse primers: 1 μL each. PCR conditions were one cycle of 94°C for two minutes, 25 cycles of 94°C for 1.5 minutes, 60°C for 1.5 minutes and 72°C for 1.5 minutes with a final extension at 72°C for 7 minutes. The following PCR primers were used heavy chain forward, 5′-ACAGG TGTCG ACTCGAGGCTGATGTCG T-3′ (SEQ ID NO:31); heavy chain reverse 5′-GCCCT TGGTGATGCTACTCGAGACGGTGACGAGGGT-3′ (SEQ ID NO:32) and light chain forward 5′-ACAGA TGCCAGTACGGTGTCGTGTGAGGAGG3′ (SEQ ED NO:33); light chain reverse

- 30 -
5'-GTTGG CCTTG GGCTG ACTTA AAACG GTGAG CTGGG T-3' (SEQ ID NO:34). The amplified light and heavy chain variable region PCR products were cloned in-frame with the appropriate leader sequence at the 5'-end and constant region at the 3'-end using In-Fusion strategy (Clontech, Palo Alto, CA) and cloned into E. coli XL10 cells from Stratagene, La Jolla, CA). The DNA sequences for the clones were confirmed by sequencing and the amino acid sequences were deduced from the DNA sequences. The amino acid sequences for RH2-18 light and heavy chains (without the leader sequences) are shown in Figure 1C. The variable regions are italicized. Figures 1D and 1E show the CDR regions for the RH2-18 light chain and heavy chain variable regions and also identify the sequence differences in the frameworks between the RH2-18 light chain and heavy chain variable regions and the corresponding regions in the germline.

The above plasmids were transfected into 293EBNA monolayer cells using FUGENE transfection reagents (FUGENE is a trademark of Fugent LLC and is available from Roche Diagnostics, Nutley, NJ). The transfected cells were incubated in OPTI-MEM serum free medium (Invitrogen) and the secreted antibodies were purified from the culture medium using protein A/G affinity chromatography. The concentration of purified antibodies was determined by OD at 280 nm and the purity by LABCHIP capillary SDS gel electrophoresis (Caliper Life Sciences, Hopkinton, MA). Figure IF shows the results of a LABCHIP electrophoresis analysis for 12 converted antibodies. Lane 2 shows the RH2-18 antibody. The antibodies purified were used for in vitro characterization as described herein. The above plasmids were also used for mass production of RH2-18 antibody and the other antibodies for in vivo animal studies described in the animal study section.

For all biological assays, rhesus Dkk-1 protein was prepared by Baculovirus expression and purified via metal-affinity resin. Isolated anti-Dkk-1 antibodies from CAT-library panning were selected on the basis of ability to bind to rhesus (and mouse) Dkk-1 proteins. To determine if an antibody inhibited Dkk-1-interactions with cell surface receptors (LRP5/6) and inhibited of Dkk-1 - function, the following assays were established: a cell-based Dkk-1-binding assay, a Dkk-1 functional assay measuring canonical Wnt-signaling, and a cell differentiation assay for Dkk-1-functional analysis using the osteoblastic differentiation marker alkaline phosphatase (ALP). The above assays were run in consecutive order to select neutralizing antibodies for (a) blocking Dkk-1 binding to LRP5/6, (b) inhibiting Dkk-1-function in Wnt signaling, and (c) neutralizing the negative Dkk-1-function on bone cell differentiation in vitro.

**EXAMPLE 2**

A time-resolved fluorescence (TRF) cell-based assay was used to show that four of the anti-Dkk-1 antibodies inhibited Dkk-1 binding to LRP5/6.

For the assay, the anti-Dkk-1 antibodies were added to HEK cells overexpressing LRP5 (HEK293hLrp5 cells) at final concentrations of 0.2, 0.6, 2.0, 6.0, and 20 nM. Anti-IL3 Receptor monoclonal antibody (8B4) was used as a negative control. Eu-labeled Dkk-1 (100 pM) was incubated
with the cells in absence or presence of the antibodies for 20 minutes. Dkk-1 bound in solution by the antibodies and thus blocked from binding to the cell surface of the HEK293hLrp5 cells was removed by 4x wash steps and Eu-labeled Dkk-1 bound to cell surface was measured by TRP signal. Eu-labeled Dkk-1 protein binding to HEK293 cells overexpressing human LRP5 or LRP6 was monitored by measuring the time-resolved fluorescence of bound ligand. The results for the top five of these antibodies (RHI-10, RH2-18, RH2-31, RH2-59, and RH2-80) are shown in Figure 2A and 2B. Figure 2A shows Eu-Dkk-1 binding to HEK293hLrp5 cells and the ability of the above five antibodies to inhibit binding of the Dkk-1 to LRP5. Figure 2A shows a titration of RH2-18 antibody inhibition of Dkk-1 binding to LRP5 over an extended dose range. Figure 2B shows that in this assay format, an effective dose of RH2-18 was about 5 nM. The data in Figures 2A and 2B shows that the inhibitory activity of the anti-Dkk-1 antibodies was substantial to complete in the low nanomolar range (4.75 nM for RH2-18 antibody). Additional analyses, using Eu-labeled recombinant mouse Dkk-1 provided similar results, indicating that the inhibitory mechanism of the selected anti-Dkk-1 antibodies was conserved for both the mouse and rhesus Dkk-1 protein.

