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(54) **Title**: HIGH-AFFINITY NUCLEIC ACID APTAMERS AGAINST SCLEROSTIN PROTEIN

(57) **Abstract**: Described are nucleic acid aptamers that are able to bind to and inhibit the function of sclerostin, which is an important negative regulator of bone growth. The aptamers have application as therapeutics for diseases of bone including osteoporosis, osteopenia, osteoarthritis and other osteoporosis-related conditions and complications.

HIGH-AFFINITY NUCLEIC ACID APTAMERS AGAINST SCLEROSTIN PROTEIN

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to provisional application Serial No. 61/349,058, filed on May 27, 2010, which is incorporated herein by reference.

FIELD

[0002] Described herein are novel nucleic acid ligands (aptamers) directed against sclerostin, which is an extracellular negative regulator of bone growth. The disclosed aptamers have promise to directly stimulate bone formation and be used as therapeutics to treat bone disease such as osteoporosis.

BACKGROUND

[0003] Nucleic acid aptamers are in vitro evolved nucleic acids that are able to bind and inhibit protein function.

[0004] Nucleic acid aptamers have been developed over the last 20 years to develop therapeutic aptamers against a variety of targets for a number of diseases including macular degeneration, HIV, cancer, cardiovascular disease, amongst others. One aptamer, pegaptanib (MACUGEN), is used clinically for the treatment of macular degeneration, discovered by Gilead Sciences, licensed to Eyetech Pharmaceuticals and marketed outside the USA by Pfizer. Other aptamer based drugs are in clinical trials against coagulation factors, growth factors, inflammation markers and other targets. To our knowledge, no aptamers have been developed in relation to osteoporosis.

[0005] Osteoporosis has a significant medical and economic impact worldwide. In developed nations, approximately 4% of the population has osteoporosis, and the economic burden to the US alone has been estimated at $14 billion annually. Currently, the majority of pharmacological agents presently used in the clinic are bisphosphonate

based antiresorptive agents, including alendronate (FOSAMAX), risedronate (ACTONEL) or ibandronate (BONIVA).

[0006] The established drug agents can be somewhat effective in controlling bone mass, but they have a number of disadvantages including poor oral absorption, esophagitis and osteonecrosis of the jaw. Therefore, there is a move toward anabolic agents that stimulate bone formation that would potentially accelerate bone growth. Recently, teriparatide (recombinant parathyroid hormone, FORTEO) was approved as the first anabolic agent to enter the clinic but there have been some concerns regarding FORTEO that it is only effective to remodel bone during the first 12 months treatment and then efficacy declines.

[0007] Sclerostin is an osteocyte-specific negative regulator of bone formation which makes it an attractive drug target for osteoporosis therapy. Amgen is developing protein-based antibodies against sclerostin for osteoporosis therapy (Human Clinical Phase 2). Novartis and Eli Lily are also developing sclerostin-blocking antibodies (Preclinical). OsteogeneX is developing small molecule inhibitors against sclerostin, currently in preclinical and lead optimization.

[0008] Antibodies generally have a number of limitations including risk of immune response, batch to batch variation and limited shelf-life. Small molecules have significant problems of binding affinity and specificity.

SUMMARY

[0009] The present invention provides aptamers, including their formulations and/or compositions, that bind to the protein sclerostin, referred to herein as "sclerostin aptamers", and methods for using such sclerostin aptamers for the treatment and prevention of osteoporosis and other related bone diseases.

[0010] The invention provides for an alternative molecular approach that stimulates bone growth by inhibiting sclerostin function and that has fewer side effects for osteoporosis

and other related diseases. This is addressed with the development of nucleic acid aptamers that target and inhibit sclerostin specifically and effectively. This invention specifically relates to aptamers that are able to bind to and inhibit the function of sclerostin, which is an important negative regulator of bone growth and implicated in bone disease such as osteoporosis. This invention claims aptamers, as a unique new composition of matter, that inhibit sclerostin function and have clear implications as therapeutics for osteoporosis and related diseases.

[0011] The present invention is directed to methods of using anti-sclerostin aptamers as therapeutics for stimulating bone formation. The method comprises administering to a human an amount of anti-sclerostin aptamers that is effective to cause an increase in the rate of bone formation.

[0012] The formulations described herein comprise a sclerostin aptamer or a pharmaceutically acceptable salt thereof. The formulations may comprise any aptamer that binds to sclerostin or a variant or a fragment thereof. Preferably, the aptamer binds to sclerostin and inhibits its activity.

[0013] The present invention also provides methods of using anti-sclerostin aptamer for treating bone-related diseases, disorders or conditions wherein the presence of sclerostin causes undesirable pathological effects. Such diseases, disorders and conditions include but not limited to osteoporosis, osteopenia, osteoarthritis, osteomalacia, osteodystrophy, osteomyeloma, bone fracture, Paget's disease, osteogenesis imperfecta, bone sclerosis, aplastic bone disorder, humoral hypercalcemic myeloma, multiple myeloma, and bone thinning following a disorder that causes or induces bone thinning. Such bone hinning diseases, disorders and conditions include but not limited to metastasis, hypercalcemia, chronic renal disease, kidney dialysis, primary hyperparathyroidism, secondary hyperparathyroidism, inflammatory bowel disease, Crohn's disease, long-term use of corticosteroids, or long-term use of gonadotropin releasing hormone (GnRH) agonists or antagonists. Subjects may be male or female of any ages.

[0014] The present invention may administer to a human subject an amount of anti-sclerostin aptamers alone or in combination with other drugs.

[0015] The present invention also provides diagnostic methods of quantifying expression of sclerostin. The anti-sclerostin aptamers may be labeled by a detectable substance including but not limited to fluorescent materials, enzymes, luminescent materials and radioactive materials. Such embodiments of the invention can be used to detect sclerostin levels in a biological sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG.1 is a drawing showing the method used for sclerostin aptamer selection.

[0017] FIG.2 is a table showing the sequences of the aptamers that were isolated from the ssDNA pool after 15 rounds of selection against sclerostin and are claimed in this invention. Conserved nucleotides are marked by asterisk.

[0018] FIG.3 is a graph showing determination of relative binding strength of aptamers against sclerostin using an aptamer enzyme-linked assay. Combinations of sclerostin aptamers, thrombin binding aptamers, GST-Sclerostin and GST protein (indicated by plus signs below the graph) were evaluated for their binding activity and cross-reactivity. The data are averaged from triplicate samples.

[0019] FIG.4 is a drawing showing the stability of modified aptamers: A.) unmodified Scl 2 aptamers and B.) 3' inverted thymidine modified Scl 2 aptamers evaluated in MC3T3-E1 cells that were supplemented with 5% FBS.

