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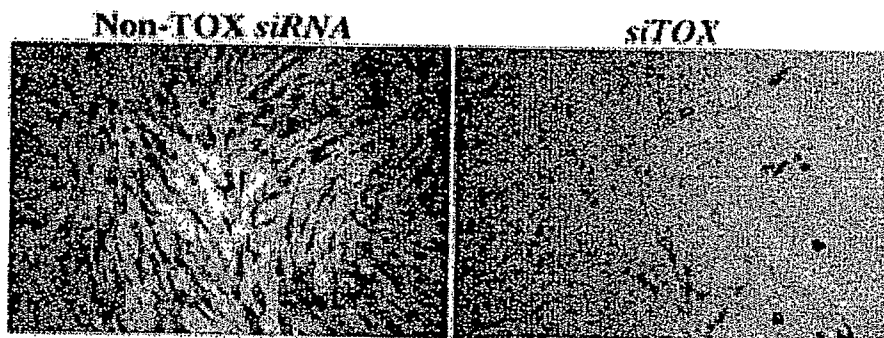
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(54) Title: CONTROLLING OSTEOGENESIS BY INHIBITION OF OSTEOGENIC SUPPRESSORS



(57) Abstract: The present invention provides methods of screening for agents that inhibit the activity or expression of one or more polypeptides that contribute to the suppression of osteogenesis. The invention also provides methods of inducing osteogenesis in a cell by administering to the cell an agent that inhibits one or more polypeptides that contribute to the suppression of osteogenesis.



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CONTROLLING OSTEOGENESIS BY INHIBITION OF OSTEOGENIC SUPPRESSORS

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 [0001] The present application claims the benefit of U.S. Application No. 60/822,184, filed August 11, 2006, the entire disclosure of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

- 10 [0002] Tissue-specific (or adult) stem cells are regarded as the source for normal tissue homeostasis and tissue repair. They also provide tremendous promise for regenerative medicine due to their capacity to proliferate and differentiate into a variety of mature cell types. Human mesenchymal stem cells (hMSCs) can differentiate into osteocytes, adipocytes, chondrocytes, muscle cells and neurons. However, the molecular mechanisms underlying these differentiating processes are poorly understood.
- 15 [0003] RNA-mediated interference (RNAi) is a highly conserved gene silencing event functioning through targeted destruction of individual mRNA by a homologous double-stranded small interfering RNA (siRNA) (Fire, A. et al., *Nature* 391:806-811 (1998)). siRNAs generated by both chemical synthesis and *in vitro* or *in vivo* transcription through vector-based expression systems have been proven very useful tools in studying gene loss-of-
- 20 function in mammalian cells (Brummelkamp, T. R. et al., *Science* 296:550-553 (2002); Caplen, N. J. et al., *Proc Natl Acad Sci U S A* 98:9742-9747 (2001); Elbashir, S. M. et al., *Nature* 411:494-498 (2001); Lee, N. S. et al., *Nat Biotechnol* 20:500-505 (2002); Miyagishi, M. et al., *Nat Biotechnol* 20: 497-500 (2002); Paddison, P. J. et al., *Genes Dev* 16:948-958 (2002); Paul, C. P. et al., *Nat Biotechnol* 20:505-508 (2002); Sui, G. et al., *Proc Natl Acad Sci U S A* 99:5515-5520 (2002); Yu, J. Y. et al., *Proc Natl Acad Sci U S A* 99:6047-6052 (2002)). While high throughput screens using genome-scale siRNA libraries have been successfully carried out in mammalian cells (Berns, K. et al., *Nature* 428:431-437 (2004); Paddison, P. J. et al., *Nature* 428:427-431 (2004); Zheng, L. et al., *Proc Natl Acad Sci U S A* 101:135-140 (2004)), effective application of arrayed synthetic siRNA library in stem cells
- 30 has not been reported. Human mesenchymal stem cells (hMSCs) can be easily isolated from adults and expanded rapidly *in vitro*. Due to their ability to differentiate into various mature cell types (Prockop, D. J. *Nat Biotechnol* 20:791-792 (2002); Ryan, J. M. et al., *J Inflamm*

(*Lond*) 2:8 (2005)) (Campagnoli, C. et al., *Blood* 98:2396-2402 (2001); Dezawa, M. et al., *Science* 309:314-317 (2005); Dezawa, M. et al., *J Clin Invest* 113:1701-1710 (2004); Pittenger, M. F. et al., *Science* 284:143-147 (1999); Pittenger, M. F. et al., *Circ Res* 95:9-20 (2004); Woodbury, D. et al., *J Neurosci Res* 61:364-370 (2000)), they have been of great
 5 interests to researchers exploring cell-based therapies for degenerative diseases including bone disorders (Dezawa, M. et al., *Science* 309:314-317 (2005); Dezawa, M. et al., *J Clin Invest* 113:1701-1710 (2004); Horwitz, E. M. et al., *Proc Natl Acad Sci U S A* 99:8932-8937 (2002); Pittenger, M. F. et al., *Circ Res* 95:9-20 (2004); Prockop, D. J. *Science* 276:71-74 (1997)). Cell fate transition from stem cell self-renewal to differentiation involves not only
 10 positive regulators but also negative regulators that normally suppress differentiation.

[0004] There is a need for methodologies that influence the differentiation path of mesenchymal stem cells, for example, into cells of an osteoblast lineage. The present invention addresses this and other needs.

BRIEF SUMMARY OF THE INVENTION

[0005] The present invention provides methods for screening for agents that promote osteogenesis by suppressing the activity or expression of one or more of the polypeptides listed in Table 1. The invention further provides methods for promoting osteogenesis in a cell by suppressing the activity or expression of one or more of the polypeptides listed in
 20 Table 1.

[0006] Accordingly, in a first aspect, the invention provides methods for identifying agents that promote osteogenesis. In some embodiments, the methods comprise:

- (a) contacting a plurality of agents to at least one of the polypeptides or polynucleotides selected from Table 1;
- 25 (b) measuring the activity of at least one of the polypeptides or polynucleotides;
- (c) selecting at least one of the plurality of agents, wherein the selected agent(s) inhibit the activity of at least one of the polypeptides or polynucleotides; and
- (d) measuring the ability of the selected agent(s) to promote osteogenesis, thereby identifying agents that promote osteogenesis. In some embodiments, the polypeptides or
 30 polynucleotides are expressed in a host cell.

[0007] In another embodiment, the methods comprise:

- (a) contacting a plurality of agents to cells expressing at least one of the polypeptides or polynucleotides selected from Table 1;
- (b) measuring the expression (*i.e.*, transcription or translation) of at least one of the polypeptides or polynucleotides;
- (c) selecting at least one of the plurality of agents, wherein the selected agent(s) inhibit the expression of at least one of the polypeptides or polynucleotides; and
- (d) measuring the ability of the selected agent(s) to promote osteogenesis, thereby identifying agents that promote osteogenesis.

10 [0008] In some embodiments of the screening methods, the measuring step (b) comprises measuring the level of transcription.

[0009] In some embodiments of the screening methods, the measuring step (d) is carried out *in vitro*. In some embodiments, the measuring step (d) is carried out *in vivo*.

15 [0010] The cells used in the contacting and measuring steps can be prokaryotic or eukaryotic. In some embodiments, the cells are mammalian cells, for example, stem cells or mesenchymal stem cells.

[0011] In a further aspect, the invention provides methods for promoting osteogenesis in a cell. In some embodiments, the methods comprise contacting the cell with an siRNA that inhibits expression of at least one of the gene products selected from Table 1.

20 [0012] In a related aspect, the invention provides methods for promoting osteogenesis in a cell. In some embodiments, the methods comprise contacting a cell with an agent that inhibits the activity of a polypeptide selected from Table 1.

25 [0013] With regard to the embodiments for promoting osteogenesis in a mammalian cell, in some embodiments, the methods are performed *in vivo*. In some embodiments, the methods are performed *in vitro*. In some embodiments, the methods are performed *ex vivo*.

[0014] In some embodiments of the screening and treatment methods, the cell is a mammalian cell. In some embodiments, the cell is a stem cell. In some embodiments, the stem cell is a mesenchymal stem cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 illustrates siRNA transfection efficiency in hMSCs was above 90%. Cells successfully transfected with siTOX underwent apoptosis, while cells transfected with non-TOX siRNA remained alive.

5 [0016] Figure 2 illustrates the identification and confirmation of siRNA hits that induced osteogenic differentiation of hMSCs: a. Alkaline phosphatase activity, as indicated by the staining, was up regulated in the hit siRNA transfected or OS treated cells as compared to the control siRNA transfected cells. b. Expression of the early (Cbfa1 and Osx but not Dlx5) and late (Bsp) osteogenic markers was differentially induced in different hit siRNA or OS treated
10 samples prepared 3 days after transfection or OS treatment. c. ALP activity was reduced in the hit siRNA and siCbfa1 co-transfected cells as compared to the hit siRNA and siCon co-transfected cells. d. Expression knockdown of targeted genes was observed at 36 hours after siRNA transfection. OS, osteogenic differentiation media; Osx, osterix.

[0017] Figure 3 illustrates enhanced alkaline phosphatase activity staining in hMSCs
15 transfected with the picked hit siRNAs as compared to cells transfected with siCon.

[0018] Figure 4 illustrates sequential treatment of hMSCs with siADK or siCon followed by the OS demonstrated the enhancing effect of siADK vs. siCon on bone cell maturation. 2+6OS, OS treatment was started 2 days after siRNA transfection and continued for 6 days. Similar abbreviation applies to 3+5OS and 4+4OS. Cells were stained with alizarin red
20 solution for calcium phosphate deposition.

[0019] Figure 5 illustrates that the majority of the osteogenic hit siRNAs enhanced bone cell maturation, but inhibited the adipogenic differentiation of hMSCs. a. All 12 selected hit siRNAs, except for siMJD and siTBX3, enhanced bone cell maturation when combined with the OS treatment, as demonstrated by alizarin red staining. b. All 12 selected hit siRNAs,
25 except for siGNAS, inhibited adipogenic differentiation of hMSCs treated with the adipogenic inducing media on the second day after siRNA transfection, based on Oil Red O staining. siCon, control siRNA; OS, osteogenic inducing media; 3+9OS, OS treatment was started 3 days after siRNA transfection and continued for 9 days.

[0020] Figure 6 illustrates that combined siKCNT1 and siSLC12a2 transfection has
30 synergistic effect on enhancing the bone cell maturation process induced by the OS (alizarin red staining). 1+13OS, cells were treated with OS one day after siRNA transfection and treatment was continued for 13 days. OS, osteogenic inducing media.

[0021] Figure 7 illustrates that intracellular cAMP signaling plays opposing roles in osteogenic vs. adipogenic differentiation in hMSCs. a. Adenylyl cyclase agonist forskolin inhibited the osteogenic effect of the OS media on hMSCs in a dose dependent manner (a-c), while adenosine kinase inhibitor 5-iodotubercidin enhanced the osteogenic effect of the OS media (d-f, g-i) and inhibited the adipogenic differentiation of hMSCs in a dose dependent manner (j-l). a-f, ALP staining; g-i, alizarin red staining; j-l, oil red o staining. b. Treatment with cAMP analogs (DB-cAMP & CPT-cAMP) or IBMX promoted hMSCs to undergo adipogenic differentiation, especially in the presence of dexamethasone (DEX). c. Enhanced cAMP level by CPT-cAMP, IBMX or PGE2 application could switch cell fate determination from osteogenic to adipogenic differentiation in hMSCs treated with the OS. Adipo, adipogenic inducing media; OS, osteogenic inducing media.