EXAMPLE 3

This example shows the neutralizing activity of the anti-Dkk-1 antibodies on Dkk-1 function in Wnt signaling. Dkk-1 is a negative regulator of the canonical Wnt-signaling through β-catenin and nuclear Lef-1/TCF. HEK293hLrp5 cells were co-transfected with a reporter plasmid with Lef-1/TCF binding sites (pTOPflash) and an expression vector encoding Lef-1. Cells transfected with pTOPflash/Lef-1 are highly responsive to Wnt-ligands as indicated by increased activity of the reporter (luciferase). Rhesus monkey Dkk-1 (50 nM) robustly inhibits pTopflash activity in this cell system. The anti-Dkk-1 antibodies RHI-10, RH2-18, RH2-31, and RH2-80 were tested here for their ability to also neutralize Dkk-1-function over a 20 hour time period. The anti-Dkk-1 antibodies were added at 10, 30, and 100 nM concentrations. The results shown in Figure 3 indicate that all of the tested anti-Dkk-1 antibodies blocked Dkk-1 binding to the LRP5 on the cell surface and thereby inhibited the functional activity of Dkk-1 in the Wnt3A signaling pathway. As shown in Figure 3, treatment with Wnt3A significantly stimulated the signaling pathway compared to the control and that Rhesus monkey Dkk-1 inhibited Wnt3A activation of the reporter readout. The antibodies neutralized the Dkk-1 effect at 30 nM and higher concentrations. Note that with all anti-Dkk-1 antibodies tested, signals could rise to greater than with Wnt3A ligand alone. This effect required addition of recombinant Dkk-1 protein to the assay system, as in a parallel assay this antibody effect on Wnt-signaling was not observed in the absence of exogenously added rhesus-Dkk-1 protein.
EXAMPLE 4

The effect of the neutralizing Dkk-1 antibodies on osteoblastic cell differentiation in vitro was tested. The mesenchymal pluripotent cell line, C3H10T1/2, differentiates towards the osteoblastic cell lineage by treatment with osteogenic factors. Wnt3A treatment over three days induces the expression of the early osteoblastic marker alkaline phosphatase (ALP). Dkk-1 inhibits Wnt3A induced differentiation as determined by measuring endogenous ALP activities. This osteoblastic cell differentiation system provides a relevant cell context and a more prolonged 3-day assay period.

Osteoblastic cell differentiation of C3H10T1/2 cells was determined by measuring the increase in endogenous ALP activities. Cells were grown to confluence in culture and treated with Wnt3A for 3 days to induce osteoblastic differentiation. Concomitant treatment with recombinant Dkk-1 inhibited Wnt3A induced ALP activity. As shown in Figure 4, when anti-Dkk-1 antibodies RH2-18, RH2-59, or RH2-80 were added at 10 nM, 30 nM or 100 nM final concentrations, the inhibitory function of Dkk-1 on osteoblastic cell differentiation was neutralized in a dose-dependent manner. The neutralizing effect of 100 nM RH2-18 on Dkk-1 was nearly complete and its stability sufficient to produce and maintain a neutralizing effect within a 3-day assay period. RH2-31 was found to have little potency/stability and was therefore excluded from further studies (data not shown). Related analyses of endogenous marker genes (TROY, IGFBP2, Axin2) induced within the first 24 hours of treatment showed a similar capacity for these antibodies to block Dkk-1 inhibition of Wnt3a-induced gene expression in this cell background.

EXAMPLE 5

An epitope map of the RH2-18 antibody was constructed. Dkk-1 protein is composed of two cysteine-rich domains located in the N-terminal and C-terminal regions, respectively. We generated deletion constructs for Dkk-1 encoding either the N-terminal or C-terminal region of rhesus-Dkk-1 and confirmed that cysteine-rich-domain-2 located in the C-terminal half of Dkk-1 is necessary and sufficient for Dkk-1 binding to the receptor LRP5/6.

The neutralizing anti-Dkk-1 antibodies disclosed herein cannot detect denatured Dkk-1 protein on Western-immunoblots. Further, they do not bind to discrete peptides derived from the Dkk-1 C-terminus. This suggested that the epitope on Dkk-1 that is recognized by the anti-Dkk-1 antibodies herein is complex (that is, topographical based and not peptide-based).

Figure 5A shows a dot-blot binding analysis of RH2-18. Rhesus Dkk-1 proteins were fused to a GFP tag (loading control). Full-length rhesus Dkk-1 protein, C-terminal region (ΔN-Dkk-1, encoding amino acid residues 159 to 266) or N-terminal region (ΔC-Dkk-1) were expressed and analyzed by dot-immunoblotting using RH2-18 antibody. Briefly, DKK1-GFP tagged variants were expressed in transiently transfected 293 cells and native conditioned media was blotted directly onto nitocellulose membranes. Bound native protein was probed with tag-antibody (anti-GFP, Abeam Inc., Cambridge,
MA) or anti-DKK1 antibody RH2-18. Bound antibodies were detected with secondary antibodies coupled to alkaline phosphatase.

The dot blot analyses showed the binding of neutralizing antibodies RH2-18 (as well as RH1-10, RH2-31, RH2-59, RH2-80) to the C-terminal region of Dkk-1 (amino acids 159- to 266). This indicated that the antibody epitope(s) maps primarily to within cysteine-rich-domain-2 of Dkk-1. Moreover, the dot blot analysis showed that the antibody binds to native protein (whereas the Western blots demonstrated no binding to denatured protein). The C-terminal domain of Dkk-1 is predicted to form a globular tertiary structure by homology model with colipase (as described below). Together these data indicate that the epitope of the ant-Dkk-1 antibodies herein and RH2-18 antibody in particular is defined by a complex epitope affected by both sequence and tertiary structure of Dkk-1 in its cysteine-rich-domain-2.