[0020] FIG.5 is a drawing showing the principle of a Wnt reporter assay and the effect of the sclerostin aptamers in cell culture. A.) Schematic showing the principles behind the reporter luciferase activity assay. B.) Effect of 3' inverted thymidine modified sclerostin aptamers on Wnt3a mediated activity in MC3T3-E1 cells. Data shown represents triplicate values of independent assays. *** represents that values are statistically

significant from each other analyzed by unpaired t-test with 95% confidence. C.) Effect of varying concentration of 3' inverted thymidine modified Scl 2 aptamers against Sclerostin functions.

[0021] FIG.6 is a graph determining the secondary structure of Scl 2 aptamers. A.) CD spectra of Scl 2 aptamers. B.) CD melting spectra of Scl 2 aptamers.

[0022] FIG.7 is a graph showing data by Isothermal Titration Calorimetry to measure the binding between sclerostin and sclerostin aptamers. Titration (top) of Scl 2 aptamer with serial injections of sclerostin. Binding isotherms (bottom) resulting from integration of raw calorimetric data after correction for the heat of aptamer dilution.

DETAILED DESCRIPTION

[0023] The invention will now be described further with reference to the following experimental procedures and results. The following experimental details are intended to be exemplary of the practice of the present invention, and should not be construed to limit the scope of the invention in anyway.

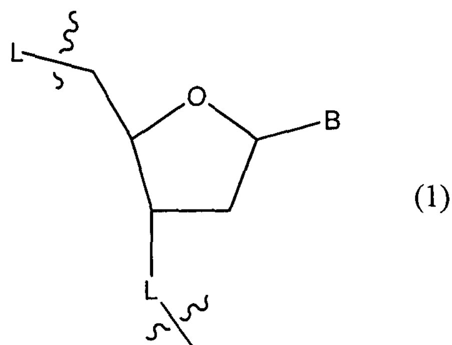
[0024] Seven different sequences of DNA aptamers were identified and claimed in this invention with details in Figure 2 using the scheme in Figure 1. Notably, aptamer Scl 1 and Scl 2 were a dominant sequence that accounted for 79% total in the pool. In addition, high level of sequence homology was observed, with a conserved motif present in almost all clones at approximately the same location in the random region (5'-GGXGGXXGGXTGGG-3') (SEQ ID NO: 1), where X is any nucleotide base.

[0025] Enzyme-linked binding assay showed specific binding of sclerostin aptamers to sclerostin. The results suggest that their relative binding strength for sclerostin were in the following order: Scl4> Scl 1=Scl 2 > Scl 3 (Figure 3). Aptamers showed negligible binding to GST, suggesting that the aptamers bound specifically to sclerostin. In addition, a thrombin binding aptamer with authentic G-quadruplex structure did not cross-react with the sclerostin protein.

[0026] Sclerostin aptamers were stabilized by capping the 3' end with 3' inverted thymidine (3'-InT) and evaluated in MC3T3-E1 cells that were supplemented with 5% FBS (Figure 4). Without any modifications, aptamers was quickly degraded by nuclease in serum as noted by the smear. In the case of 3' inverted thymidine aptamer, the oligo remained intact for 28 hr, suggesting that the stability of aptamers can be greatly enhanced.

[0027] Several different kinds of modifications can also be made to the aptamers to reduce exonuclease degradation and increase lifetime in the serum of an individual. Degradation can occur with intramuscular, intravenous and oral administration of the aptamer. Modification of the 3' end of the aptamer with inverted thymidine, deoxythymidine nucleotide, and polyethylene glycol (PEG) can reduce degradation of the oligonucleotide aptamer increases stability of the aptamer. In one embodiment, PEG has an average molecular weight from about 20 to 80 kDa.

[0028] Further, the phosphodiester linkages of the deoxyribose-phosphate backbone of the aptamer can also be modified to improve stability. As used through this document, the term "aptamer" refers to a molecule having repeating units of the structure shown in Formula 1. Wavy lines demarcate one nucleotide and/or repeat unit from a neighboring nucleotide and/or repeat unit.



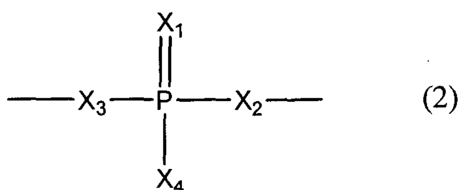
(1)

[0029] Each repeat unit of Formula 1 has a deoxyribose moiety linked to one of the common nucleotide bases (B): guanine, thymine, cytosine, adenine and/or uracil. The base (B) for each repeating unit is independent from the other repeat units. The

nucleotide sequences disclosed herein describe the order of appearance of bases (B) in an aptamer from the repeat unit on the 5' end of the aptamer to the 3' end of the aptamer.

[0030] "L" is a linker group that links the deoxyribose moiety of adjacent repeat units. In the well-known structure of DNA, the L group is a phosphate group PO_4H, which can exist as a salt or in a neutral protonated form. The deoxyribose moiety together with the linker group forms the backbone of the aptamer, where the nucleotide base "B" varies independently barriers between repeat units. The majority of the linker groups (L) forming the repeat units of Formula 1 in the aptamer are phosphate groups. As such, a majority of the backbone of the aptamer can be referred to as a deoxyribose-phosphate backbone. Many nuclease enzymes exist that can degrade oligonucleotide molecules without specificity for the specific nucleotide base sequence of the oligonucleotide molecule. Without wishing to be bound by any one particular theory, linker groups "L" other than phosphate can be incorporated into an oligonucleotide or aptamer to prevent degradation by nucleases.

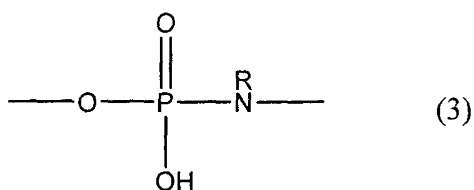
[0031] In one embodiment, L can be replaced with a group as shown in Formula 2, where X_{1-4} are independently O or S. X_2 and X_3 can be bonded to either the 3' carbon or the 5' carbon of a deoxyribose moiety. In one embodiment, X_1 is O and X_4 is O that can be either protonated or unprotonated. In another embodiment, one or more of X_2 and/or X_3 is S and X_1 and X_4 are O, where O can be either protonated or unprotonated. Where one of X_2 and/or X_3 are S, the aptamer can be referred to as having a thioester linkage in the deoxyribose-phosphate backbone.