[0022] Figure 8 illustrates that CREB activity is required for both osteogenic and adipogenic differentiation of hMSCs. a. Examination of CREB protein activity by Western blot demonstrated increased pCREB level in cells transfected with hit siRNAs compared to cells transfected with siCon or un-treated cells. b. Cells transfected with CRE (CREB responding element) decoy, which competes with endogenous CRE in target genes, are less responsive to the stimulation of adipogenic inducing media (Adipo) or osteogenic inducing media (OS).

[0023] Figure 9 illustrates additional signaling pathways involved in osteogenic differentiation of hMSCs. a. PLZF1 expression was only induced in siMJD and OS treated but not other hit siRNA treated samples collected on day 7 or day 12 after siRNA transfection. b. Inhibition of ERK1/2 or p38 signaling pathway by PD98059 or SB202190, respectively, inhibited the osteogenic inducing effect of OS on hMSCs. c. Phosphorylated form of p38 was up regulated in cells transfected with siDUSP6. OS, osteogenic inducing media.

[0024] Figure 10 illustrates that ATP has a dosage-dependent inhibitory effect on OS-induced osteogenesis of hMSCs, as well as on siP2RY11 and siSLC12a2 induced osteogenesis of hMSCs in combination with the OS treatment. OS and ATP were added on the third day after siRNA transfection and continued for a week. Cells were stained for alkaline phosphatase activity. OS, osteogenic inducing media.

[0025] Figure 11 illustrates that siCOMP promotes both adipogenic and osteogenic differentiation of hMSCs. The top two rows (second row panel are enlarged views of the

corresponding upper panels) show that siCOMP treatment enhances adipogenesis of hMSCs induced by the adipogenic inducing media (Adipo). Cells were treated with Adipo on the third day after siRNA transfection and treatment was continued for 12 days before cells were stained with Oil Red O. The bottom row shows siCOMP induces osteogenic differentiation of hMSCs. Cells were assayed for alkaline phosphatase activity 9 days after siRNA transfection. OS, osteogenic inducing media.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

10 [0026] A synthetic siRNA library targeting 5,000 human genes was screened to identify the endogenous repressors of osteogenic specification, which upon silencing initiate differentiation of hMSCs into osteoblasts. This screen yielded 53 suppressors of osteogenic specification in hMSCs. *See*, Table 1. Furthermore, cAMP was identified to play reverse roles in osteogenesis vs. adipogenesis. The present invention finds use in modulating the genetic network controlling osteogenesis and in treating bone diseases.

II. Definitions

[0027] "Osteogenesis," as used herein, refers to proliferation of bone cells and growth of bone tissue (*i.e.*, synthesis and deposit of new bone matrix) from undifferentiated stem cells and cells of osteoblast lineage. Osteogenesis also refers to differentiation or transdifferentiation of progenitor or precursor cells into bone cells (*i.e.*, osteoblasts). Progenitor or precursor cells can be pluripotent stem cells including, *e.g.*, mesenchymal stem cells. Progenitor or precursor cells can be cells pre-committed to an osteoblast lineage (*e.g.*, pre-osteoblast cells) or cells that are not pre-committed to an osteoblast lineage (*e.g.*, pre-adipocytes or myoblasts).

25 [0028] The term "agent" refers to any compound useful in the screening assays described herein. An agent can be, for example, an organic compound, a polypeptide (*e.g.*, a peptide or an antibody), a nucleic acid (*e.g.*, DNA, RNA, double-stranded, single-stranded, an oligonucleotide, antisense RNA, small inhibitory RNA, micro RNA, a ribozyme, etc.), an oligosaccharide, a lipid. Usually, the agents used in the present screening methods have a molecular weight of less than 10,000 daltons, for example, less than 8000, 6000, 4000, 2000 daltons.

[0029] The term “test compound” or “drug candidate” or “modulator” or grammatical equivalents as used herein describes any molecule, either naturally occurring or synthetic, e.g., protein, oligopeptide (e.g., from about 5 to about 25 amino acids in length, preferably from about 10 to 20 or 12 to 18 amino acids in length, preferably 12, 15, or 18 amino acids in length), small organic molecule, polysaccharide, lipid, fatty acid, polynucleotide, RNAi or siRNA, asRNA, oligonucleotide, etc. The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a sufficient range of diversity. Test compounds are optionally linked to a fusion partner, e.g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a “lead compound”) with some desirable property or activity, e.g., inhibiting activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

[0030] The terms “short-inhibitory RNA” and “siRNA” interchangeably refer to short double-stranded RNA oligonucleotides that mediate RNA interference (also referred to as “RNA-mediated interference,” or RNAi). RNAi is a highly conserved gene silencing event functioning through targeted destruction of individual mRNA by a homologous double-stranded small interfering RNA (siRNA) (Fire, A. *et al.*, *Nature* **391**:806-811 (1998)). Mechanisms for RNAi are reviewed, for example, in Bayne and Allshire, *Trends in Genetics* (2005) 21:370-73; Morris, *Cell Mol Life Sci* (2005) 62:3057-66; Filipowicz, *et al.*, *Current Opinion in Structural Biology* (2005) 15:331-41.

[0031] The term “activity” refers to the commonly recognized functioning of a polypeptide known in the art. For example, the polypeptides listed in Table I have commonly recognized activities as enzymes, receptors, cell adhesion molecules, intracellular signaling mediators, etc. Assays for measuring the activities of the listed proteins, including enzymatic activity assays, receptor signaling assays, receptor-ligand binding assays, cell migration assays, are known in the art. The activity of a protein can also be correlated with its expression, for example, the transcription or translation of the polypeptide.

[0032] The term “promote” refers to the increase in a measured parameter (e.g., activity, expression, osteogenesis) in a treated cell (tissue or subject) in comparison to an untreated

cell (tissue or subject). A comparison can also be made of the same cell or tissue or subject between before and after treatment. The increase is sufficient to be detectable. In some embodiments, the increase in the treated cell is at least about 50%, 60%, 70%, 80%, 90%, 1-fold, 2-fold, 3-fold, 4-fold or more in comparison to an untreated cell.

5 [0033] The term “inhibit” refers to the decrease in a measured parameter (*e.g.*, activity, expression, osteogenesis) in a treated cell (tissue or subject) in comparison to an untreated cell (tissue or subject). A comparison can also be made of the same cell or tissue or subject between before and after treatment. The decrease is sufficient to be detectable. In some
10 embodiments, the decrease in the treated cell is at least about 50%, 60%, 70%, 80%, 90%, or completely inhibited in comparison to an untreated cell. In some embodiments the measured parameter is undetectable (*i.e.*, completely inhibited) in the treated cell in comparison to the untreated cell.

[0034] A “stem cell,” as used herein, refers to any mammalian pluripotent cell or multipotent cell or progenitor cell or precursor cell that is capable of differentiating into
15 multiple cell types. Stem cells suitable for use in the methods of the present invention include those that are capable of differentiating into cells of osteoblast lineage, *e.g.*, osteoblasts. Suitable multipotent cells or precursor cells for use in the methods of the present invention include, for example, mesenchymal stem cells, pre-osteoblast cells, pre-adipocyte cells, and myoblast cells.

20 [0035] “Differentiate” or “differentiation,” as used herein, refers to the process by which precursor or progenitor cells (*i.e.*, stem cells) differentiate into specific cell types, *e.g.*, osteoblasts. Differentiated cells can be identified by their patterns of gene expression and cell surface protein expression. Typically, cells of an osteoblast lineage express genes including, for example, alkaline phosphatase, collagen type I, osteocalcin, and osteoponin.
25 Typically, cells of an osteoblast lineage express bone specific transcription factors including, for example, Cbfa1/Runx2 and Osx (*see, e.g.*, Olsen, *et al*, 2000 *supra* and Nakashima *et al.*, Cell 108(1):17-29 (2002). Additional transcription factors that are involved in osteoblast differentiation include, *e.g.*, gsc, Dlx1, Dlx5, Msx1, Cart1, Hoxa1, Hoxa2, Hoxa3, Hoxb1, rae28, Twist, AP-2, Mf1, Pax1, Pax3, Pax9, TBX3, TBX4, TBX5, and Brachyury (*see, e.g.*,
30 Olsen *et al*, 2000 *supra*).

[0036] “Transdifferentiation” refers to the process by which precursor or progenitor cells (*i.e.*, stem cells) pre-committed to cell types of one lineage differentiate into specific cell

types of another lineage, *e.g.*, pre-adipocytes transdifferentiate into osteoblasts or myoblasts transdifferentiate into osteoblasts. Transdifferentiated cells can be identified by their patterns of gene expression and cell surface protein expression. Typically, cells of an osteoblast lineage express genes including, for example, alkaline phosphatase, collagen type I, osteocalcin, and osteopontin. Typically, cells of an osteoblast lineage express bone specific transcription factors including, for example, Cbfa1/Runx2 and Osx (*see, e.g.*, Olsen *et al.*, 2000 *supra* and Nakashima *et al.*, *supra*). Additional transcription factors that are involved in osteoblast differentiation include, *e.g.*, gsc, Dlx1, Dlx5, Msx1, Cart1, Hoxa1, Hoxa2, Hoxa3, Hoxb1, rae28, Twist, AP-2, Mf1, Pax1, Pax3, Pax9, TBX3, TBX4, TBX5, and Brachyury (*see, e.g.*, Olsen *et al.*, 2000 *supra*).

[0037] A “solid support,” as used herein in connection with inducing osteogenesis, refers to a three-dimensional matrix or a planar surface on which the stem cells can be cultured. The solid support can be derived from naturally occurring substances (*i.e.*, protein based) or synthetic substances. For example, matrices based on naturally occurring substances may be composed of autologous bone fragments or commercially available bone substitutes as described in *e.g.*, Clokie *et al.*, *J. Craniofac. Surg.* 13(1):111-21 (2002) and Isaksson, *Swed. Dent. J Suppl.* 84:1-46 (1992). Suitable synthetic matrices are described in, *e.g.*, U.S. Pat. Nos. 5,041,138; 5,512,474, and 6,425,222. For example, biodegradable artificial polymers, such as polyglycolic acid, polyorthoester, or polyanhydride can be used for the solid support. Calcium carbonate, aragonite, and porous ceramics (*e.g.*, dense hydroxyapatite ceramic) are also suitable for use in the solid support. Polymers such as polypropylene, polyethylene glycol, and polystyrene can also be used in the solid support. Cells cultured and differentiated on a solid support that is a three-dimensional matrix typically grow on all of the surfaces of the matrix, *e.g.*, internal and external. Cells cultured and differentiated on a solid support that is planar typically grow in a monolayer.

[0038] The term “plurality” refers to two or more.

III. Methods of Screening for Agents that Promote Osteogenesis

Contacting one or more polypeptides with one or more agents

[0039] The present invention provides for methods of screening comprising contacting at least one of the polypeptides listed in Table 1 with one or more agents that are candidates to inhibit one or more of the polypeptides listed in Table 1. In some embodiments, a plurality of different agents are screened, for example, 50, 100, 250, 500, 1000, 10,000 or more different

agents. The polypeptides can be in a host cell, in a cell extract, or purified (*e.g.*, partially, substantially or completely), as desired.