Additional analyses of the C-terminal domain using site-directed mutagenesis (alanine-scanning method) identified amino acid residues S187 to V188, R203 through K208, and E241 through L243 as the Dkk-1 amino acid-residues most important for RH2-18 antibody binding to Dkk-1. In this regard, mutations to these residues caused a striking reduction in the capacity of the antibody to bind to the mutant Dkk-1 in the dot blot analyses (See Figure 5B). Figure 5B shows a loss of RH2-18 binding by various amino acid substitutions in the Dkk-1 C-terminal domain as determined by dot-blot binding analyses. Rhesus Dkk-1 proteins were fused to a GFP tag (loading control). Full-length rhesus Dkk-1 protein, C-terminal region (ΔN-Dkk-1, encoding amino acid residues 159 to 266) or N-terminal region (ΔC-Dkk-1, encoding residues 1 to 158) were expressed and analyzed by dot-immunoblotting using RH2-18. Alanine-substitutions were introduced in ΔN-Dkk-1 and the amino acid-residues affected are indicated. From independent structure/function analysis of Dkk-1 by alanine-scanning, amino acid residues R203, H204 (F205) within the C-terminal domain were identified as necessary for Dkk-1 binding to LRP6 (See Identification of DKK1 Residues Necessary for Interaction with LRP5/6. Lipfert et al., J. Bone Miner. Res. 21: S99 (2006)). Thus, these residues are important for both Dkk-1 function (binding to LRP5/6) and for binding the RH2-18 antibody. The overlap between the key amino acid residues required for Dkk1 interaction with LRP5 and the amino acid residues for Dkk1 binding to RH2-18 antibody provide a rationale for the inhibitory activity of the RH2-18 antibody.

Interpretation of the effects of the mutations shown in Figure 5B is best considered in the context of a three-dimensional model of the second cysteine-rich domain (See Figure 5C). Figure 5C shows a structural-homology model of Dkk-1 C-terminal domain (amino acid residues 187 to 266) indicating the amino acid residues necessary for binding of Dkk-1 to the RH2-18 antibody as determined by alanine-scanning (See Figure 5C). Substitutions of amino acid residues found to result in diminished antigen-antibody interaction in immunoblotting experiments using non-denatured protein are amino acid residues S187 to V188, R203 through K208, E241, and L243), which appear to play a role in the formation of a complex epitope. Amino acid residues R171 to L174, which are outside the amino acid sequence shown in the C-terminal homology model, were also found to contribute to the complex
epitope. In addition, substituting amino acid C22O with alanine also resulted in a loss of RH2-18 binding to Dkk-1.

Thus, the RH2-18 antibody binds a complex epitope comprising amino acid residues from different discrete regions of the second cysteine-rich domain were identified as being necessary for antibody binding: amino acid residues S187 and V188, both in a region preceding the first finger domain, and amino acids R.203, H204, F205, W206, S207 = and K.208, of which comprise the first finger of the domain. Further towards the C-terminus of the domain, and preceding the second finger, are E241 and L243, which were also required for RH2-18 binding to Dkk-1. Finally, Cys220 is necessary for RH2-18 binding to Dkk-1. Cys220 "predicted to play an important role in establishing a proper tertiary structure, RH2-18 to bind the Dkk-1, again an indication of the complex nature of this epitope. Together, the data suggest that RH2-18 epitope is defined by a topographical surface of Dkk-1 C-terminal region that includes but is not limited to the first finger domain A202-I209 of Dkk-1.

A comparison of the amino acid sequences of human Dkk-1, -2, -4, and Rhesus monkey Dkk-1 (Figure 6A) shows a lack of conservation of amino acid residues located within Dkk-1 that were identified as important for RH2-18 binding to Dkk-1 as described above. Amino acid residues in Dkk-1 that were deemed to be necessary for RH2-18 binding are enclosed within the blue boxes. Note the lack of sequence conservation between Dkk-1 and Dkk-2 and Dkk-4 for amino acid residues: Arg17i-Leu174, Seri87 to Vali88, Ser207 and GI11241. Based on the epitope mapping of residues in Dkk-1 that are necessary for binding of RH2-18 and based on the lack of sequence conservation between Dkk-1 and Dkk-2 and Dkk-4 at amino acid residues Arg17i to Leu174, Seri87, Vali88, Ser207, and Glu241. RH2-18 and other antibodies sharing a similar epitope are predicted to show a high degree of selectivity for Dkk-1.

The Dkk-1 amino acid sequence is closely related to that of Dkk-2 and Dkk-4 with 50% and 45% identity at the amino acid level, respectively. Cross-reactivity of RH2-18 against rhesus monkey Dkk-2 (using a chimeric protein consisting of the N-terminal region of the rhesus Dkk-1 fused to the C-terminal region of the rhesus Dkk-2 with myc and His-6 tags at the C-terminal end of the Dkk-2 C-terminal region; see SEQ ID NO:71) and cyromolgus monkey Dkk-4 (SEQ ID NO:70) was tested by dot-blot analysis using recombinant proteins. Native recombinant rhesus monkey Dkk-1, Dkk-2, and cyromolgus monkey Dkk-4 proteins (0.1 ng to 100 ng) were used. A non-related recombinant protein was loaded as a control for non-specific assay signal (HIS protein). The dot blots were probed with RH2-18. Figure 6B shows that there was little or no detectable specific binding of RH2-18 to Dkk-2 or Dkk-4. Selectivity of the antibody towards Dkk-1 was minimally 100-fold. In this assay format, the detection limit for binding of RH2-18 to rhesus Dkk-1 was found to be about 1 ng. Signal strength at all concentrations of these Dkk isoforms was comparable to that towards a non-related HIS-tagged protein. Consistent with the epitope mapping and the sequence alignment data, the results showing that there was little to no cross-reactivity of the antibody to these proteins suggest there would be a low probability that RH2-18 would bind to Dkk-2 or Dkk-4. These data indicate RH2-18 and similar antibodies recognize a
novel, complex, three dimensional epitope covering several discrete regions of Dkk-1, which indicates that RH2-18 and similar antibodies are unique.