(2)

[0032] In another embodiment, the linker group "L" is an amide-containing group as shown in Formula 3, where R is one or more of hydrogen and a substituted or

unsubstituted C_1-C_{20} hydrocarbyl group. A hydrocarbyl group is a carbon containing group that is straight or branched, saturated or unsaturated, cyclic or non-cyclic, aromatic or non-aromatic, where with carbon can be bonded with 1 or more heteroatoms including O, N, S and halides. Where the linker group "L" is a group having Formula 3, the aptamer can be referred to as having an amide linkage in the deoxyribose-phosphate backbone. The "NR" group of Formula 3 can be bonded to either the 3' carbon or the 5' carbon of a deoxyribose moiety. In one embodiment, R is methoxymethyl or methoxyethyl.



(3)

[0033] In one embodiment, the aptamer has from about 20 to about 50 nucleotide bases and/or repeat units. In other embodiment, the aptamer has from about 14 to about 50 nucleotide bases and/or repeat units. In another embodiment, the aptamer has from about 30 to about 35 nucleotide bases and/or repeat units. In one embodiment, the aptamer has from about 1 to about 15 repeat units having a linker "L" selected from Formulae 2-3. In another embodiment, the aptamer has from about 1 to about 10 repeat units having a linker "L" selected from Formulae 2-3. In another embodiment, the aptamer has from about 1 to about 5 repeat units having a linker "L" selected from Formulae 2-3. In yet another embodiment, the aptamer has more than 10 repeat units having a linker "L" selected from Formulae 2-3. Linker groups in repeat units not selected from formulae 2-3 are phosphate

[0034] In one embodiment, the aptamer has from about 10 to about 100% of the repeat units having a linker "L" selected from Formulae 2-3. In another embodiment, the aptamer has from about 10 to about 70% of the repeat units having a linker "L" selected from Formulae 2-3. In yet another embodiment, the aptamer has from about 10 to about 50% of the repeat units having a linker "L" selected from Formulae 2-3. In still another

embodiment, the aptamer has from about 10 to about 30% of the repeat units having a linker "L" selected from Formulae 2-3. In a further embodiment, the aptamer has from about 10 to about 20% of the repeat units having a linker "L" selected from Formulae 2-3. Linker groups in repeat units not selected from formulae 2-5 are phosphate.

[0035] Many nucleases are exonucleases that degrade oligonucleotides from the 5' or 3' end. As such, in one embodiment a linker group L selected from Formula 2-3 is located within about 5 repeat units from the 5' or the 3' end of the apatmer. In another embodiment, a linker group L selected from Formula 2-3 is located within about 3 repeat units from the 5' or the 3' end of the apatmer. In yet another embodiment, a linker group L selected from Formula 2-3 is located is part of the repeat unit on the 5' or the 3' end of the apatmer.

[0036] Degradation of the aptamers can also be reduced by the inclusion of modified nucleotide bases (B). The pyrimidine nucleotide bases, cytosine, thymine and uracil can be replaced with alkylated pyrimidines. Examples of alkylated pyrimidines include pseudoisocytosine; N4,N4-ethanocytosine; 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil; 5-fluorouracil; 5-bromouracil; 5-carboxymethylaminomethyl-2-thiouracil; 5-carboxymethylaminomethyl uracil; dihydrouracil; 1-methylpseudouracil; 3-methylcytosine; 5-methylcytosine; 5-methylaminomethyl uracil; 5-methoxy amino methyl-2-thiouracil; 5-methoxycarbonylmethyluracil; 5-methoxyuracil; uracil-5-oxyacetic acid methyl ester; psuedouracil; 2-thiocytosine; 5-methyl-2 thiouracil, 2-thiouracil; 4-thiouracil; 5-methyluracil; N-uracil-5-oxyacetic acid methylester; uracil 5-oxyacetic acid; 2-thiocytosine; 5-propyluracil; 5-propylcytosine; 5-ethyluracil; 5-ethylcytosine; 5-butyluracil; 5-pentyluracil; 5-pentylcytosine; methylpsuedouracil; and 1-methylcytosine. The purine nucleotide bases, adenine and guanine, can be replaced by alkylated purines. Examples alkylated purines include 8-hydroxy-N6-methyladenine; inosine; N6-isopentyl-adenine; 1-methyladenine; 1-methylguanine; 2,2-dimethylguanine; 2-methyladenine; 2-methylguanine; N6-methyladenine; 7-methylguanine; 2 methylthio-N6-isopentenyladenine; and 1-methylguanine.

Aptamer Sequences and Properties

[0037] An aptamer is an oligonucleotide that binds to a non-nucleic acid biological target. In a double-stranded DNA molecule, the nucleotide bases form intermolecular pyrimidine-purine pairs through the well-known Watson-Crick base paring. Aptamers are believed to recognize non-nucleic acid biological targets through bonding of the nucleotide bases with non-nucleic acid molecules. The aptamers can be single-stranded, double-stranded, or form intramolecular base-pairing in portions of the aptamer sequence. Seven different aptamer sequences were identified as capable of binding to sclerostin, which is illustrated in detail in the following working examples.

The term "anti-sclerostin activity," “sclerostin inhibitor,” "antagonist," "neutralizing," and "downregulating" refer to a compound (or its property, as appropriate) which acts as an inhibitor of sclerostin relative to sclerostin activity in the absence of the same inhibitor. The term “variant” refers to a polynucleotide or aptamer that differs in nucleotide sequence from a “parent” polynucleotide or aptamer by virtue of addition, deletion and/or substitution of one or more nucleotide bases in the parent sequence. A variant polynucleotide or aptamer possesses a similar or identical function to the parent polynucleotide or aptamer. A variant polynucleotide or aptamer has a similar nucleotide base sequence to a parent and satisfies at least one of the following: a polynucleotide or aptamer having a nucleotide base sequence that is one or more of at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, and at least about 98% identical. Identity with respect to SEQ ID NOS: 2-8 is defined herein as the percentage of nucleotide bases in a candidate or variant sequence that are identical with the parent sequence, after aligning the sequences to achieve the maximum percent sequence identity. None of 5' -terminal and/or 3'-terminal additions shall be construed as affecting sequence identity nor shall the chemical linkage of the 3' or 5' end of any aptamer to a non-nucleotide group be construed as affecting sequence identity.

[0038] In one embodiment, a variant is an aptamer containing one of SEQ ID NO: 2-8 where additional nucleotide repeat units are inserted or added on the 5' or 3' end of the aptamer. In another embodiment, a variant is an aptamer containing one of SEQ ID NO: 2-8 where one or more pyrimidine nucleotide bases is substituted for another pyrimidine nucleotide base or a modified pyrimidine nucleotide base and/or one or more purine nucleotide bases is substituted for another purine nucleotide base or a modified purine nucleotide base.