[0040] Screening assays for inhibitory agents of the polypeptides of Table 1 wherein the polypeptides are not in a host cell can be used when measuring, for example, binding activity or enzymatic activity. For example, the ability of a candidate agent to inhibit the binding of a natural substrate or ligand can be measured. In other embodiments, the ability of a candidate agent to inhibit enzymatic consumption of substrate or accumulation of product can be measured.

[0041] In some embodiments, the assays are designed to screen large combinatorial libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (*e.g.*, in microtiter formats or in microwell plates in robotic assays). The combinatorial libraries can be completely random, or comprise members that contain a core structure based on one or more promising lead compounds. The combinatorial libraries can be completely synthetic or can include some or all members that are derived from naturally occurring sources, including, for example, bacteria, fungi, plants, insects and vertebrate (*e.g.*, *Xenopus* (frog) or *Anguilla* (eel)) and non-vertebrate animals (*e.g.*, *Strongylocentrotus* (sea urchin) or mollusks). *See also*, Boldi, *Combinatorial Synthesis of Natural Product Based Libraries*, 2006, CRC Press.

[0042] In one embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such “combinatorial chemical libraries” or “ligand libraries” are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional “lead compounds” or can themselves be used as potential or actual therapeutics.

[0043] A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical “building blocks” such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (*i.e.*, the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

[0044] Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. *See, for example*, U.S. Patent Nos. 5,663,046; 5,958,792; 6,185,506; 6,541,211; 6,721,665, the disclosures of which are hereby incorporated herein by reference. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see*,
5 *e.g.*, U.S. Pat. No. 5,010,175; Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991); Houghton, *et al.*, *Nature* 354:84-88 (1991); and *Combinatorial Peptide Library Protocols*, Cabilly, ed., 1997, Humana Press. Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (*e.g.*, PCT Publication No. WO 91/19735), encoded peptides (*e.g.*, PCT Publication WO 93/20242), random bio-
10 oligomers (*e.g.*, PCT Publication No. WO 92/00091), benzodiazepines (*e.g.*, U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic
15 syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994); *Combinatorial Libraries: Synthesis, Screening and Application Potential*, Cortese, ed., 1995, Walter De Gruyter Inc; and Obrecht and Villalgorido, *Solid-Supported Combinatorial and Parallel Synthesis of Small-Molecular-Weight Compound Libraries*, 1998, Elsevier Science Ltd), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates
20 (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, *infra*, Sambrook and Russell, *infra* and U.S. Patent Nos. 6,955,879; 6,841,347; 6,830,890; 6,828,098; 6,573,098; and 6,399,334), peptide nucleic acid libraries (*see, e.g.*, U.S. Pat. Nos. 5,539,083; 5,864,010 and 6,756,199), antibody libraries (*see, e.g.*, Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see*,
25 *e.g.*, Liang *et al.*, *Science*, 274:1520-1522 (1996); U.S. Pat. No. 5,593,853; and *Solid Support Oligosaccharide Synthesis and Combinatorial Carbohydrate Libraries*, Seeberger, ed., 2004, John Wiley & Sons (E-book)), small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum C&EN, January 18, page 33 (1993) and U.S. Pat. No. 5,288,514; isoprenoids, U.S. Pat. No. 5,569,588; thiazolidinones and metathiazanones, U.S. Pat. No. 5,549,974; pyrrolidines,
30 U.S. Pat. Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Pat. No. 5,506,337, and the like). *See also*, *Combinatorial Library Design and Evaluation: Principles, Software Tools, and Applications in Drug Discovery*, Ghose, *et al.*, eds., 2001, Marcel Dekker; *Molecular Diversity and Combinatorial Chemistry: Libraries and Drug Discovery*, Chaiken

and Janda, eds., 1996, Oxford Univ Pr.; and *Combinatorial Library Methods and Protocols*, English, ed., 2002, Humana Press.

[0045] Devices for the preparation of combinatorial libraries are commercially available (see, e.g., Advanced Chem Tech, Louisville KY., Symphony, Rainin, Woburn, MA., Applied Biosystems, Foster City, CA., Millipore, Bedford, MA and Caliper Life Sciences, Hopkinton, MA).

[0046] In some embodiments, the screening assays can be conveniently carried out in multiwell plates (e.g., 96-well, 384-well, etc.) wherein each agent to be screened is individually tested in a single well. In some embodiments, two or more candidate agents are tested in a single reaction mixture.

Agents

[0047] The agents used in the screening methods can be, for example, small organic compounds (e.g., molecular weight less than 10,000 daltons, for example, less than 8000, 6000, 4000, 2000 daltons), lipids, sugars, polypeptides, nucleic acids (e.g., oligonucleotides, DNA, RNA, ribozymes, short inhibitory RNA (siRNA), micro RNA (miRNA), etc.). Typically, the amount of inhibitory agent contacted with the cells is from about 0.05 nM to about 50 μ M, for example, about 1 nM to about 1 μ M, about 0.1 μ M to about 50 μ M, or about 0.05 nM, 0.1 nM, 0.5 nM, 1.0 nM, 1.5 nM, 25 nM, 100 nM, 1.0 μ M, 10 μ M, or 50 μ M.

Organic compounds

[0048] In some embodiments, the one or more agents are small organic compounds. Essentially any chemical compound can be screened as a potential inhibitor of one or more of the polypeptides of Table 1 in the assays of the invention. Most preferred are generally compounds that can be dissolved in aqueous or organic (especially DMSO-based) solutions and compound which fall within Lipinski's "Rule of 5" criteria. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on multiwell plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma-Aldrich (St. Louis, Mo.); Fluka Chemika-Biochemica Analytika (Buchs Switzerland), as well as numerous providers of small organic molecule libraries ready for screening, including Chembridge Corp. (San Diego, CA), Discovery Partners International (San Diego, CA), Triad Therapeutics (San Diego, CA), Nanosyn

(Menlo Park, CA), Affymax (Palo Alto, CA), ComGenex (South San Francisco, CA), Tripos, Inc. (St. Louis, MO), Reaction Biology Corp. (Malvern, PA), Biomol Intl. (Plymouth Meeting, PA), TimTec (Newark, DE), and AnalytiCon (Potsdam, Germany), among others.

5 *Polypeptides and Antibodies*

[0049] In some embodiments, the one or more agents are polypeptides (including but not limited to peptides having 8-30 amino acids, antibodies, etc.). Polypeptide libraries useful for screening are commercially available from numerous sources, for example, from Cambridge Peptides, Cambridge, United Kingdom; JPT Peptide Technologies, Berlin, Germany; 10 Bio-Synthesis, Lewisville, TX; and Prestwick Chemical, Washington, DC. Methods for producing peptide libraries are also well known in the art. *See, for example, Synthetic Peptides: A User's Guide*, Grant, ed., 2002, Oxford University Press; Benoiton, *Chemistry of Peptide Synthesis*, 2005, CRC Press; Jones, *Amino Acid and Peptide Synthesis*, 2002, Oxford University Press. Peptide synthesizers are commercially available, for example, from 15 TechniKrom, Inc., Evanston, IL; Applied Biosystems, Foster City, CA; and Advanced Automated Peptide Protein Technologies (AAPPT), Louisville, KY.

[0050] In some embodiments, the one or more agents are antibodies, including polyclonal or monoclonal antibodies, Fab fragments, single chain antibodies (scFv), complementary regions from combinatorial libraries, etc. A combinatorial antibody library useful for 20 screening purposes is available from MorphoSys, Martinsried/Planegg, Germany. Methods for producing antibody libraries are known in the art.

Inhibitory Oligonucleotides

[0051] In some embodiments, the one or more agents are inhibitory oligonucleotides, including antisense oligonucleotides, ribozymes, short inhibitory RNA (siRNA), micro RNA 25 (miRNA). Libraries of randomized oligonucleotides are commercially available from, for example, Integrated DNA Technologies (IDT), Coralville, IA; Ambion, Austin, TX and Qiagen, Valencia, CA. In one embodiment, the inhibitory oligonucleotides inhibit the activity or expression level of one or more of the polypeptides listed in Table 1.

Antisense Oligonucleotides

[0052] An "antisense" oligonucleotide corresponds to an RNA sequence as well as a DNA sequence coding therefor, which is sufficiently complementary to a particular mRNA molecule, for which the antisense RNA is specific, to cause molecular hybridization between the antisense RNA and the mRNA such that translation of the mRNA is inhibited. Such hybridization can occur under *in vitro* and *in vivo* conditions. The antisense molecule must have sufficient complementarity to the target gene so that the antisense RNA can hybridize to the target gene (or mRNA) and inhibit target gene expression regardless of whether the action is at the level of splicing, transcription, or translation. In some embodiments, the complementary antisense sequence is about 15-30 nucleotides in length, for example, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides, or longer or shorter, as desired. The antisense components of the present invention may be hybridizable to any of several portions of the target cDNA, including the coding sequence, 3' or 5' untranslated regions, or other intronic sequences, or to target mRNA.

[0053] Antisense oligonucleotides can include sequences hybridizable to any of several portions of the target DNA, including the coding sequence, 3' or 5' untranslated regions, or other intronic sequences, or to target mRNA.

Small Inhibitory RNA Oligonucleotides

[0054] siRNA technology relates to a process of sequence-specific post-transcriptional gene repression which can occur in eukaryotic cells. In general, this process involves degradation of an mRNA of a particular sequence induced by double-stranded RNA (dsRNA) that is homologous to that sequence. siRNA can be effected by introduction or expression of relatively short homologous dsRNAs. For screening purposes, the double stranded oligonucleotides used to effect inhibition of expression, at either the transcriptional or translational level, can be of any convenient length. siRNA molecules are typically from about 15 to about 30 nucleic acids in length, for example, about 19-25 nucleic acids in length, for example, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 nucleic acids in length. Optionally the dsRNA oligonucleotides can include 3' overhang ends. Exemplary 2-nucleotide 3' overhangs can be composed of ribonucleotide residues of any type and can be composed of 2'-deoxythymidine residues, which lowers the cost of RNA synthesis and can enhance nuclease resistance of siRNAs in the cell culture medium and within transfected cells (*see*, Elbashir et al., 2001, *Nature* 411:494-8).

[0055] Longer dsRNAs of 50, 75, 100 or even 500 base pairs or more can also be utilized in certain embodiments of the invention. Exemplary concentrations of dsRNAs for effecting inhibition are about 0.05 nM, 0.1 nM, 0.5 nM, 1.0 nM, 1.5 nM, 25 nM or 100 nM, although other concentrations can be utilized depending upon the nature of the cells treated, the gene target and other factors readily discernable to the skilled artisan.

[0056] Exemplary dsRNAs can be synthesized chemically or produced *in vitro* or *in vivo* using appropriate expression vectors. Exemplary synthetic RNAs include 21 nucleotide RNAs chemically synthesized using methods known in the art. Synthetic oligonucleotides are preferably deprotected and gel-purified using methods known in the art (*see, e.g.,* Elbashir, *et al.*, 2001, *Genes Dev.* 15:188-200). Alternatively the dsRNAs can be transcribed from a mammalian expression vector. A single RNA target, placed in both possible orientations downstream of an appropriate promoter for use in mammalian cells, will transcribe both strands of the target to create a dsRNA oligonucleotide of the desired target sequence. Any of the above RNA species will be designed to include a portion of nucleic acid sequence represented in a target nucleic acid.