EXAMPLE 6

The affinity of the RH2-18 antibody for human Dkk-1 and Rhesus Dkk-1 was determined by measuring RH2-18 binding kinetics by surface plasmon resonance in a Biacore 3000 instrument according to manufacturer's instructions (Biacore, Inc., Piscataway, NJ). Several independent affinity studies were performed using different RH2-18 antibody lots against human Dkk-1 and rhesus Dkk-1. The calculated Kd values for each experiment ranged from 202 to 269 pM for binding to human Dkk-1, with a mean value of 251 pM. For rhesus monkey Dkk-1, the range was between 771 to 934 pM and the mean value was 858 pM. -

The quality of the RH2-18 antibodies was assessed by size exclusion chromatography in comparison to other well characterized mABs. The results suggest excellent stability of RH2-18 antibodies as there was no apparent aggregation.

EXAMPLE 7

RH2-18 was evaluated in in vivo pharmacodynamic and efficacy studies.

Genetic proof-of-concept data exists that indicates that disrupting the Dkk-1/Wnt interaction in the developing skeleton causes increased bone mass. In addition, an anti-Dkk-1 antibody developed by Amgen was osteoanabolic when injected to rats in a three week study (30 mg/kg, twice weekly s.c.) (See, DKKI Inhibition Increases Bone Mineral Density in Rodents Grisanti M et ah, J. Bone Miner. Res. 21: S25 (2006). Bone mass increases were seen in both growing and adult mice dosed in a similar fashion and for a similar period of time. An in vivo proof-of-concept study was undertaken with RH2-18 to validate the phenotype pharmacologically. This study was performed in growing mice with a plan to later test the response in the adult skeleton. Thus, the purpose of the study was to establish that RH2-18 antibodies, which neutralized all tested Dkk-1 functions in vitro, increased bone mass in the growing skeleton. The tested hypothesis, therefore, was that RH2-18 increases bone mass in a dose-effect fashion in the long bone of growing mice.

Five week old C57BL/6J female mice were obtained and acclimated to the animal facility for one week. RH2-18 antibody was administered subcutaneously (s.c.) in 0.1 mL phosphate buffer per mouse. There were 11 mice per group. Mice were treated twice weekly for four consecutive weeks with 0, 0.5, 1.5, or 5 mg/kg RH2-18 antibody, or 0.4 mg/kg PTH (1-34) (s.c. 3X/wk). At necropsy, femurs and vertebrae were dissected free and fixed in 70% ethanol. Whole femurs were scanned by Piximus (GE/Lunar; Schenectady, NY) dual energy X-ray absorptiometry. The femurs were subdivided into a distal region of interest (ROI), located 0 to 3 mm from the distal end, and a central region of interest, located 5 to 10 mm from the distal end. The central ROI is composed of 100% cortical bone, while the distal ROI is about 20% trabecular bone. Piximus software calculates bone mineral
density (BMD, mg/cm²) for whole bone (WFBMD), distal femur (DFBMD), and central femur (CFBMD). The results are shown in Figures 7 through 9.

Figure 7 shows that the distal femur bone mineral density (BMD) was increased 5.2 to 8.7% in a dose effect fashion by RH2-18 antibody, in the dose range 0.5 to 5 mg/kg. This BMD change most likely represents effects on both cancellous and cortical bone. Figure 8 shows that whole femur BMD was increased 4.7 to 4.8% by RH2-18 antibody, in the dose range 1.5 to 5 mg/kg. This BMD change most likely represents effects on both cancellous and cortical bone. Figure 9 shows that central femur BMD was increased 3.2 to 3.5% in a dose effect fashion by RH2-18 antibody, in the dose range 1.5 to 5 mg/kg. This BMD change represents effects primarily on cortical bone.

These results indicate that administering RH2-18 antibody to the mice over a four week period caused in a dose dependent manner a significant increase in bone mass in growing female mice.

It can be concluded from the results that administering RH2-18 antibody (which blocks the interaction of Dkk-1 and Wnt) to mice causes high bone density in growing mice. Therefore, it is possible to increase bone mass in the growing skeleton using antibodies such as RH2-18 to neutralize the Dkk-1/Wnt signaling blockade.

EXAMPLE 8

The canonical Wnt signaling cascade regulates intestinal epithelial cell proliferation. Genetic lesions in the genes for cytoplasmic signaling intermediates (β-catenin, APC, axin) cause enhanced transcriptional activity, which is associated with more than 90% of all colorectal cancer. To date, no such tumorigenic mutations have been described for cell surface receptors or secreted intermediates that regulate this pathway, including Dkk-1. Nonetheless, the possible tumorigenic effect of antibody that neutralizes Dkk-1 was evaluated.

Dkk-1 antibody should only exert effects on tissues where Dkk-1 is expressed. Thus, the tissue distribution of Dkk-1 is an important factor in determining safety. In mice, Dkk-1 is mainly expressed in bone (about 64-fold over the next highest expressing tissue) as shown in Table 6 below.

<table>
<thead>
<tr>
<th>mouse</th>
<th>Bone</th>
<th>Bladder</th>
<th>Intestine</th>
<th>Liver</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT value</td>
<td>26</td>
<td>32</td>
<td>36</td>
<td>34</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 6 shows CT values for Dkk-1 expression in select tissues of mouse. For reference, CT is the threshold cycle. It is the PCR cycle in real-time quantitative PCR assays at which a statistically significant increase in reporter fluorescence can be detected above the background. CT values in the mid-upper 30s represent very low to no expression. The mRNA level is defined as low with 30<CT<40, medium 30<CT<25, high 25<CT<15. As shown above, expression of Dkk-1 was very low or not at all in bladder, intestine, liver, and uterus tissue and moderate in bone.
In human tissues, the expression levels of Dkk-1 in the same tissue were observed to vary depending on the donor. Expression was generally at minimal detection levels in normal intestine, colon or liver (CT values in the high 30s (that is, undetectable), where Wnt signaling abnormality is associated with the tumorigenesis. Dkk-1 CT values on average were in the high 20s for human bladder, cervix, stomach, and uterus (tissues not associated with Wnt/β-catenin-induced tumors), while they were around 30 for several of the bone samples we obtained from patients after knee replacement. In this regard, Dkk-1 expression was highest in tissues not typically associated with Wnt signaling-induced tumors. Conversely, tissues where cytoplasmic mutations can be associated with tumorigenesis showed no substantial Dkk-1 expression.