[0039] The aptamers taught herein can be administered to a patient in a composition containing the aptamer or a salt thereof and a pharmaceutically acceptable carrier. For example, the aptamer can be combined with a water or alcohol containing media for administration. Similarly, the aptamer can be administered in tablet form together with a binder such as a sugar- or starch-based binder. Generally, speaking, the invention can be administered directly to a mammalian subject using any route known in the art, including e.g., by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular, or intradermal), inhalation, transdermal (topical) application, rectal administration, or oral administration. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., Remington's Pharmaceutical Sciences, 17th ed., 1989). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions

are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

[0040] The compounds of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

[0041] The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxy-ethylaspartamidephenol, or polyethyl-eneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

[0042] While at least one embodiment of the present invention has been shown and described, it is to be understood that many changes and modifications may be made thereunto without departing from the philosophy and scope of the invention as defined in the appended claims.

Examples

Example 1: Preparation of Sclerostin

[0043] The present invention describes aptamers that bind to sclerostin. Sclerostin was obtained by cloning cDNA of SOST obtained from *Mus musculus* 6 days neonate head cDNA. The coding region of SOST was amplified by PCR with the forward primer 5'-

GTATGTATGAATTCATGCATGCAGCCCTCACTAGCCCC-3′ (SEQ ID NO: 9) and the reverse primer 5′-GTATGTATCTCGAGCTAGTAGGCGTTCTCCAGCT-3′ (SEQ ID NO: 10). The PCR product was gel purified, digested with *EcoR1/Xho*I and ligated with a similarly digested pGEX-4T1 vector to make the plasmid pGEX-SOST.

[0044] For heterologous expression of sclerostin, 2 liters of LB broth supplemented with ampilicin (50 μg/ml) were inoculated with saturated pGEX-SOST/BL21 (DE3) culture (1/200 dilution) and grown at 37 °C until A_{600} = 0.6. Protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (0.25 mM), and cultures were incubated at 25 °C for 4 h. After cooling to 4 °C, the cells were harvested by centrifugation and resuspended in buffer A: phosphate buffer saline (PBS; pH7.3) with protease inhibitors, 1 g of wet cell pellet/5 ml of buffer.

[0045] For purification of sclerostin, cells were lysed by sonication and then centrifuged (30 min, 30,000 × *g*), and the supernatant was filtered and then applied to 5ml GSTrap HP columns. The 5-ml column was washed with 40 ml of buffer A, then 50 ml Buffer B (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione (Calbiochem) to elute the protein. Pure fractions (by SDS-PAGE) were combined and stored at 4 °C for short term or frozen at −80 °C for long term storage.

Example 2: Preparation and identification of the Sclerostin aptamers

[0046] Sclerostin aptamers were selected by magnetic separation using sclerostin immobilized on GST-magnetic beads. The starting point of the selection process was a random degenerate ssDNA library (SelexApt) that was chemically synthesized and HPLC purified. (SelexApt: 5′-CCG TAA TAC GAC TCA CTA TAG GGG AGC TCG GTA CCG AAT TC-(N30)-AAG CTT TGC AGA GAG GAT CCT T-3′) (SEQ ID NO: 11). Another way of describing SEQ ID NO: 11 is that SEQ ID NO: 11 is SEQ ID NO: 12 coupled to SEQ ID NO: 14 with another sequence therebetween (the N30 sequence). Primers that anneal to the 5′- and 3′-sequences flanking the degenerate region of SelexApt used during the selection and cloning were: "SelexF", 5′-CCG TAA TAC GAC

TCA CTA TAG GGG AGC TCG GTA CCG AAT TC-3′ (SEQ ID NO: 12); "SelexR", 5′-AAG GAT CCT CTC TGC AAA GCT T-3′ (SEQ ID NO: 13); in non-biotinylated and 5′-biotinylated forms, respectively (HPLC purified). 1 nmol of DNA library was incubated with GST-sclerostin immobilized on GST magnetic beads for 30 min at room temperature. The unbound DNA was separated and removed by washing with phosphate buffered saline (PBS). The bound sequences were eluted with 10 mM reduced glutathione (GSH) and PCR amplified using biotinylated primers. Single-stranded DNA pool was obtained by streptavidin-magnetic bead purification.

[0047] Iterations of 15 cycles were performed with counter selection against magnetic beads at rounds 3, 6, 9 and 12. During the last round of SELEX, the recovered DNA molecules were PCR amplified using non-biotinylated primers and cloned into pCR-Blunt II TOPO vectors (Invitrogen) and sequenced. Multiple sequence alignment was performed by clustalW2.

[0048] For aptamer-enzyme linked assays, 96 well plates prepacked with glutathione sepharose media (GE healthcare) were coated with 500 ng purified proteins (GST-sclerostin or GST) in 200 μl coating buffer (50 mM Tris-Cl pH 8.5, 100 mM NaCl and 100 mM KCl) for 1.5 hr at room temperature. The wells were washed 4 times with coating buffer. Biotinylated oligodeoxynucleotides (Scl 1, 2, 3, 4 aptamers, thrombin binding aptamer and oligodeoxynucleotide 35-mer random sequence) were heated to 90°C and then cooled quickly to 4°C. 50 nM aptamers were incubated with protein in the 96 well plate overnight at 4°C shaking gently. Wells were washed 6 times with 200 μl of coating buffer for each wash 10 min on a plate vortex. Streptavidin horseradish peroxidase was diluted 1:2000 in buffer and 200 μl aliquots applied to each well. Strips were incubated for 30 min at room temperature and washed again as described above. Then, 150 μl of Turbo- 3,3′,5,5′-tetramethylbenzidine (TMB) was added to each well and incubated for 20 min at room temperature in the dark. The reaction was quenched by addition of 150 μl of 1M H_2SO_4 and the protein bound aptamer-streptavidin complexes were quantified by determining the absorbance at 450 nm.