[0057] The specific sequence utilized in design of the siRNA oligonucleotides can be any contiguous sequence of nucleotides contained within the expressed gene message of the target. Programs and algorithms, known in the art, may be used to select appropriate target sequences. *See*, the Ambion website at ambion.com. In addition, optimal sequences can be selected utilizing programs designed to predict the secondary structure of a specified single stranded nucleic acid sequence and allowing selection of those sequences likely to occur in exposed single stranded regions of a folded mRNA. Methods and compositions for designing appropriate siRNA oligonucleotides may be found, for example, in U.S. Pat. No. 6,251,588, the contents of which are incorporated herein by reference.

Ribozymes

[0058] Ribozymes are enzymatic RNA molecules capable of catalyzing specific cleavage of RNA. The composition of ribozyme molecules preferably includes one or more sequences complementary to a target mRNA, and the well known catalytic sequence responsible for mRNA cleavage or a functionally equivalent sequence (*see, e.g.,* U.S. Pat. No. 5,093,246, which is incorporated herein by reference in its entirety). Ribozyme molecules designed to catalytically cleave target mRNA transcripts can also be used to prevent translation of subject target mRNAs.

[0059] While ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy target mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. Preferably, the target mRNA has the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art.

[0060] Gene targeting ribozymes necessarily contain a hybridizing region complementary to two regions, each of at least 5 and preferably each 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 contiguous nucleotides in length of a target mRNA. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense mRNA.

[0061] With regard to antisense, siRNA or ribozyme oligonucleotides, phosphorothioate oligonucleotides can be used. Modifications of the phosphodiester linkage as well as of the heterocycle or the sugar may provide an increase in efficiency. Phosphorothioate is used to modify the phosphodiester linkage. An N3'-P5' phosphoramidate linkage has been described as stabilizing oligonucleotides to nucleases and increasing the binding to RNA. Peptide nucleic acid (PNA) linkage is a complete replacement of the ribose and phosphodiester backbone and is stable to nucleases, increases the binding affinity to RNA, and does not allow cleavage by RNase H. Its basic structure is also amenable to modifications that may allow its optimization as an antisense component. With respect to modifications of the heterocycle, certain heterocycle modifications have proven to augment antisense effects without interfering with RNase H activity. An example of such modification is C-5 thiazole modification. Finally, modification of the sugar may also be considered. 2'-O-propyl and 2'-methoxyethoxy ribose modifications stabilize oligonucleotides to nucleases in cell culture and *in vivo*.

[0062] Inhibitory oligonucleotides can be delivered to a cell by direct transfection or transfection and expression via an expression vector. Appropriate expression vectors include mammalian expression vectors and viral vectors, into which has been cloned an inhibitory oligonucleotide with the appropriate regulatory sequences including a promoter to result in expression of the antisense RNA in a host cell. Suitable promoters can be constitutive or development-specific promoters. Transfection delivery can be achieved by liposomal transfection reagents, known in the art (e.g., Xtreme transfection reagent, Roche, Alameda,

CA; Lipofectamine formulations, Invitrogen, Carlsbad, CA). Delivery mediated by cationic liposomes, by retroviral vectors and direct delivery are efficient. Another possible delivery mode is targeting using antibody to cell surface markers for the target cells.

Cells

5 [0063] In some embodiments, the one or more polypeptides of Table 1 are in a host cell. The activity of one or more of the polypeptides in Table 1 can be measured in any cell capable of expressing the one or more polypeptides, endogenously or recombinantly. The cells can be prokaryotic or eukaryotic. Exemplary cells include bacterial cells (*e.g.*, *E. coli*, *Bacillus*), plant cells (*e.g.*, *Arabidopsis*, *Brassica*, tobacco), insect cells, and mammalian
10 cells. Recombinant expression of polypeptides in different host cell systems is known in the art. *See*, for example, Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, 3rd Ed., 2001, Cold Spring Harbor Laboratory Press; Ausubel, *et al.*, *Current Protocols in Molecular Cloning*, 1987-2006, John Wiley Interscience. Appropriate host cells and expression vectors for recombinant expression of one or more of the polypeptides of Table 1
15 are commercially available, for example, from Invitrogen, Carlsbad, CA; Promega, Madison, WI; and Qiagen, Valencia, CA.

[0064] In some embodiments, the mammalian cells can be stem cells, typically mesenchymal stem cells (MSCs), pre-osteoblasts, or cells of other lineages such as, for example, pre-adipocytes or myoblasts. Methods for isolation and differentiation of human
20 and animal MSCs have been described (*see, e.g.*, U.S. Pat. Nos. 5,942,225 and 5,486,359; and Pittenger *et al.*, *Science* 284:143 (1999)).

[0065] Mesenchymal stem cells ("MSC") are capable of differentiating into the mesenchymal cell lineages, including bone, cartilage, adipose, muscle, stroma, including hematopoietic supportive stroma, and tendon, and play important roles in repair and
25 regeneration (*see, e.g.*, Olsen, 2000, *Ann. Rev. Cell Dev. Biol.* 16:191). MSCs are identified by specific cell surface markers which are identified with unique monoclonal antibodies as described in *e.g.*, U.S. Pat. No. 5,643,736.

[0066] Human mesenchymal stem cells (MSC) can be obtained by isolating pluripotent mesenchymal stem cells from other cells in the bone marrow or other MSC source. Bone
30 marrow cells can be obtained from iliac crest, femora, tibiae, spine, rib or other medullary spaces. Other sources of human mesenchymal stem cells include embryonic yolk sac, placenta, umbilical cord, fetal and adolescent skin, blood, adipose tissue, and muscle satellite

cells. Typically, cells from a tissue specimen containing mesenchymal stem cells are cultured in growth medium containing growth factors that (1) stimulate mesenchymal stem cell growth without differentiation, and (2) allow for the selective adherence of only the mesenchymal stem cells to a substrate surface. After culturing the cells for a suitable amount of time, non-adherent matter can be removed from the substrate surface, thus providing an expanded population of mesenchymal stem cells. Thus, homogeneous MSC populations can be obtained by positive selection of adherent marrow or periosteal cells which are free of markers associated with either hematopoietic cell or differentiated mesenchymal cells.

[0067] The cells to be differentiated into cells of an osteoblast lineage can be derived from any suitable mammal. For example, the cells can be obtained from rodents including, for example, mice, rats, guinea pigs, and rabbits; non-rodent mammals such as, for example, cats, dogs, pigs, sheep, horses, cows, and goats; primates including, for example, chimpanzees and humans. The cells to be differentiated can be primary cells or can be cells maintained in culture. If the cells are maintained in culture, they are contacted, for example, with the compounds/compositions of the present invention between, *e.g.*, the 5th and 15th passage in culture. Techniques and methods for establishing a primary culture of cells for use in the methods of the invention are known to those of skill in the art (*see, e.g.*, Humason, *Animal Tissue Techniques*, 4th Edition, W. H. Freeman and Company (1979), and Ricciardelli, *et al.*, (1989) *In Vitro Cell Dev. Biol.* 25: 1016).

General Culturing Methods

[0068] Culture of host cells expressing one or more polypeptides of Table 1 utilizes routine techniques in the field of cell culture. Suitable cell culture methods and conditions can be determined by those of skill in the art using known methodology (*see, e.g.*, Freshney *et al.*, *Culture of Animal Cells* (4th Edition, 2000); Ausubel, *et al.*, *supra*; Sambrook and Russell, *supra*; *Insect Cell Cultures: Fundamental and Applied Aspects*, Vlak, *et al.*, eds. 1996, Kluwer Academic Pub.; and Evans, *et al.*, *Plant Cell Culture*, 2003, Taylor & Francis. In general, the cell culture environment includes consideration of such factors as the substrate for cell growth, cell density and cell contract, the gas phase, the medium, and temperature.

[0069] Plastic dishes, flasks, or roller bottles can be used to culture cells according to the methods of the present invention. Suitable culture vessels include, for example, multi-well plates, petri dishes, tissue culture tubes, flasks, roller bottles, and the like.

Measuring the activity or expression level of the polypeptide

[0070] The activity of any of the polypeptides listed in Table 1 can be measured according to known methods based the polypeptides' known function. For example, for the polypeptides with known enzymatic activity (e.g., adenylate cyclase, adenosine kinase, serine/threonine kinase, phospholipase), the consumption of substrate and/or the accumulation of product in the enzymatic reaction can be measured. For the polypeptides that are known receptors (e.g., olfactory receptors, integrins, potassium channels), downstream intracellular signaling or binding to ligand can be measured. Such assays are known in the art.

[0071] The level of expression of the polypeptides listed in Table 1 can be measured according to methods well known in the art, and described herein. Levels of expression can be measured at the transcriptional and/or translational levels. At the translational level, expression of one or more of the proteins listed in Table 1 can be measured using immunoassays including immunohistochemical staining, western blotting, ELISA and the like with an antibody that selectively binds to the particular protein or a fragment thereof. Detection of the protein using protein-specific antibodies in immunoassays is known in the art (see, e.g., Harlow & Lane, *Using Antibodies: A Laboratory Manual* (1998); Coligan, et al., eds., *Current Protocols in Immunology* (1991-2006); Goding, *Monoclonal Antibodies: Principles and Practice* (3rd ed. 1996); and Kohler & Milstein, *Nature* 256:495-497 (1975). At the transcriptional level, mRNA can be detected by, for example, amplification, e.g., PCR, LCR, or hybridization assays, e.g., northern hybridization, RNase protection, or dot blotting, all methods known in the art. The level of protein or mRNA is detected, for example, using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies. These assays are well-known to those of skill in the art and described in, e.g., Ausubel, et al., eds., *Current Protocols In Molecular Biology* (1987-2006).

[0072] Modulation (e.g., increase or decrease) of transcriptional levels can also be measured using promoter-reporter gene fusion constructs. For example, the promoter region of a gene encoding a polypeptide in Table 1 can be fused (i.e., operably linked) to the coding sequence of a polypeptide that produces a detectable signal. Reporter constructs are well known in the art. Exemplary reporter sequences include, for example, fluorescent proteins (e.g., green, red, yellow), phosphorescent proteins (e.g., luciferase), antibiotic resistance proteins (e.g., β -lactamase), enzymes (e.g., alkaline phosphatase).

Selecting the agent that inhibits the activity of the polypeptide

[0073] Inhibition of polypeptide activity of one or more of the polypeptides listed in Table 1 can be measured by comparison to polypeptide activity of the same polypeptide that has not been contacted with one or more candidate agents (inside or outside of a cell).

5 Polypeptide activity that is inhibited will be, *e.g.*, at least about 10%, 25% or 50% less in a treated sample (or reaction mixture) in comparison to an untreated sample. In some embodiments, polypeptide activity can be inhibited at least about 60%, 70%, 80%, 90%, or even completely inhibited, in comparison to polypeptide activity in an untreated sample.

10 [0074] Similarly, inhibition of polypeptide expression of one or more of the polypeptides listed in Table 1, at the transcriptional or translational level, can be measured by comparison to polypeptide expression levels of the same polypeptide in a cell that has not been contacted with one or more candidate agents. In some embodiments, polypeptide expression levels that are inhibited will be, *e.g.*, at least about 10%, 25% or 50% less in a treated cell in comparison to an untreated cell. In some embodiments, polypeptide expression levels can be inhibited at
15 least about 60%, 70%, 80%, 90%, or even completely inhibited, in comparison to polypeptide expression levels in an untreated cell.