For human cell lines, Dkk-1 CT values were in the middle 20s for MG-63, Caco-2, MCF-7, SW480 and HCT16 cells, while they were in the 30s range in HEK-293, SW48 and DLD1 cells. The latter two cell lines are derived from colon cancer tissue with cytoplasmic Wnt signaling pathway mutations. In human tissue samples, Dkk-1 tended to be up-regulated in tumors as compared to their paired normal tissues. In these studies, Dkk-1 expression in these cells was measured and compared to that of other proliferative markers (Ki-67, PCNA, E2F1 and IGFBP-3) by real-time quantitative PCR analysis using a TAQMAN system (Applied Biosystems, Foster City, CA). All data CT values were converted to fold induction vs. the corresponding normal samples. In summary, Dkk-1 tissue distribution in mice Dkk-1 showed that the mRNA for this gene is mainly expressed in bone, while it is expressed in bone, bladder and cervix in humans. Dkk-1 is highly up-regulated in most human tumors or cancer cell lines, as were several known proliferative markers.

To monitor the effects of anti-Dkk-1 antibodies on proliferation of normal tissues in vivo, the levels of the proliferation markers identified above (Ki-67, PCNA, E2F1) and genes associated with cell hyperplasia (IGFBP-3, Dkk-1) in mice treated for four weeks (proof-of-concept study described in Example 7) were quantified. These markers were chosen as a measure of cell proliferation in selected tissues (intestine, liver, bladder, uterus). Quantitative PCR analysis using a TAQMAN system did not reveal any consistent or substantial difference in the expression of the proliferation markers in anti-Dkk-1 antibody-treated treated vs. control samples after four weeks of treatment.

Based on the higher expression of Dkk-1 in colon cancer cell lines, the effects of anti-Dkk-1 antibody on transcription and proliferation were assessed in vitro. RH2-80 antibody (30 µg/mL) was tested in a proliferation assay using colon cancer cell lines exhibiting constitutively active Wnt signaling - SW480, HCT16, SW48, DLD1, and HEK293. MG-63 osteosarcoma cells and non-specific antibody 8B4 were used as controls. For measuring cell proliferation, the cells were cultured according to American Type Culture Collection (ATCC) protocols. The day before performing the proliferation assay, freshly prepared cells were seeded at 2 to 5 x 10^3 cells/well of 96-well Cytostar scintillating plate in 100 µL of the corresponding cell medium. On the following day, 0.5 µCi/mL of [methyl-14C]thymidine was added to each well along with either RH2-80 antibody, 8B4 antibody, or Wnt3A. Cell growth was measured using a 1450 MICROBETA Jet (Wallac Inc., Gaithersburg, MD) at 1 day, 2
days, 3 days, and 5 days after adding the antibody. Greater integration of [methyl-14C]thymidine into cells, correlated with greater light detected within the Cytostar plate on MICROBETA Jet. Cells were replenished with fresh media containing treatment on the third day. Data shown in the Figure 10B represented day 3 treatment.

RH2-80 antibody (30 µg/mL) was tested in a TOPFlash transcription assay using colon cancer cell lines exhibiting constitutively active Wnt signaling - SW480, HCT16, SW48, DLD1, and HEK293. MG-63 osteosarcoma cells and non-specific antibody 8B4 were used as controls.

For measuring Wnt/DKK1 signaling in the assay, cells were seeded at 25,000 cells/well in 96-well plate in 100 µL of the complete cell medium according to ATCC protocol the day before transfection. On the day of transfection, 60 µL of FuGene 6, 375 ng pTopFlash, 80 ng pTKrenilla, and 5 µg pcDNA3.1-LEFI were added to a final volume of 600 µL OPTIMEM. The mixture was incubated for 1 hour at room temperature. Then, 1.4 µL of OPTIMEM was added to the above mixture and mixed well gently. Then, 20 µL of the DNA mixture was added to each well of 96-well plate. The plate was gently tapped and put back to the 37°C incubator. The day after transfection, cells were treated with the either RH2-80 antibody, 8B4 antibody, or Wnt3A. About 24 hours after treatment, a Dual-luciferase assay was performed according to Promega protocol. Luciferase signal was normalized to renilla signal first before calculating the induction. Fold induction was obtained by comparing to control signal.

Results of the transcription and proliferation assays using HCT16 cells are shown in Figures 10A and 10B. In general, the results indicated that many cells had high baseline transcription levels that could not be further enhanced by Wnt treatment. For all cell lines, RH2-80 alone did not enhance LEF-I/TCF promoter activity. This lack of responsiveness was seen in cells that did respond to Wnt3a treatment, such as HCT16. Separate analyses showed that the anti-Dkk-1 antibody RH2-80 did not alter the expression of endogenous Wnt target genes, including myc, jun, PPARδ, FGF18, COX2, IGF-1, and IGF-2. Further, there was no induction of Wnt signaling components such as Dkk-1, Dkk-2, Dkk-4, LRP5, LRP6, Sost, WIF1, and CTGF in any of the six tested cell lines.