[0049] Seven different aptamer sequences were identified as capable of binding to sclerostin:

5'-GTTTCCAAAGCCGGGGGGGTGGGATGGGTT-3' (SEQ ID NO: 2) (Scl 1);

5'-TTGCGCGTTAATTGGGGGGGTGGGTGGGTT-3' (SEQ ID NO: 3) (Scl 2)

5'-TGCCTTGTTATTGTGGTGGGCGGGTGGGAC-3' (SEQ ID NO: 4) (Scl 3);

5'-GGGGGGGGTGGGGTGGGTCAATATTCTCGTC-3' (SEQ ID NO: 5) (Scl 4);

5'-TTGCGCGTTAATTGGGGGGGTGGGTGGGTT-3' (SEQ ID NO: 6) (Scl 5);

5'-CCCTCCAAAGCGGGGGGGGTGGGTGGGCAG-3' (SEQ ID NO: 7) (Scl 6); and

5'-TTCTGTCACATGTGGGGGGGGGGGTGGGTT-3' (SEQ ID NO: 8) (Scl 7).

[0050] SEQ ID NOS: 2-8 all contain SEQ ID NO: 1 as a conserved sequence. In addition to SEQ ID NOS: 2-8, variants of SEQ ID NOS: 2-8 are also believed to have anti-sclerostin activity.

<u>**Example 3: Modification of the the Sclerostin aptamers**</u>

[0051] Aptamers modified to be conjugated with inverted thymidine on the 3' end inhibit sclerostin function in cell culture. We employed lymphoid enhancer factor/T-cell factor (LEF/TCF) luciferase reporter assay to study the effects of aptamers on Wnt mediated activity in osteoblast MC3T3-E1 cells which is considered to be an *in vitro* model of bone development. Both LEF and TCF are nuclear transducers of an activated Wnt/β-catenin pathway as they interact with β-catenin (Figure 5A).

[0052] To further investigate the modified aptamers, TOP flash luciferase reporter contains three Wnt-specific binding sites for TCF/LEF transcription factors and the firefly luciferase reporter (F*luc*) under the control from herpex simplex virus thymidine kinase; whilst the FOP flash construct is identical to the TOP construct with three TCF binding sites that are mutated and thus serves as a negative control. The luciferase gene is driven by the promoter which is specifically activated by the binding of β-catenin through the activation of Wnt. We initially compared the 3' InT aptamers using Wnt-reporter assay (Figure 5B).

[0053] For T-cell factor luciferase reporter assays, MC3T3-E1 cells were seeded in 24-well plate and transiently transfected with either 100 ng of TOP-Wnt induced luciferase plasmid or FOP (control plasmid) using Lipofectamine reagent. Wnt3a (800ng), Sclerostin (800ng) expression vectors were co-transfected when needed. 10 ng of Renilla luciferase vector was co-transfected to correct for transfection efficiency. 6 hr post-transfection, medium were changed to antibiotics containing medium supplemented with appropriate amount of aptamers and incubated for 24 hr. Cells were lysed with 100μl of passive lysis buffer and 20μl was used for analyses. Luciferase assays were performed using a luciferase reporter system.

Example 4: Effects of the Sclerostin aptamers against sclerostin

[0054] Aptamer Scl 2 significantly inhibited sclerostin function in Wnt signaling, restoring the luciferase activity similar to the Wnt control. In the presence of Wnt, the signaling pathway is activated, having a large luciferase signal. Sclerostin is an antagonist of canonical Wnt signaling, binding to LRP5/6. So, the luciferase activity decreased. The efficacy of the aptamers was tested at fixed concentration at 1.5μM.

[0055] To further study the effect of 3' InT aptamer Scl 2 against sclerostin's antagonistic effect on Wnt signaling, 3' InT aptamers Scl 2 was added in varying concentration from 0.1 μM to 1.5 μM. With increasing concentration, the aptamer specifically blunts the antagonistic effect of sclerostin against Wnt signaling. The inhibitory effects of aptamers can be saturated at 1.5 μM.

[0056] For aptamer stability assessment, 1 μM of Scl 2 and 3' inverted thymidine modified Scl 2 were added to MC3T3-E1 cells at 80% confluency. Cells were grown in 6 well plates and with 2ml complete medium (α-MEM, supplemented with 5% FBS, penicillin/Streptomycin and fungizone) at 37°C supplemented with 5% CO_2. At time points indicated, 10 μl of medium was loaded onto urea-PAGE and electrophoese. Gels were stained with 1:10000 SYBR Gold for 20 min and images observed under UV.

[0057] For Circular Dichroism, oligonucleotides (10 μM) were resuspended in Tris-HCl (10 mM, pH7.5) buffer that contained KCl (100 mM). Samples were heated at 90°C for 5 min, followed by gradual cooling to room temperature. CD spectra were collected on a JASCO J810 spectropolarometer (JASCO, MD, USA) equipped with a water-jacketed cell holder at 310 nm – 220 nm, by using 4 scans at 100 nm min-1, 1s response time, 1nm bandwidth. Quartz cells with an optical path length of 1mm were used for the measurement. The scans of the buffer alone were subtracted from the average scans for the sample. CD melting curves obtained at wavelength 260 nm allowed an estimation of melting temperature, Tm, the mid-point temperature of the unfolding process.

[0058] Biophysical properties of sclerostin aptamers were characterized by circular dichroism to experimentally demonstrate whether G-quadruplex structure was formed in the aptamer sequence. In their CD spectra, aptamer Scl 2 showed a positive maximum peak near 265nm (Figure 6A). This is a spectroscopic evidence of parallel G-quadruplex structure. Moreover, we determined the thermal stability of the G-quadruplex structure of aptamer Scl 2 by melting CD and showed that the Tm value of aptamer Scl 2 is 75oC, suggesting that the structure is very stable that is suitable for potential therapeutic use in the cellular environment (Figure 6B).

The G-quadruplex structure is a square arrangement of four guanine nucleotide bases. The four guanine nucleotide bases forming the G-quadruplex structure can come from one DNA aptamer strand or two or more DNA aptamer strands. That is, the G-quadruplex structure can be an intramolecular structure or an intermolecular structure.

[0059] For Isothermal Titration Calorimetry, equilibrium binding studies between anti-sclerostin aptamers and scleorstin are performed on MicroCal VP-ITC. In a typical ITC experiment, 15 μM GST-sclerostin or 20 μM GST was loaded into the cell with 200 μM aptamer or random sequence in the titrating syringe. GST-sclerostin and GST were dialyzed into the PBS buffer with a MWCO of 10,000. The titration experiments were performed at 25°C with an initial 0.2 μl injection, followed by thirty 1.2 μl injections. The spacing between injections was 200 s. The stirring speed during the titration was 900 rpm.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

[0060] Sclerostin-aptamer interaction was investigated by studying the thermodynamics of the interaction of aptamer Scl 2 with sclerostin by Isothermal Titration Calorimetry. In the upper panel of figure 7 the calorimetric titrations of the aptamers into sclerostin solution conducted at 25°C. An exothermic heat pulse was observed after each injection of aptamers into the protein solution. The binding stoichiometry was fitted using simple single site binding model. Our results showed that the binding stoichiometry (n) of aptamer-protein complexes clearly indicates that in solution $n = 0.91 \pm 0.02$ molecules of Scl 2 aptamer bind to one Sclerostin molecule. The dissociation constant for the interaction of aptamer Scl 2 with sclerostin is 500nM that is a competitive value in DNA-protein interactions. In addition, the values of ΔH and ΔS reveal that the binding processes are enthalpically driven with a favorable enthalpy of reaction (ΔH) at 25°C of -10.9 kcal/mol offset by an unfavorable entropy of reaction ($T\Delta S$ = -2.3 kcal/mol).