[0075] In other embodiments, the inhibition of polypeptide activity or expression in the presence of one or more test agents is compared to polypeptide activity or expression level in the presence of a known inhibitor. In this case, same or similar polypeptide activity or
20 expression levels indicates that the one or more test agents are inhibitors.

[0076] In some embodiments, selectivity or specificity of the inhibitory agents can be measured by administering the agent to a cell that does not recombinantly or endogenously express any of the polypeptides of Table 1. Agents that specifically inhibit a polypeptide of Table 1 will generally not elicit any detectable response in a cell that does not express the
25 polypeptide.

Detecting Osteogenesis

[0077] Induction of osteogenesis, *in vitro* or *in vivo*, can be detected using any method known in the art. For example, by detecting expression of osteoblast-specific proteins, detecting expression of bone-specific transcription factors, and detecting changes in bone
30 density. Osteoblast-specific proteins include, for example, alkaline phosphatase (ALP), collagen type I, osteocalcin, and osteopontin (*see, e.g., Olsen et al., Annu. Rev. Cell. Dev.*

Biol. 16:191 (2000)). In some embodiments, expression of alkaline phosphatase is detected as an indicator of osteogenesis. Bone specific transcription factors include, for example, Cbfa1/Runx2, gsc, Dlx1, Dlx5, Msx1, Cart1, Hoxa1, Hoxa2, Hoxa3, Hoxb1, rae28, Twist, AP-2, Mf1, Pax1, Pax3, Pax9, TBX3, TBX4, TBX5, and Brachyury (*see, e.g., Olsen et al*,
5 2000, *supra*). Typically, expression of Cbfa1/Runx2 is detected as an indicator of osteogenesis.

Detection of Osteoblast-Specific Proteins

[0078] Expression of osteoblast-specific proteins can be detected by measuring the level of the osteoblast-specific protein or mRNA. The level of particular osteoblast-specific proteins
10 can conveniently be measured using immunoassays including immunohistochemical staining, western blotting, ELISA and the like with an antibody that selectively binds to the particular osteoblast specific proteins or a fragment thereof. Detection of the protein using protein-specific antibodies in immunoassays is known to those of skill in the art (*see, e.g., Harlow & Lane, Using Antibodies: A Laboratory Manual* (1998); Coligan, *et al.*, eds., *Current*
15 *Protocols in Immunology* (1991-2006); Goding, *Monoclonal Antibodies: Principles and Practice* (3rd ed. 1996); and Kohler & Milstein, *Nature* 256:495-497 (1975)). For measurement of mRNA, amplification, *e.g.,* PCR, LCR, or hybridization assays, *e.g.,* northern hybridization, RNase protection, dot blotting, are preferred. The level of protein or mRNA is detected, for example, using directly or indirectly labeled detection agents, *e.g.,*
20 fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies. These assays are well-known to those of skill in the art and described in, *e.g., Ausubel, et al.*, eds., *Current Protocols In Molecular Biology* (1987-2006).

[0079] Typically, expression of the osteoblast specific-protein, alkaline phosphatase, is used to detect differentiated osteoblasts. Expression of alkaline phosphatase (ALP) is
25 correlated with osteogenesis. ALP hydrolyzes inorganic pyrophosphates to phosphates and promotes the formation of hydroxyapatite crystals in bone matrix. Deactivating mutations of ALP cause osteomalacia, characterized by poorly mineralized bones and frequent bone fractures, indicating that ALP plays a significant role in bone formation (*see, e.g., Hessle, et al., Proc. Natl. Acad. Sci. USA* 99:9445(2002)). ALP is a highly active and stable enzyme,
30 making direct assays of its enzymatic activity convenient. In addition, direct histochemical staining of cells can conveniently be used to detect ALP.

[0080] Osteogenesis can also be detected by detecting extracellular matrix (ECM) mineralization. Calcium phosphate deposits associated with ECM mineralization can be detected by staining cells with alizarin red solution, as described herein. The extent and intensity of red staining can then be evaluated.

5 *Enzymatic Activity*

[0081] For direct assays of ALP activity, cells can be plated in multiwell plates (*e.g.*, 96-well, 384-well, 1536-well) and treated with an appropriate amount of one or more candidate agents, either alone or with other growth factors (*e.g.*, BMP-4) and then incubated at 37°C. in 5% CO₂. After an appropriate incubation time, the media is removed and lysis
10 buffer is added into each well. After an appropriate incubation time in lysis buffer, alkaline phosphatase substrate solution (*e.g.*, 2'-[2'-benzothiazoyl]-6'-hydroxybenzothiazole phosphate (BBTP)) is added to each well. After an appropriate incubation time at room temperature, the plates are read on a plate reader using methods known in the art.

Immunohistochemical Detection

15 [0082] For direct immunohistochemical staining of cells to detect ALP, cells are seeded in multiwell assay plates at a suitable density and treated with an appropriate amount of a candidate agent, either alone or with other growth factors (*e.g.*, BMP-4) for an appropriate time. Cells are then fixed in a 10% formalin solution. The fixed cells are washed again and stained with a reagent specific for ALP (*e.g.*, an antibody specific for ALP or a
20 colorimetric ALP substrate) using methods known to those of skill in the art (*see, e.g.*, Harlow & Lane, *supra*; Coligan, *supra*; Goding, *supra*; and Kohler & Milstein, *supra*). Photographic images of the cells are taken and ALP positive cells are counted manually from the images.

Detection of Bone-Specific Transcription Factors

25 [0083] Expression of bone-specific transcription factors can be detected using reporter gene assays. These assays are well known to those of skill in the art and are described in, *e.g.*, Ausubel, *et al.*, *supra*. Detecting levels of expression of the bone specific transcription factor Cbfa1/Runx2 can be used to detect osteogenesis. Cbfa1/Runx2 plays an essential role in osteoblast differentiation transgenic mice lacking the Cbfa1/Runx2 gene die shortly after
30 birth due to loss in bone formation (*see, e.g.*, Ducy *et al.*, *Cell* 89:747 (1997) and Komori *et al.*, *Cell* 89:755 (1997)).

[0084] Reporter genes including, for example, chloramphenicol acetyltransferase, firefly luciferase, bacterial luciferase, fluorescent proteins (*e.g.*, green, yellow, purple), or β -galactosidase can be used in the reporter gene assays. The reporter construct is typically transiently or stably transfected into a cell. The promoter region of the relevant gene (*e.g.*, Cbfa1/Runx2) is typically amplified by PCR appropriate primers. The resulting PCR product is inserted into a suitable cloning vector, amplified and sequenced. The resulting plasmid is digested with appropriate restriction enzymes and the resulting fragment is inserted into a vector comprising a reporter gene.

Transiently Transfected Cells

[0085] For reporter gene assays with transiently transfected cells, the cells can be seeded in a multiwell plate at an appropriate density (*e.g.*, about 30,000 cells/well) in growth medium and incubated for a suitable time. Plasmid DNA is transfected into the cells using a suitable transfection reagent. After a suitable time (*e.g.*, about 8 hours), the transfected cells are plated into multiwell assay plates (*e.g.*, Corning) and treated with an appropriate amount of a candidate agent. The cells are incubated for an appropriate period of time (*e.g.*, several days, *e.g.*, 2, 3 or 4 days), then the reporter gene activity in the cells is assayed using methods known to those of skill in the art.

Stably Transfected Cells

[0086] For reporter gene assays with stably transfected cells, the cells can be seeded in a multiwell plate at an appropriate density (*e.g.*, about 30,000 cells/well) in growth medium and incubated for a suitable time. An appropriate amount of reporter plasmid and a vector comprising a selectable marker (*e.g.*, an antibiotic resistance gene) are co-transfected into the cells using an appropriate transfection reagent. After an appropriate incubation time, cells are seeded in culture dishes and an appropriate amount of antibiotic is added to the culture medium. Fresh antibiotic is added at appropriate intervals. The antibiotic resistant colonies are pooled to yield the stably transfected cells. The transfected cells are plated into multiwell assay plates (*e.g.*, Corning) and treated with an appropriate amount of a candidate agent. The cells are incubated for an appropriate period of time (*e.g.*, several days, *e.g.*, 2, 3 or 4 days), then the reporter gene activity in the cells is assayed using methods known to those of skill in the art.

[0087] The screening methods of the present invention are well-suited to high throughput screening. Numerous agents can be concurrently screened using multiwell plates (*e.g.*,

96-well, 192-well, 384-well, 768-well, 1536-well, etc.) and automated systems. Automated systems of use in the present screening methods are purchasable from, for example, Thermo LabSystems, Waltham, MA; Caliper Life Science, Hopkinton, MA; Beckman Coulter, Inc., Fullerton, CA; and Invitrogen Corp., Carlsbad, CA.

5 **IV. Methods of Delivering siRNA Oligonucleotides to a Cell**

[0088] Compositions comprising one or more siRNA oligonucleotides that inhibit one or more of the polypeptides listed in Table 1 can be delivered to a mammalian cell to promote osteogenesis. The cell can be contacted with one or more siRNA oligonucleotides, *in vitro*, *ex vivo*, or *in vivo*, using methods well known in the art and described herein.

10 [0089] In some embodiments, the mammalian cell is contacted with a composition comprising one or more of the siRNA sequences that inhibit the expression of one or more of the polypeptides listed in Table 1. In one embodiment, the mammalian cell is contacted with a composition comprising one or more siRNA oligonucleotides that inhibit the expression of one or more polypeptides selected from the group consisting of GNAS (Human GNAS
15 complex locus, transcript variant 3 (as of August 9, 2007), isoform b of the alpha subunit of Gs, NM_080426), ADCY8 (adenylate cyclase 8, NM_001115), ADK (adenosine kinase, NM_001123), P2RY11 (purinergic receptor P2Y, G-protein coupled, 11, NM_002566), TBX3 (T-box 3 or ulnar mammary syndrome, NM_005996), BIRC4 (baculoviral IAP repeat-containing 4, NM_001167), BCL2L2 (BCL2-like 2, NM_004050), SLC12A2 (solute carrier
20 family 12, member 2, NM_001046), KCNT1 (potassium channel, subfamily T, member 1, XM_029962.2), GBDR1 (putative glial blastoma cell differentiation-related, NM_016172), DUSP6 (dual specificity phosphatase 6, NM_001946) and MJD (Machado-Joseph disease or ataxin 3, NM_004993). In some embodiments, the mammalian cell is contacted with a composition comprising one or more siRNA oligonucleotide sequences listed in Table 1.

25 [0090] For *in vitro* or *in vivo* delivery to a cell, compositions can comprise one or more siRNA molecules for direct transfection, for example, in double-stranded or hairpin configurations. Alternatively, compositions can comprise one or more vectors, for example, plasmid mammalian expression vectors or viral expression vectors, which express one or more siRNA sequences, for example, in double-stranded or hairpin configurations.