In the cell proliferation studies, the results showed that RH2-80 (at 30 µg/mL) did not enhance any cell growth in each of the tested cell lines. Assays were performed in the presence of serum and in the absence or presence of supplemental Wnt3a treatment. The results for effect of RH2-80 antibody on HCT16 cell proliferation are shown in Figure 11B. Parallel analyses tested RH2-80 and RH2-59 antibodies (both at 16 µg/mL) for an effect on the growth of MG-63, HCT16, and SW480 cells. No growth enhancement was observed in serum-containing medium. No stimulation of cell growth was observed by RH2-80 antibody treatment of MG-63 and HCT16 cells when tested in OPTIMEM serum free medium in comparison to vehicle (N.S.). Significance was observed in comparisons between RH2-80 and RH2-59 (anti-Dkk-1 antibody with similar efficacy) and with 20C2HA (negative control), both of which trended towards slight antiproliferative effects vs. the vehicle control (N.S.). Repeat analyses showed no antibody effects in HCT16 cells (seeded at higher initial density) and in SW480 cells in
In this regard, there was no downward trend in growth for cells treated with RH2-59 or 20C2HA, and again, RH2-80 performed identically to the vehicle control.

Further study of the anti-Dkk-1 antibodies in SCED mice of human colon cancer xenografts was also performed. In this xenograph model for tumor growth, phosphate-buffered saline (PBS) was used as the vehicle, RH2-18 antibody expressed in a Pichia strain at 5 mpk was used as the test group, while Wnt3a at 0.02 mpk was used as a positive control. Subcutaneous injection of 1x10^6 HCT16 cells in 100 µL PBS into the right and left flank of about six-week-old NOD.CB 17-Prkdcscid/J (SCED) mice. Treatments were followed on the second day after injection and continued twice week for a total of seven treatments. Tumors were isolated after about 3.5 weeks. Percent of tumor weight was obtained by combing both tumors from one mouse and dividing by the mouse's total body weight. Statistics were performed using Student's t Test. The results are shown in Figure 11. Wnt3a significantly increased tumor growth vs. the PBS (Vehicle) treated group by about two-fold. The antibody did not significantly stimulate tumor growth vs. vehicle treatment. Two of four animals in the vehicle group showed some evidence of tumor cells in the abdomen, which was not observed in the other groups. This possible HCT16 cell infiltration into the abdomen may have artificially reduced the apparent tumor size in these mice due to cell loss in the region of interest and thus, could have lowered the tumor size in these groups.

***

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.
WHAT IS CLAIMED IS:

1. An isolated antibody or immunologically functional fragment thereof, comprising:
   (a) one or more light chain (LC) complementary determining regions (CDRs)
   (i) an LC CDR1 with at least 80% sequence identity to SEQ ID NO: 12,
   (ii) an LC CDR2 with at least 80% sequence identity to SEQ ID NO: 13, and
   (iii) an LC CDR3 with at least 80% sequence identity to SEQ ID NO: 14;
   (b) one or more heavy chain (HC) CDRs selected from the group consisting of
   (i) an HC CDR1 with at least 80% sequence identity to SEQ ID NO: 9,
   (ii) an HC CDR2 with at least 80% sequence identity to SEQ ID NO: 10, and
   (iii) an HC CDR3 with at least 80% sequence identity to SEQ ID NO: 11; or,
   (c) one or more LC CDRs of (a) and one or more HC CDRs of (b), wherein the
   antibody or immunologically functional fragment thereof can specifically bind a Dkk-1 polypeptide.

2. The isolated antibody or immunologically functional fragment of claim 1, that comprises:
   (a) one or more light chain (LC) complementary determining regions (CDRS)
   (i) an LC CDR1 with at least 90% sequence identity to SEQ ID NO: 12,
   (ii) an LC CDR2 with at least 90% sequence identity to SEQ ID NO: 13, and
   (iii) an LC CDR3 with at least 90% sequence identity to SEQ ID NO: 14;
   (b) one or more heavy chain (HC) CDRs selected from the group consisting of
   (i) an HC CDR1 with at least 90% sequence identity to SEQ ID NO: 9,
   (ii) an HC CDR2 with at least 90% sequence identity to SEQ ID NO: 10, and
   (iii) an HC CDR3 with at least 90% sequence identity to SEQ ID NO: 11; or,
   (c) one or more LC CDRs of (a) and one or more HC CDRs of (b), wherein the
   antibody or immunologically functional fragment thereof can specifically bind a Dkk-1 polypeptide.

3. The isolated antibody or immunologically functional fragment of claim 2, that comprises:
   (a) one or more light chain (LC) complementary determining regions (CDRS)
   (i) an LC CDR1 with the sequence as set forth in SEQ ID NO: 12,
   (ii) an LC CDR2 with the sequence as set forth in SEQ ID NO: 13, and
   (iii) an LC CDR3 with the sequence as set forth in SEQ ID NO: 14;
(b) one or more heavy chain (HC) CDRs selected from the group consisting of
(i) an HC CDR1 with the sequence as set forth in SEQ ID NO: 9,
(ii) an HC CDR2 with the sequence as set forth in SEQ ID NO: 10, and
(iii) an HC CDR3 with the sequence as set forth in SEQ ID NO: 11; or,
5
(c) one or more LC CDRs of (a) and one or more HC CDRs of (b), wherein the
antibody or immunologically functional fragment thereof can specifically bind a Dkk-1 polypeptide.

4. The isolated antibody or immunologically functional fragment of claim 3 that
comprises the LC CDR3 with the amino acid sequence of SEQ ID NO: 14 or the HC CDR3 with the
10 amino acid sequence of SEQ ID NO: 11.

5. The isolated antibody or immunologically functional fragment of claim 4 that
comprises the LC CDR3 with the amino acid sequence of SEQ ID NO: 14 and the HC CDR3 with the
15 amino acid sequence of SEQ ID NO: 11.

6. The isolated antibody or immunologically functional fragment of claim 1 that
comprises at least two CDRs from the CDRs listed in (a) and (b).