[0061] All patents, patent applications, provisional applications, and publications referred to or cited herein, including those listed below, are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

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CLAIMS

What is claimed is:

1. A composition comprising:

A DNA aptamer comprising an oligonucleotide having the sequence 5'-GGXGGXXGGXTGGG-3' (SEQ ID NO: 1) or a salt thereof,

wherein the aptamer inhibits sclerostin

preferably, the aptamer has from about 14 to about 50 nucleotide repeat units;
preferably, the aptamer is conjugated with one or more selected from deoxythymidine nucleotide, inverted thymidine and polyethylene glycol;
preferably, the aptamer is an oligonucleotide having a backbone formed of deoxyribose-phosphate linkages;
preferably, the aptamer is an oligonucleotide having one or more deoxyribose-phosphate linkages stabilized by one or more selected from a thioester linkage and an amide linkage;
preferably, the aptamer has a parallel G-quadruplex structure.

2. An aptamer capable of binding sclerostin, the aptamer selected from the group comprises of one of the following sequences:
5'-GTTTCCAAAGCCGGGGGGGTGGGATGGGTT-3' (SEQ ID NO: 2) (Scl 1);
5'-TTGCGCGTTAATTGGGGGGGTGGGTGGGTT-3' (SEQ ID NO: 3) (Scl 2)
5'-TGCCTTGTTATTGTGGTGGGCGGGTGGGAC-3' (SEQ ID NO: 4) (Scl 3);
5'-GGGGGGGGTGGGGTGGGTCAATATTCTCGTC-3' (SEQ ID NO: 5) (Scl 4);
5'-TTGCGCGTTAATTGGGGGGGTGGGTGGGTT-3' (SEQ ID NO: 6) (Scl 5);
5'-CCCTCCAAAGCGGGGGGGGTGGGTGGGCAG-3' (SEQ ID NO: 7) (Scl 6); and
5'-TTCTGTCACATGTGGGGGGGGGGGTGGGTT-3' (SEQ ID NO: 8) (Scl 7),
or a variant of one of SEQ ID NOS: 2-8.

3. The aptamer of claim 2, wherein the aptamer is conjugated with one or more selected from deoxythymidine nucleotide, inverted thymidine and polyethylene glycol.

4. The aptamer of claim 3, wherein the aptamer is conjugated at the 3' end with one or more selected from deoxythymidine nucleotide, inverted thymidine and polyethylene glycol.

5. The aptamer of claim 2, wherein the aptamer is an oligonucleotide having a backbone formed of deoxyribose-phosphate linkages.

6. The aptamer of claim 2, wherein the aptamer is an oligonucleotide having one or more deoxyribose-phosphate linkages stabilized by one or more selected from a thioester and an amide linkage.

7. The aptamer of claim 2, wherein the aptamer has a parallel G-quadruplex structure.

8. The composition of claim 1 or the aptamer according to any one of claims 2-7 for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects, said treating comprising: administering the composition of claim 1 and or the aptamer according to any one of claims 2-7 to an individual, wherein the individual requires treatment for a bone-related disease, disorder, or condition wherein sclerostin causes undesirable pathological effects,
preferably, wherein the composition further comprises a pharmaceutical carrier.

9. The composition or the aptamer of claim 8, wherein the aptamer is selected from the group consisting of an oligonucleotide having one of the following sequences:
5'-GTTTCCAAAGCCGGGGGGGTGGGATGGGTT-3' (SEQ ID NO: 2) (Scl 1);
5'-TTGCGCGTTAATTGGGGGGGTGGGTGGGTT-3' (SEQ ID NO: 3) (Scl 2)
5'-TGCCTTGTTATTGTGGTGGGCGGGTGGGAC-3' (SEQ ID NO: 4) (Scl 3);
5'-GGGGGGGGTGGGGTGGGTCAATATTCTCGTC-3' (SEQ ID NO: 5) (Scl 4);
5'-TTGCGCGTTAATTGGGGGGGTGGGTGGGTT-3' (SEQ ID NO: 6) (Scl 5);
5'-CCCTCCAAAGCGGGGGGGGTGGGTGGGCAG-3' (SEQ ID NO: 7) (Scl 6); and
5'-TTCTGTCACATGTGGGGGGGGGGGTGGGTT-3' (SEQ ID NO: 8) (Scl 7).
or a variant of one of SEQ ID NOS: 2-8.

10. The composition or the aptamer of claim 9, wherein the aptamer is conjugated at the 3' end with one or more selected from deoxythymidine nucleotide, inverted thymidine and polyethylene glycol;
preferably, the aptamer is an oligonucleotide having a backbone formed of deoxyribose-phosphate linkages;
preferably, the aptamer is an oligonucleotide having one or more deoxyribose-phosphate linkages stabilized by one or more selected from a thioester and an amide linkage;
preferably, the bone-related diseases, disorders or conditions comprises at least one selected from the group consisting of osteoporosis, osteopenia, osteoarthritis, osteomalacia, osteodystrophy, osteomyeloma, bone fracture, Paget's disease, osteogenesis imperfecta, bone sclerosis, aplastic bone disorder, humoral hypercalcemic myeloma, multiple myeloma, and bone thinning;
preferably, the bone-related diseases, disorders or conditions comprises osteoporosis.