30 [0091] For transfection, a composition comprising one or more siRNA molecules (within or without vectors) can comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically

acceptable formulations. Methods for the delivery of nucleic acid molecules are described, for example, in Gilmore, *et al.*, *Curr Drug Delivery* (2006) 3:147-5 and Patil, *et al.*, *AAPS Journal* (2005) 7:E61-E77, each of which are incorporated herein by reference. Delivery of siRNA molecules is also described in several U.S. Patent Publications, including for example, 2006/0019912; 2006/0014289; 2005/0239687; 2005/0222064; and 2004/0204377, the disclosures of each of which are hereby incorporated herein by reference. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, by electroporation, or by incorporation into other vehicles, including biodegradable polymers, hydrogels, cyclodextrins (*see, for example* Gonzalez *et al.*, 1999, *Bioconjugate Chem.*, 10, 1068-1074; Wang *et al.*, International PCT publication Nos. WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)acid (PLGA) and PLGA microspheres (*see for example* U.S. Pat. No. 6,447,796 and US Patent Application Publication No. 2002/130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). In another embodiment, the nucleic acid molecules of the invention can also be formulated or complexed with polyethyleneimine and derivatives thereof, such as polyethyleneimine-polyethyleneglycol-N-acetylgalactosamine (PEI-PEG-GAL) or polyethyleneimine-polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) derivatives.

[0092] Examples of liposomal transfection reagents of us with this invention include, for example: CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N,N,N-tetramethyl-N,N,N,N-tetrapalmit-y-spermine and dioleoyl phosphatidylethanolamine (DOPE) (GIBCO BRL); Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-tri-methyl-ammoniummethylsulfate) (Boehringer Mannheim); Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL); and (5) siPORT (Ambion); HiPerfect (Qiagen); X-treme GENE (Roche); RNAicARRIER (Epoch Biolabs) and TransPass (New England Biolabs).

[0093] In some embodiments, the siRNA sequence is delivered into the cell cloned into a mammalian expression vector. Mammalian expression vectors suitable for siRNA expression are commercially available, for example, from Ambion (*e.g.*, pSilencer vectors), Austin, TX; Promega (*e.g.*, GeneClip, siSTRIKE, SiLentGene), Madison, WI; Invitrogen, Carlsbad, CA;

InvivoGen, San Diego, CA; and Imgenex, San Diego, CA. Typically, expression vectors for transcribing siRNA molecules will have a U6 promoter.

[0094] In some embodiments, the siRNA sequence is delivered into the cell cloned into a viral expression vector. Viral vectors suitable for delivering siRNA molecules to a cell include adenoviral vectors, adeno-associated vectors, and retroviral vectors (including lentiviral vectors). Viral vectors developed for delivering and expressing siRNA oligonucleotides are commercially available from, for example, GeneDetect, Bradenton, FL; Ambion, Austin, TX; Invitrogen, Carlsbad, CA; Open BioSystems, Huntsville, AL; and Imgenex, San Diego, CA.

10 V. Administration and Formulation

[0095] Agents (*e.g.*, siRNA, organic compounds, polypeptides) that inhibit the activity and/or expression of one or more of the polypeptides listed in Table 1 can be used to induce osteogenesis in mammalian cells. A mammalian cell is contacted with an inhibitory agent, whereupon the mammalian cell differentiates into a cell of an osteoblast lineage. The mammalian cell can be contacted with an inhibitory agent (or a composition thereof) either *in vivo*, *ex vivo* (both described below) or *in vitro* (described above).

In Vivo Induction of Osteogenesis

[0096] The inhibitory agents as well as compositions thereof can conveniently be used to induce osteogenesis *in vivo*. The compounds and compositions of the present invention are administered to an individual, *e.g.*, a mammal such as a human, in an amount effective to induce differentiation of mammalian cells into cells of an osteoblast lineage. In view of their ability to induce osteogenesis, the inhibitory agents are useful for treating bone disorders and diseases, including osteoporosis, rickets, osteomalacia, McCune-Albright syndrome, and Paget's disease. In one embodiment, the compounds and compositions of the present invention are used to treat osteoporosis. In one embodiment, the compounds and compositions of the present invention are used to increase bone density. In one embodiment, the compounds and compositions of the present invention are used to increase bone density and reduce bone loss. In some embodiments, the pharmaceutical composition comprises one or more siRNA oligonucleotides selected from those listed in Table 1.

[0097] One of skill in the art will appreciate that the compositions of the present invention can be used alone or in combination with other compounds and therapeutic regimens to

induce osteogenesis. For example, the inhibitory agents can be administered in conjunction with bone morphogenetic proteins ("BMPs") or other anti-resorptive medications that affect the bone remodeling cycle. Suitable bone morphogenetic proteins include, for example, BMP-2, BMP-4, and BMP-7. Suitable anti-resorptive medications include, for example, bisphosphonates such as, for example, alendronate sodium and risedronate sodium; hormones, including, for example, calcitonin and estrogens, and selective estrogen receptor modulators, including, for example, raloxifene.

[0098] An effective amount of the composition will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of the composition; the LD50 of the composition; and the side-effects of the composition at various concentrations. Typically, the amount of the composition administered will range from about 0.01 to about 20 mg per kg, for example about 0.05 to about 15 mg per kg, for example about 0.1 to about 10 mg per kg body weight. Generally, lower doses are initially administered and incrementally increased until an appropriately efficacious dose is reached.

[0099] The compositions can be administered, for example, orally or parenterally, for example, intravenously, intramuscularly, intraperitoneally, subcutaneously, or directly into bone tissue. Oral administration is the preferred method of administration. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials. In some embodiments, the compositions are delivered by an implantable pump.

[0100] The compositions of the present invention are typically formulated with a pharmaceutically acceptable carrier before administration to an individual or subject. 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: The Science and Practice of Pharmacy*, University of the Sciences in Philadelphia, 21st Edition, 2005, Lippincott, Williams & Wilkins).

[0101] Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound or composition suspended in diluents, including water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an

appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of the following: lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, *e.g.*, sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

[0102] The compositions of the present invention can be in formulations suitable for other routes of administration, such as, for example, intravenous infusion, intraperitoneally, subcutaneously, intramuscularly, directly into bone. The formulations include, for example, aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets.

[0103] The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. For example, if the compositions of the present invention are administered to treat or prevent osteoporosis, the dose administered to the patient should be sufficient to prevent, retard, or reverse decreases in bone density. The dose will be determined by the efficacy of the particular composition employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular composition in a particular patient.

[0104] The inhibiting agents, including siRNA compositions, can be administered once or repeatedly, as needed. For example, the inhibitory agents can be administered at regular intervals (*e.g.*, twice daily, daily, twice weekly, weekly, monthly) for an extended period of time (*e.g.*, 7 days, 14 days, 21 days, 1 month, 2 months, 3 months, 6 months, 9 months, 12 months, or more). Administration of the inhibitory agents can be more or less often over time, as needed.

Ex Vivo Induction of Osteogenesis

[0105] Differentiated osteoblast cells can be administered to a subject by any means known to those of skill in the art. In one embodiment of the invention, differentiated osteoblast cells on an intact solid support (*e.g.*, a three-dimensional matrix or a planar surface) can be
5 administered to the subject, *e.g.*, via surgical implantation. Alternatively, the differentiated osteoblast cells can be detached from the matrix, *i.e.*, by treatment with a protease, before administration to the subject, *e.g.*, intravenously, subcutaneously, intramuscularly, intraperitoneally or directly into bone.

[0106] In some embodiments of the present invention, mesenchymal stem cells are
10 extracted from a human and subsequently contacted with an agent that inhibits one or more of the polypeptides of Table 1 for proliferation and differentiation into cells of an osteoblastic cell lineage. Cells can be extracted from the subject to be treated, *i.e.*, autologous (thereby avoiding immune-based rejection of the implant), or can be from a second subject, *i.e.*, heterologous. In either case, administration of cells can be combined with an appropriate
15 immunosuppressive treatment.

[0107] Osteoblast cells differentiated according to the methods of the present invention may be administered to a subject by any means known in the art. Suitable means of administration include, for example, intravenous, subcutaneous, intramuscular, intraperitoneal, into bone and surgical implantation.

[0108] The cells may be in formulations suitable for administration, such as, for example, aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include
20 suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets.
25

[0109] For surgical implantation, differentiated cells can be left on an intact solid support, *e.g.*, a three-dimensional matrix or planar surface. The matrix or planar surface is surgically implanted into the appropriate site in a subject. For example, a patient needing a bone graft can have differentiated cells on an intact solid support surgically implanted.

[0110] In determining the effective amount of the cells to be administered in the treatment or prophylaxis of conditions owing to diminished or aberrant osteoblasts, the physician

evaluates cell toxicity, transplantation reactions, progression of the disease, and the production of anti-cell antibodies. For administration, osteoblast cells differentiated according to the methods of the present invention can be administered in an amount effective to provide osteoblasts to the subject, taking into account the side-effects of the osteoblasts at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

Detection of Bone Density

[0111] To assess the effect of the compositions of the present invention on bone density, a baseline measurement of bone density in an individual who will receive treatment may taken.

Bone density is periodically measured at suitable intervals during and after administration of an inhibitory agent of the invention (e.g., one or more siRNA oligonucleotides selected from those listed in Table 1). Methods and devices for measuring bone density are well known in the art and are described in, e.g., U.S. Pat. Nos. 6,436,042; 6,405,068; 6,320,931; 6,302,582; 6,246,745; 6,230,036; 6,213,934; 6,102,567; 6,058,157; 5,898,753; 5,891,033; 5,852,647; 5,817,020; 5,782,763; 5,778,045; 5,749,363; 5,745,544; 5,715,820; 5,712,892; 5,572,998; and 5,480,439.

VI. Therapeutic and Diagnostic Uses

[0112] The methods and compositions of the present invention find use in treating bone disorders and diseases resulting from defective or deficient osteoblast formation.

Exemplified bone diseases that develop as a result of a deficient or defective osteoblast differentiation include, for example, osteoporosis, rickets, osteomalacia, McCune-Albright syndrome, and Paget's disease. *See, for example*, Chapters 333-334 of Kasper, *et al.*, *Harrison's Principles of Internal Medicine*, 16th Edition, 2004, McGraw-Hill.

[0113] The present methods are also useful for, *inter alia*, *in vitro* bone culturing and analysis of osteogenesis.

[0114] The following examples are intended to illustrate, not limit, the present invention.

EXAMPLES

Example 1

Experimental Procedures

Cell Culture, Transfection and High Throughput Screen

- 5 [0115] The human mesenchymal stem cells (hMSCs, PT-2501) were purchased from Cambrex Inc. and cultured as instructed by the supplier. Cells were expanded to passage 5 before being used for siRNA library screen. Briefly, diluted Xtreme-siRNA transfection reagent (cat# 4 476 115 00, Roche) (0.12 μ l Xtreme in 14 μ l DMEM) was mixed with pre-spotted siRNA (14 ng/well) in each well of 384-well plates for one hour. Cells (4000/well) in
- 10 60 μ l media (no antibiotics) were then added using automated dispenser (Multidrop 384, Thermo LabSystems) (siRNA final concentration at around 15 nM). Transfection was allowed to continue for 8 hours before first media renewal. After 7 days of culture, cells were stained for alkaline phosphatase (ALP) activity (cat# 86R-1KT, Sigma). Post-screen assays were done in 96-well plates and siRNA was transfected at the concentration of 30 nM
- 15 at the cell density of 8000 cells/well. CRE decoy oligonucleotide and control oligonucleotide (phosphorothioate bond modification on all nucleotides) (Park, Y. G. et al., *J Biol Chem* 274:1573-1580 (1999)) were transfected into cells similarly as siRNA using Xtreme-siRNA transfection reagent, except that the final DNA concentration was 90 nM. Control siRNA (siCon) used in the screen contains a pool of 50 scrambled sequences that have at least 4
- 20 mismatches to known human transcripts and ESTs, and at least 2-3 mismatches to the whole human genome. siCon used in target characterization after primary screen was purchased from Dharmacon (D-001210-01-05), which was bioinformatically designed to have ≥ 4 mismatches with known human and mouse genes. siTOX was also purchased from Dharmacon (D-001500-01-05).
- 25 siCBFA1: UAGUAGAGAUUAUGGAGUGCtg (SEQ ID NO:1) (antisense);
GCACUCCAUAUCUCUACUAtt (SEQ ID NO:2) (sense).
- CRE decoy 24-mer palindrome: 5'-TGACGTCATGACGTCATGACGTCA-3' (SEQ ID NO:3).
- CRE mismatch control: 5'-TGTGGTCATGTGGTCATGTGGTCA-3' (SEQ ID NO:4).