7. The isolated antibody or immunologically functional fragment of claim 6 that
comprises at least three CDRs from the CDRs listed in (a) and (b).

8. The isolated antibody or immunologically functional fragment of claim 7 that
comprises at least four CDRs from the CDRs listed in (a) and (b).

9. The isolated antibody or immunologically functional fragment of claim 8 that
comprises at least five CDRs from the CDRs listed in (a) and (b).

10. The isolated antibody or immunologically functional fragment of claim 9 that
comprises all six of the CDRs listed in (a) and (b).

11. The isolated antibody or immunologically functional fragment of claim 1 that is
a domain antibody.

12. The isolated antibody or immunologically functional fragment of claim 1 that
dissociates from the Dkk-1 polypeptide with a Kd of about 269 pM or less.
13. The isolated antibody or immunologically functional fragment of claim 1 that is a monoclonal antibody.

14. The isolated antibody or immunologically functional fragment of claim 1 that is a scFv, a Fab, a Fab' or a (Fab')2.

15. The isolated antibody or immunologically functional fragment of claim 1 that is a human or humanized antibody.

16. An isolated antibody or immunologically functional fragment thereof, comprising:
   (a) a light chain variable region (VL) having at least 80% sequence identity with SEQ ID NO:4;
   (b) heavy chain variable region (VH) having at least 80% sequence identity with SEQ ID NO:8; or
   (c) a VL of (a) and a VH of (b).

17. The isolated antibody or immunologically functional fragment of claim 16, that consists of two identical VH and two identical VL.

18. The isolated antibody or immunologically functional fragment of claim 16, wherein the VL has at least 90% sequence identity with SEQ ID NO:4; and the VH has at least 90% sequence identity with SEQ ID NO:8.

19. The isolated antibody or immunologically functional fragment of claim 18, that consists of two identical VH and two identical VL.

20. The isolated antibody or immunologically functional fragment of claim 16, wherein the VL has at least 95% sequence identity with SEQ ID NO:4; and the VH has at least 95% sequence identity with SEQ ID NO:8.

21. The isolated antibody or immunologically functional fragment of claim 20 that consists of two identical VH and two identical VL.

22. The isolated antibody or immunologically functional fragment of claim 21 wherein the VL has the amino acid sequence of SEQ ID NO:4; and the VH has the amino acid sequence of SEQ ID NO:8.
23. The isolated antibody or immunologically functional fragment of claim 22 that consists of two identical VH and two identical VL-

24. The isolated antibody or immunologically functional fragment of claim 22 that comprises:
   (a) a light chain comprising the amino acid sequence of SEQ ID NO:3;
   (b) a heavy chain comprising the amino acid sequence of SEQ ID NO:7; or
   (c) a light chain comprising the amino acid sequence of SEQ ID NO:3 and a heavy chain comprising the amino acid sequence of SEQ ID NO:7.

25. The isolated antibody or immunologically functional fragment of claim 24 that consists of two identical light chains and two identical heavy chains.

26. The isolated antibody or immunologically functional fragment of claim 16 that is a monoclonal antibody.

27. The isolated antibody or immunologically functional fragment of claim 16 that is a scFv, a Fab, a Fab' or a (Fab').sub.2.

28. The isolated antibody or immunologically functional fragment of claim 16 that is a human or humanized antibody.

29. An isolated antibody or an immunologically functional fragment thereof that specifically binds a mature human Dkk-1 protein consisting of amino acids 32-266 of SEQ ID NO:35 and having a tertiary structure established by a disulfide bond between cysteine residues 220 and 245, wherein the antibody binds to an epitope comprising a loop consisting of the amino acids between cysteine residues 201 and 210 of SEQ ID NO:35.

30. The isolated antibody or immunologically functional fragment of claim 29 that is a monoclonal antibody.

31. The isolated antibody or immunologically functional fragment of claim 29 that is a scFv, a Fab, a Fab' or a (Fab').sub.2.

32. The isolated antibody or immunologically functional fragment of claim 29 that is a human or humanized antibody.
33. An antibody or an immunologically functional fragment thereof that competes with an antibody of claim 24 for specific binding to a Dkk-1 polypeptide.

34. The isolated antibody or immunologically functional fragment of claim 33 that competes with an antibody that consists of two identical heavy chains and two identical light chains, wherein the heavy chains consist of the amino acid sequence set forth in SEQ ID NO:3 and the light chains consist of the amino acid sequence set forth in SEQ ID NO:7.

35. The isolated antibody or immunologically functional fragment of claim 34 that dissociates from the Dkk-1 polypeptide with a Kd of about 269 pM or less.

36. A nucleic acid encoding (a) a light chain CDR with the amino acid sequence as set forth in SEQ ID NO:14; and/or (b) a heavy chain CDR with the amino acid sequence as set forth in SEQ ID NO:1 1, wherein the nucleic acid encodes an antibody or an immunologically functional fragment thereof.

37. A nucleic acid comprising a sequence that encodes the VH, the VL or both the V_H and the V_L of the antibody or immunologically active fragment of claim 16.

38. A nucleic acid comprising a nucleic acid segment encoding the VH, VL or both the V_H and the V_L of the antibody or immunologically active fragment of claim 22.

39. An expression vector comprising the nucleic acid of claim 37.

40. An isolated cell comprising the expression vector of claim 39.

41. A method of producing an antibody or an immunologically active fragment thereof comprising the step of culturing a cell according to claim 40.

42. A composition comprising an antibody or immunologically functional fragment thereof according to claim 1 and a component selected from the group consisting of a buffer, a pharmaceutically acceptable diluent, a carrier, a solubilizer, an emulsifier, and a preservative.