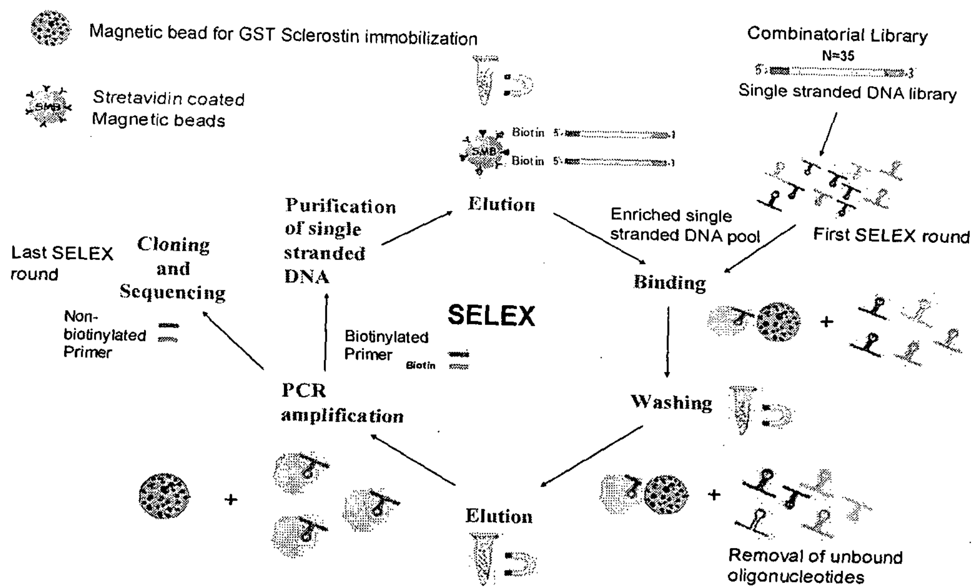

FIG.1

Aptamer clone	Core region of the aptamer sequences (5' to 3')	No. of nucleotides	Percentage
Scl 1	GTTTCCAAAGCCGGGGGGGTGGGATGGGTT------------	30	55%
Scl 2	TTGCGCGTTAATTGGGGGGGTGGGTGGGTT------------	30	24%
Scl 3	TGCCTTGTTATTGTGGTGGGCGGGTGGGAC------------	30	7%
Scl 4	-----------GGGGGGGGTGGGGTGGGTCAATATTCTCGTC	31	3%
Scl 5	TTGCGCGTTAATTGGGGGGGTGGGTGGGTT------------	30	3%
Scl 6	CCCTCCAAAGCGGGGGGGGTGGG-TGGGCAG-----------	30	3%
Scl 7	TTCTGTCACATGTGGGGGGGGGGGTGGGTT------------	30	3%
	** ** ** ****		