Calcium Phosphate Staining

[0116] Cells were rinsed with PBS twice, stained in alizarin red solution (cat# vw3611-2, VWR) for 8 minutes at room temperature, washed with PBS again, fixed in 10% formalin solution (cat# HT-5014, Sigma) for 20 minutes, then rinsed with water and air dried.

5 Oil Red O staining

[0117] Cells were rinsed with PBS twice, fixed in 10% formalin solution for 20 minutes, rinsed with PBS twice and dH₂O once, washed with propylene glycol for 5 minutes, and stained with Oil red in propylene glycol (I2722A, Newcomer Supply) for 30 minutes. Cells were then washed with 85% propylene glycol for 5 minutes, rinsed with dH₂O 3 times and
10 preserved in 50% glycerol.

Compound Treatment

[0118] 8-CPT-cAMP, Na (cat# 116812), 5-Iodotubercidin (cat# 407900), Prostaglandin E₂ (PGE₂, cat# 538904), SB 202190 (cat# 559388) and U0126 (cat# 662005) were purchased from Calbiochem; N₆,2'-O-Dibutyryl-cAMP (cat# D0260-5MG), forskolin (cat# F6886),
15 ascorbic acid 2-phosphate (cat# 49752-10G), β-glycerophosphate (G-6251), dexamethasone (D8893), 3-isobutyl-1-methylxanthine (IBMX, I5879) and Insulin (I 9278) were purchased from Sigma/Aldrich/Fluka.

RNA preparation and RT-PCR

[0119] Total RNA from each sample was prepared from around 5 x 10⁴ cells using
20 RNAeasy mini kit from Qiagen and further treated with Turbo DNA-Free (cat# 1907, Ambion) to prevent DNA contamination. Reverse transcription was carried out using SuperScript First-Strand Synthesis System for RT-PCR (cat# 11904-018, Invitrogen) or Qiagen OneStep RT-PCR kit (cat# 210210) as instructed. PCR primers used were as follows:

GAPDH: Forward 5'-GAA GGT GAA GGT CGG AGT C-3' (SEQ ID NO:5); Reverse 5'-
25 GAA GAT GGT GAT GGG ATT TC-3' (SEQ ID NO:6).

ALPL: Forward 5'-TGG AGC TTC AGA AGC TCA ACA CCA-3' (SEQ ID NO:7); Reverse 5'-ATC TCG TTG TCT GAG TAC CAG TCC-3' (SEQ ID NO:8).

RUNX2: Forward 5'-TCT TCA CAA ATC CTC CCC-3' (SEQ ID NO:9); Reverse 5'-TGG ATT AAA AGG ACT TGG TG-3' (SEQ ID NO:10).

OSX: Forward 5'-CCT ATG TAC CAG GAG TAA TGA ATA G-3' (SEQ ID NO:11);
Reverse 5'-CTC CTA GCT CTT TAA GTT CTT TCT C-3' (SEQ ID NO:12).

DLX5: Forward 5'-GAG AAG GTT TCA GAA GAC TCA GTA-3' (SEQ ID NO:13);
Reverse 5'-CTA GAA CAG CAA AAC ACA GTA GTC-3' (SEQ ID NO:14).

5 BSP: Forward 5'-GAG AAT ACC ACA CTT TCT GCT AC-3' (SEQ ID NO:15); Reverse 5'-
AAG TAG CTG TAC TCA TCT TCA TAG G-3' (SEQ ID NO:16).

MJD: Forward 5'-AGC ACA ACT AAA AGA GCA AAG AGT C-3' (SEQ ID NO:17);
Reverse 5'-CTC ATA GCA TCA CCT AGA TCA CTC C-3' (SEQ ID NO:18).

10 BIRC4: Forward 5'-GTT TCA GCA TCA ACA CTG GC-3' (SEQ ID NO:19); Reverse 5'-
TCC GTG CTT CAT AAT CTG CC-3' (SEQ ID NO:20).

P2RY11: Forward 5'-GTG TCC ACC CTC TAC TCT ACA T-3' (SEQ ID NO:21); Reverse
5'-CTC CAC TCT CTC TAC TTG GTT CT-3' (SEQ ID NO:22).

SLC12A2: Forward 5'-GGT GTC TAT CTC TTG ACC TTG T-3' (SEQ ID NO:23); Reverse
5'-GAC CTG GTG TCT AGT GTT AAG TG-3' (SEQ ID NO:24).

15 TBX3: Forward 5'-ATA ACT GAG ATT GCT GTG GG-3' (SEQ ID NO:25); Reverse 5'-
AGA GAG GGG GAA AAA TAC AG-3' (SEQ ID NO:26).

BCL2I2: Forward 5'-GCT GAG GCA GAA GGG TTA TG-3' (SEQ ID NO:27); Reverse 5'-
ATA GAG CTG TGA ACT CCG CC-3' (SEQ ID NO:28).

20 KCNT1: Forward 5'-TTC TGG AAG TTA GAA GCA GC-3' (SEQ ID NO:29); Reverse 5'-
ACC GTA CAA ACC AGT AAG GA-3' (SEQ ID NO:30).

ADK: Forward 5'-CCA GAG TCA GTA TTA AAG GTG G-3' (SEQ ID NO:31); Reverse 5'-
GAG ACC AGT TGA GAC AGA AAA CAD CY-3' (SEQ ID NO:32).

G_sα: Forward 5'-ATC TCT GTG ATC CTG TTC CTC-3' (SEQ ID NO:33); Reverse 5'-GTG
AAA TGA GGG TAG CAG TAG T-3' (SEQ ID NO:34).

25 DUSP6: Forward 5'-TAG ATA CAG GCA GTA GGT TTG C-3' (SEQ ID NO:35); Reverse
5'-CTC TCT TTG GCT CCT CTA TAT G-3' (SEQ ID NO:36).

GBDR1: Forward 5'-GAG AGA CTT CCA GAC AGA ACT C-3' (SEQ ID NO:37); Reverse
5'-CAT CTA TCA CCT CTT TCT CGT C-3' (SEQ ID NO:38).

PLZF: Forward 5'-TCT CAA ACG CCA CCT GCG CTC ACA T-3' (SEQ ID NO:39);
Reverse 5'-CAC TGG CAG GGC GAG GCG CCG TTG T-3' (SEQ ID NO:40).

Western Blot

[0120] Cell lyses buffer contains 20mM Tris pH8.0, 1mM EDTA, 150mM NaCl and 0.5% NP-40, and before usage, protease inhibitor cocktail (Cat# p8340, Sigma, 1:100 dilution) and phosphatase inhibitor cocktail 2 (Cat# p5726, sigma, 1:100 dilution) were added. Proteins (60µg/well) were separated in 12% Novex Tris -Glycine gel (Cat# EC6008 box, Invitrogen) and transferred to Nitrocellulose membrane (Cat# LC2001, Invitrogen) using the XCell II blot module system (Cat# EI9051, Invitrogen). The membrane was blocked in 5% milk/PBST for 1 hour at room temperature, incubated over night at 4 degree with primary antibody, washed 3 times with PBST (0.05% Tween 20 in PBS), followed by one hour incubation with secondary antibody in 5% milk/PBST and 3 times wash with PBST at room temperature. For antibody against pCREB however, PBST was substituted with PBS in all steps except for the last one. Antibody-bound proteins were detected utilizing ECL Western blotting detection reagent (Amersham Biosciences).

[0121] Anti-phospho-CREB was purchased from Upstate Biotechnology (cat# 06-519); Anti-phospho-p38 was purchased from Cell signaling technology (cat# 9910).

Results

[0122] After testing of various commercially available lipofection reagents, we found that Xtreme siRNA transfection reagent from Roche was the most effective in hMSCs, providing over 90% transfection efficiency and minimum cellular toxicity (Figure 1). This highly effective siRNA transfection method was then implemented into a high throughput screen that was based on an enzymatic assay of alkaline phosphatase (ALP), an early marker for osteogenic differentiation (Rodan, G. A. et al., *Cell* 89:677-680 (1997)). Fifty five hits that gave rise to a significant increase of ALP activity on day 7 after siRNA transfection in hMSCs were identified and confirmed (Figure 2a & Table 1).

TABLE I

Target ID	Symbol	5pDNA	siRNA sequence	3pDNA	SEQ ID NO: (siRNA, siRNA/DNA)	Definition
NM_000516	GNAS	AA	UCGAAGAUGAGGACUACU	TT	41, 42	GNAS complex locus
NM_001115	ADCY8	CA	GGAGAUCAACAAGCAUUC	TT	43, 44	adenylate cyclase 8
NM_001123	ADK	CA	GCCACACAAAGCAGCAACA	TT	45, 46	adenosine kinase
NM_002566	P2RY11	CA	UGC GGUGUCUACAACGUGGA	TG	47, 48	purinergic receptor P2Y, G-protein coupled, 11
NM_030959	OR12D3	AA	UUGGCCUGUAGUGACACAU	TA	49, 50	olfactory receptor, family 12, subfamily D, member 3
NM_001005170	OR52I2	TA	UGGCAUGAGGACCAAAACAA	TT	51, 52	Olfactory receptor
NM_001005238	OR51G2	CA	UCCCGGGCAACUGCACA AU	TC	53, 54	Olfactory receptor
XM_291440.1	OR2M4	CA	UGAGACCAGCUUCUAAACA	TA	55, 56	olfactory receptor
NM_001005221	OR4F29	TA	CUGGCCAGUCUCUCCUUC	TT	57, 58	Olfactory receptor
NM_002209	ITGAL	CA	GACCUGCAGGAUGACACAU	TT	59, 60	integrin, alpha L
NM_002203	ITGA2	GC	UGCUGGUGUUAGCGCUCAG	TC	61, 62	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
NM_002203	ITGA2	TG	CUGCUGGUGUUAGCGCUCA	GT	63, 64	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
NM_021146	CDT6	CA	GCACCAAGGACAAGGACAA	TG	65, 66	angiopoietin-like factor
NM_012098	ANGPTL2	AA	UGCGGGUGACUCCUUUACA	TG	67, 68	angiopoietin-like 2
XM_029962.2	KCNT1	CA	UGCGAGCCAAAGAUGGACAA	TG	69, 70	potassium channel, subfamily T, member 1