43. A composition comprising an antibody or immunologically functional fragment thereof according to claim 16 and a component selected from the group consisting of a buffer, a pharmaceutically acceptable diluent, a carrier, a solubilizer, an emulsifier, and a preservative.
44. A method of treating a disease in an individual comprising administering to the individual an effective amount of the antibody or immunologically active fragment thereof of claim 1, wherein the disease is selected from the group consisting of arthritis, diseases responsive to stem cell renewal, inflammatory diseases, neurological diseases, ocular diseases, renal diseases, pulmonary diseases, bone disorders, and skin diseases.

45. The method of claim 44, wherein the disease is selected from the group consisting of rheumatoid arthritis, psoriatic arthritis, and osteoarthritis.

46. The method of claim 44, wherein the disease is osteoporosis.

47. A method of stimulating growth of bone in an individual comprising administering to the individual an effective amount of the antibody or immunologically active fragment thereof of claim 1.
FIGURE 1B

L-001874698 IgG2M4 898 bp

HCMV Intron A Promoter
Leader
BGH pA
OriP
Kan
Amino Acid Sequences of Rh2-18

Rh2-18 Antibody Light Chain Amino Acid Sequence

1  QSVLTQPPSV SGAPGQRVTI SCTGSSSNIG AGYDVHWWAQ LPGTAPKLLI
51  YGYSNRRPSGV PDRFSGSKSG ASASLAINTGL RPDEADYYC QSYDNSLSSY
101  VFGGGTQLTV LSQPKANPTV TLFPSSSEEL QANKATLVCL ISDFYPGAVT
151  VAWKADGSPV KAGVETTKPS KQSNKYYAS SYLSLPEQW KSHRSYSCQV
201  THEGSTVEKT VAPTECS

Rh2-18 Antibody Heavy Chain Amino Acid Sequence

1  EVQLVQSGAE VKKPGASVKV SCKASGYTFT DYYIHWVRQA FGQGLEWMGW
51  IHSNSGATTY AQKFAQAVTM SRDTSSTAY MELSRLESDD TAMYFCSRED
101  YWGQGTLVTV SSASTKGPSV PFLAPCSRST SESTAALGCL VKDYFPEPV
151  VSWNSGALTQ GVHTFPAVLQ SSSLGSLSSV VTVTSSNGFT QTYTCNVDHK
201  PSNTKVDKTV ERKCCVECP CPAPPVAGPS VFLFPPKPKD TLMISRTPEV
251  TCVVDVSQE DPEVQFWNYV DGVEHNAKT KPREEQFNST FRVSVSSTVL
301  HQDMLNGKEY KCKVSNKGLP SSIKTISKT KGQPREPQVY TLPSPREEMT
351  KNSQSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPMLED SDGSFFLYSK
401  LTVDKSRRWQQ GNVFSCSVMH EALHNHYTQK SLSLPQG

FIGURE 1C
Light chain variable region sequence

L-001874698
VL1 14-7A
OSVLTQPPSV SGAPGQRTVIT S\textbf{T}G\textbf{S}\textbf{S}N\textbf{I}G AGYDVH\textbf{W}YQQ LPGTAPKLLI

L-001874698
VL1 14-7A
YG\textbf{S}N\textbf{R}PSGV PD\textbf{R}FSGSKSG AS\textbf{A}S\textbf{L}A\textbf{I}TLGL RP\textbf{C}DE\textbf{A}DYYC QSYDNSLSSY

L-001874698
(JL7)
VFGG\textbf{G}TQLTV LS
A VFGG\textbf{G}TQLTV L

FIGURE 1D
**Heavy chain variable region sequence**

L-001874698  
EVQLVQSGAE VKKPGASVKV SCKASGYTFT **DYIHWVRQA PGQGLEWMGW**

VH1 1-3 1-02  
QVQLVQSGAE VKKPGASVKV SCKASGYTFT **GYMYHWVRQA PGQGLEWMGW**

**HCDR1**

L-001874698  
IHSNSGATTY **AQKFOARVTM** SRDTSSSTAY MELSRLESDD TAMYFCSR

VH1 1-3 1-02  
INPNSSGNTNY **AQKFOARVTM** TRDTSISTAY MELSRLESDDD TAVYYCAR

**HCDR2**

L-001874698  
**-ED YWGQGTLVTV SS**

(JH4)  
**YFD YWGQGTLVTV SS**

**HCDR3**

**FIGURE 1E**
FIGURE 4
FIGURE 5A

Loading control

RH2-18

38
Central Femur BMD

% from Vehicle

Mean±SEM

CFBMD (mg/cm²)

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- **diff from 0 (P<.0001)**
  - o

- **diff from 0 (P<.05)**
  - p

RH2-18 598 (mpk, SC, 2X/wk) (SC, 3X/wk)

**FIGURE 9**
FIGURE 11

% of Tumor/Body Weight

Wnt3a

RH2-18 (G)

Veh

*
### A. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both national classification and IPC:

- INV. C07K16/18
- A61K39/395
- A61P19/00

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols):

- C07K A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched:

Electronic data base consulted during the international search (name of data base and, where practical, search terms used):

- EPO-Internal
- WPI Data
- BIOSIS
- EMBASE

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C

See patent family annex

- Special categories of cited documents
- "Y" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents such combination being obvious to a person skilled in the art
- "A" member of the same patent family

Date of the actual completion of the international search: 30 June 2008

Date of mailing of the international search report: 18/07/2008

Name and mailing address of the ISA:

European Patent Office, P B 5818 Patentlaan 2
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Tel (+31-70) 340-2040, Tx 31 651 epp nl
Fax (+31-70) 340-3016

Authorized officer: Perez-Mato, Isabel

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Form PCT/ISA/210 (continuation of second sheet) (April 2005)
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. √ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 44-47 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ D No protest accompanied the payment of additional search fees.
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