FIG.2

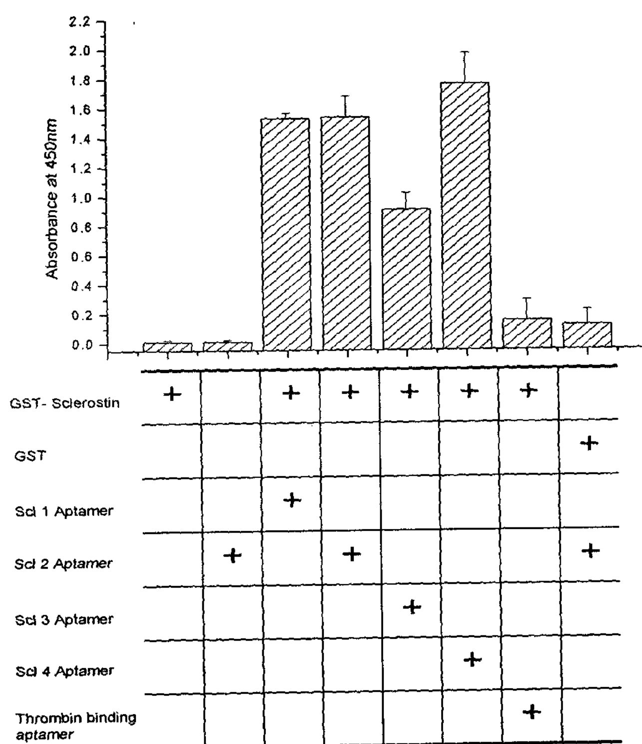

FIG.3

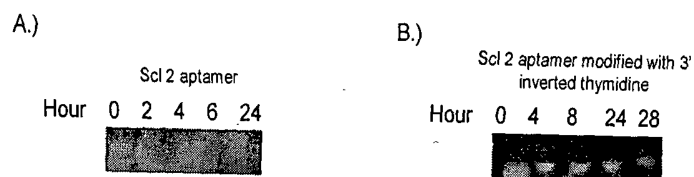

FIG.4

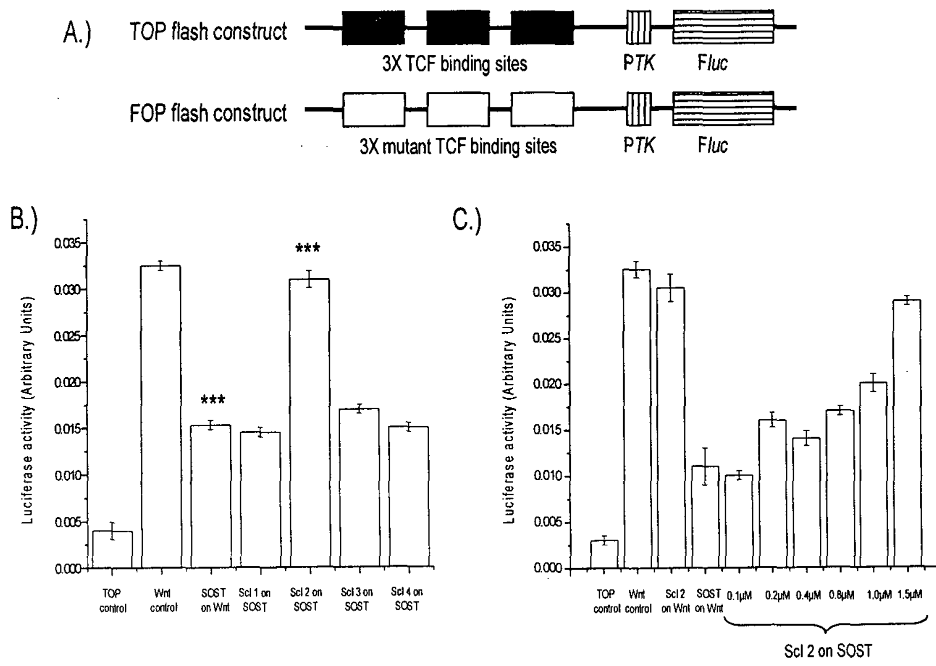

FIG.5

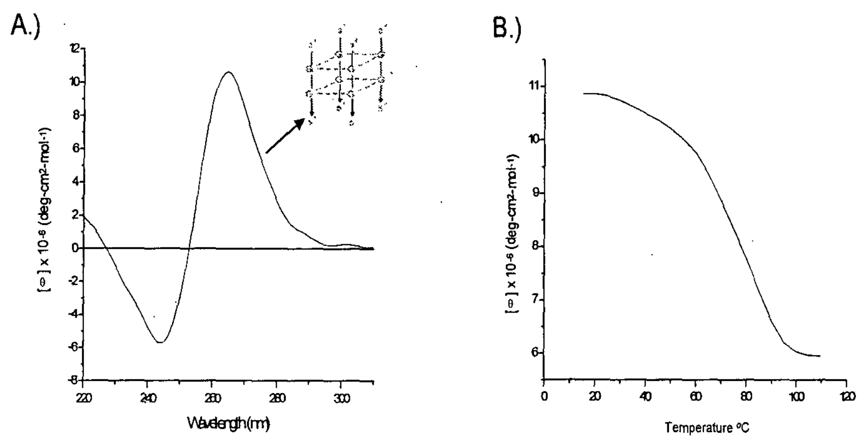

FIG.6

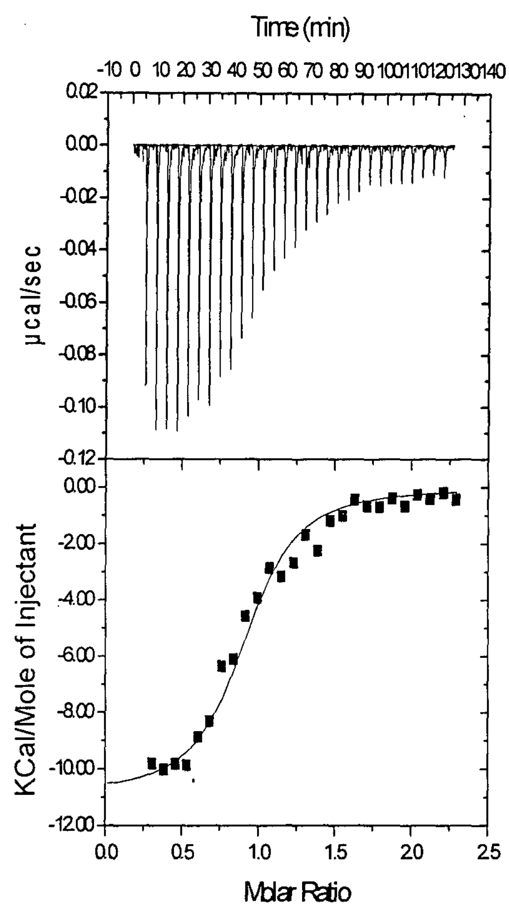

FIG.7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2011/000879

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N, 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 practicable, search terms used)

DATABASES: CPRSABS, CNABS, CJFD, CSCD, SIPONPL, CNKI, DWPI, SIPOABS, CPEA, ISI WEB OF KNOWLEDGE, CA, BA, MEDLINE, PUBMED, GENBANK+EMBL+DDBJ
SEARCH TERMS: aptamer, sclerostin, SOST, sequences search on the sequences of SEQ ID NOs: 1-3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	Ka To SHUM et al., Identification of a DNA aptamer that inhibits sclerostin's antagonistic effect on Wnt signaling, BIOCHEM. J. 28 February 2011 (28.02.2011), Vol. 434, No. 3, pages 493-501, ISSN: 0264-6021, see abstract, experimental, results, and figures 1-6.	1, 2-10 (partially)
A	CN1835974A(CELLTECH R & D INC), 20 September 2006 (20.09.2006), see the whole document	1, 2-10 (partially)
A	CN101657096A(DONEGAN J J et al.), 24 February 2010 (24.02.2010) , see the whole document	1, 2-10 (partially)

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" 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

"&"document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 Aug. 2011(10.08.2011)	**25 Aug. 2011 (25.08.2011)**

Name and mailing address of the ISA/CN
The State Intellectual Property Office, the P.R.China
6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China
100088
Facsimile No. 86-10-62019451

Authorized officer
YU Na
Telephone No. (86-10)62411084

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2011/000879

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US7648965B2(UNIV YALE et al.), 19 January 2010 (19.01.2010) , see the whole document	1, 2-10 (partially)
A	Lige Wu et al., Sclerostin: a new BMP inhibitor, Section Endocrinol Foreign Med Sci, 30 September 2005 (30.09.2005), Vol. 25, No. 5, pages 319-321, ISSN: 1003-5435, see the whole document	1, 2-10 (partially)

Box No. Ⅰ Nucleotide and/or amino acid sequence(s) (Continuation of item item1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

   a. a sequence listing filed or furnished

   ☐ on paper

   ☒ in electronic form

   b. time of filing or furnishing

   ☐ contained in the applicantion as filed

   ☒ filed together with the application in electronic form

   ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2011/000879

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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:

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).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

See extra sheet

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 fee.
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.: 1, 2-10(partially)
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.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2011/000879

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/115 (2010.01) i
A61K 31/711 (2006.01) i
A61K 31/7125 (2006.01) i
A61P 19/10 (2006.01) i
A61P 19/08 (2006.01) i

Box No. III Observations where unity of invention is lacking:

This International Searching Authority found eight groups of inventions in this international application, as follows:

1. claims:1,8(partially)
   a composition comprising a DNA aptamer, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 1 or a salt thereof; and the said composition for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

2. claims:2-10(all partially)
   an aptamer capable of binding sclerostin, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 2; and the said aptamer for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

3. claims:2-10(all partially)
   an aptamer capable of binding sclerostin, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 3; and the said aptamer for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

4. claims:2-10(all partially)
   an aptamer capable of binding sclerostin, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 4; and the said aptamer for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

5. claims:2-10(all partially)
   an aptamer capable of binding sclerostin, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 5; and the said aptamer for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

6. claims:2-10(all partially)
   an aptamer capable of binding sclerostin, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 6; and the said aptamer for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

7. claims:2-10(all partially)
   an aptamer capable of binding sclerostin, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 7; and the said aptamer for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

8. claims:2-10(all partially)
   an aptamer capable of binding sclerostin, wherein the aptamer comprises an oligonucleotide having the sequence of SEQ ID NO: 8; and the said aptamer for the treatment of bone-related diseases, disorders or conditions wherein sclerostin causes undesirable pathological effects.

Although the aptamers of the groups 2-8 capable of binding sclerostin, the nucleotide sequences of them are different. That is to say, groups 2-8 do not have the same or corresponding special technical feature, and do not linked by a single general inventive concept. For groups 1 and 2-8, the nucleotide sequences of them are different, and the structure units among them are also different. That is to say, groups 1-8 do not have the same or corresponding special technical feature, and do not linked by a single general inventive concept. Therefore, they do not meet the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
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