Target ID	Symbol	5pDNA	siRNA sequence	3pDNA	SEQ ID NO: (siRNA, siRNA/DNA)	Definition
NM_001046	SLC12A2	AA	CCUCUUCGUGGCUACAUCU	TA	71, 72	solute carrier family 12 (sodium/potassium/chloride transporters), member 2 mitochondrial carrier family protein
NM_018843	MCFP	AA	UGGACUCAUGGAUCAUCUA	TG	73, 74	B-cell CLL/lymphoma 2
NM_000633	BCL2	CA	UGUGUGUGGAGAGCGUCA	CC	75, 76	BCL2-like 2 nuclear receptor subfamily 2, group E, member 3 nuclear receptor subfamily 2, group E, member 3 putative leukocyte platelet- activating factor receptor chemokine (C-C motif) ligand 5 dual specificity phosphatase 6
NM_004050	BCL2L2	CA	CCCAGGUCUCCGAUGAACU	TT	77, 78	serine/threonine kinase 10
NM_016346	NR2E3	CA	GCAGCAGCGGGAAGCACUA	TG	79, 80	putative dipeptidase
NM_016346	NR2E3	CA	GAGGAUGCUGAUGAGAAUA	TT	81, 82	phospholipase A2, group IID
NM_022571	HUMNP2	CA	CGCUCAGCGUGGCGCUCUAU	CT	83, 84	Cytochrome P450
NM_002985	CCL5	AA	UGGGUUCGGGAGUACAUA	AC	85, 86	aldo-keto reductase family 1, member C2
NM_001946	DUSP6	AA	CUGUGGUGUCUUGGUACA	TG	87, 88	member C2
NM_005990	STK10	TA	GAGCACGAAACCCAGAAAC	TG	89, 90	methoxymethyl Coenzyme A
NM_022355	LOC64174	CA	UCGGGAUUGGUGGAGAUUA	TG	91, 92	mutase
NM_012400	PLA2G2D	TA	CCAGAAAGCGACUGCGUUUC	TA	93, 94	dopamine beta-hydroxylase
NM_178134	CYP4Z1	CA	UCCCUAUGCCUUAUACCA	TT	95, 96	(dopamine beta- monooxygenase)
NM_001354	AKR1C2	CA	AGCCAGGGCUCUAGUACAA	GC	97, 98	
NM_000255	MUT	TA	GCUGAGGGAAUACCUAAAC	TT	99, 100	
NM_000787	DBH	GA	CCACGUACUGGUGCUACA	TA	101, 102	

Target ID	Symbol	5pDNA	siRNA sequence	3pDNA	SEQ ID NO: (siRNA, siRNA/DNA)	Definition
NM_012253	TKTL1	TA	UCCGUGUCAUCGACCUGUU	TA	103, 104	transketolase-like 1
NM_000137	FAH	AA	CUUCGGAAAGUGUGCAUUA	TC	105, 106	fumarylacetoacetate hydrolase (fumarylacetoacetase)
NM_006502	POLH	TA	UGCCAGAAACACAUGGACUA	TC	107, 108	polymerase (DNA directed), eta
NM_078487	CDKN2B	AA	GGUGCGACAGCUCCUGGAA	GC	109, 110	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
NM_003671	CDC14B	CA	GAGCAGCCUUCUCCAAACU	TC	111, 112	CDC14 cell division cycle 14 homolog B (S. cerevisiae)
NM_004993	MJD	AA	CAGAUGCAUCGACCAAAC	TT	113, 114	Machado-Joseph disease (ataxin 3)
NM_001167	BIRC4	CA	UGUGCUACACAGUCAUUAC	TT	115, 116	baculoviral IAP repeat- containing 4
NM_004403	DFNA5	AA	AGUCUCCCCACUGCUUUCU	TG	117, 118	deafness, autosomal dominant 5
NM_002636	PHF1	CA	UGCUGGGUAUGAAGCUUUC	TC	119, 120	PHD finger protein 1
NM_005996	TBX3	AA	UGCCAAAGAGGAUGUACAU	TC	121, 122	T-box 3 (ulnar mammary syndrome)
NM_014819	PJA2	AA	CAGGUAGUGAGGCCAAAAG	TT	123, 124	praja 2, RING-H2 motif containing
AF447582 <i>See also,</i> NM_001025105 and NM_001892	HLCDGP1	TA	UGCUAGCAUCAUGCACAUC	TT	125, 126	down-regulated in lung cancer (PRO2975, casein kinase 1, alpha 1 (CSNK1A1))
NM_016172	GBDR1	AA	CCCGAAAACAUUGCUAGCA	TT	127, 128	putative glioblastoma cell differentiation-related

Target ID	Symbol	5pDNA	siRNA sequence	3pDNA	SEQ ID NO: (siRNA, siRNA/DNA)	Definition
XM_292126.2	LOC34154 ₉	CA	GAGCAGAUGGCCUGGAGAU	TC	129, 130	similar to semaphorin cytoplasmic domain-associated protein 3B
NM_080873	ASB11	CA	UGGAGAUCCUGCUGGCAAA	TA	131, 132	ankyrin repeat and SOCS box- containing 11
XM_497244	IFIT-1	AA	AUCCAAAAGAUGCACACAU	TA	133, 134	similar to Interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1)
NM_000095	COMP	CA	CGGUCACGGGAUGACGACUA	TG	135, 136	cartilage oligomeric matrix protein (pseudoachondroplasia)
NM_178833	LOC13330 ₈	AA	CCAACAGAAAGGAAGUAUUC	TT	137, 138	hypothetical protein BC009732
NM_153361	MGC4210 ₅	AA	CGGAUAGGCUUCUACCGAA	TT	139, 140	hypothetical protein MGC42105
NM_032921	MGC1587 ₅	AA	UGUGCUUCAGCCUGGACAA	TG	141, 142	hypothetical protein MGC15875
NM_152267	FLJ38628	CA	GCACUUUCGAGUGCAACAU	CT	143, 144	hypothetical protein FLJ38628
BF591642	IMAGE: 3568335	CA	UGGGCUCACUCUCGCAACCA	GG	145, 146	clone IMAGE:3568335 3'
gi:11683966 <i>See also,</i> AK096500 and DA771261.						<i>See also,</i> cDNA FLJ39181 fls, clone OCBBF2004235.
XM_054936.7	LOC11497 ₁	TA	CCUGAUUCAGGUGCACAAA	TG	147, 148	hypothetical protein LOC114971
XM_045308.6	DKFZP727 G051	AA	GCACUUGACAGGCCACACAU	TT	149, 150	DKFZP727G051 protein

[0123] Among the primary siRNA hits, the corresponding genes encode proteases, kinases, ion channels, protein receptors, ligands, transcription factors, extracellular matrix proteins and hypothetical proteins etc., some of which are members of the same gene family (integrin family, angiopoietin family, adenylate cyclase family and olfactory receptor family) (Table 1). While the majority of the identified genes have not been implicated in bone development, two genes, TBX3 (T-box 3) and GNAS, have been found to cause skeletal abnormalities when mutated in mouse and human, respectively (Bianco, P. et al., *J Bone Miner Res* **15**:120-128 (2000); Davenport, T. G. et al., *Development* **130**:2263-2273 (2003); Eddy, M. C. et al., *J Bone Miner Res* **15**:2074-2083 (2000); Shore, E. M. et al., *N Engl J Med* **346**:99-106 (2002); Weinstein, L. S. et al., *N Engl J Med* **325**:1688-1695 (1991)). To verify the screen, we picked twelve targeted genes (Figure 3), including GNAS (Human GNAS complex locus, transcript variant 2, isoform b of the alpha subunit of Gs, NM_080426), ADCY8 (adenylate cyclase 8, NM_001115), ADK (adenosine kinase, NM_001123), P2RY11 (purinergic receptor P2R, G-protein coupled, 11, NM_002566), TBX3 (T-box 3 or ulnar mammary syndrome, NM_005996), BIRC4 (baculoviral IAP repeat-containing 4, NM_001167), BCL2L2 (BCL2-like 2, NM_004050), SLC12A2 (solute carrier family 12, member 2, NM001046), KCNT1 (potassium channel, subfamily T, member 1, XM_029962.2), GBDR1 (putative glial blastoma cell differentiation-related, NM_016172), DUSP6 (dual specificity phosphatase 6, NM_001946) and MJD (Machado-Joseph disease or ataxin 3, NM_004993), to further characterize their function in osteogenic differentiation of hMSCs.

[0124] To confirm that the induced ALP activity was derived from the bone-specific isozyme ALPL (Weiss, M. J. et al., *Proc Natl Acad Sci U S A* **83**:7182-7186 (1986)), RT-PCR analysis using the ALPL specific primers was carried out on hMSC samples collected on day 4 after siRNA transfection. As shown in Figure 2b, similar to samples treated with the optimal osteogenic inducing media (OS, a mix of 0.05 mM Ascorbic acid 2-phosphate, 10 mM glycerophosphate and 0.1 μ M Dexamethasone in cell culture media) (Pittenger, M. F. et al., *Science* **284**:143-147 (1999)), samples transfected with the hit siRNAs, except for siTBX3 and siMJD, generated increased ALPL transcripts as compared to the control samples transfected with nonspecific siRNAs (siCon). To further confirm the osteogenic identity of the transfected hMSCs, we also examined the expression of several additional early (CBFA1, DLX5, Osterix/OSX) and one late (Bone-specific Sialoprotein or BSP) stage osteogenic markers (Acampora, D. et al., *Development* **126**:3795-3809 (1999); Ducy, P. et

al., *Cell* **89**:747-754 (1997); Nakashima, K. et al., *Cell* **108**:17-29 (2002); Wang, D. et al., *J Bone Miner Res* **14**:893-903 (1999)). Except for DLX5, which appeared unchanged among all tested samples, CBFA1, OSX and BSP were differentially up regulated in the hit siRNA and OS treated samples as compared to the siCon treated sample (Figure 2b), suggesting that the osteogenic specification of hMSCs induced by the different hit siRNAs has progressed to different stages. CBFA1/RUNX2, a master transcription factor required for bone cell fate determination and maturation in mouse (Ducy, P. et al., *Cell* **89**:747-754 (1997)), is normally expressed in hMSCs (Figure 2b).

[0125] To examine whether the hit siRNA induced osteogenic cell fate commitment requires the function of CBFA1, each hit siRNA was co-transfected with either the CBFA1-specific siRNA or the siCon in hMSCs, and ALP activity was examined. While the co-treatment with the siCon did not cause any noticeable change in ALP activity as compared to the single hit siRNA treatment, the co-treatment with the CBFA1 siRNA reduced the level of ALP activity induced by the hit siRNAs or osteogenic inducing media (OS) (Figure 2c), suggesting that the hit siRNA induced osteogenic cell fate commitment in hMSCs also requires the function of CBFA1. To confirm that the induced ALPL expression was not caused by off-target effect from the transfected hit siRNAs, RT-PCR was performed on corresponding siRNA targeted genes with the RNA samples prepared at 36 hours after siRNA transfection. As compared to the control samples, the reduced transcript level of the targeted gene in the corresponding hit siRNA transfected hMSCs affirmed the specificity (Figure 2d). In addition, the knockdown of the targeted genes was also observed at 72 hours after siRNA transfection.

[0126] Bone cell maturation is accompanied by extracellular matrix (ECM) mineralization (John Bilezikian et al., *Principles of bone biology*, Vol 1, 2nd edn: Academic Press) (2002)).

To examine whether the osteogenic differentiation of hMSCs induced by the hit siRNAs can further proceed to the maturation stage, hit siRNA treated cells were cultured for 20 days. No ECM mineralization was detected based on alizarin red staining that detects calcium phosphate deposits, a major component of mineralized ECM, indicating that the expression knockdown by the hit siRNAs primarily functions to induce early osteogenic specification of hMSCs and the late stage osteoblast maturation may require additional factors. Since OS treated hMSCs undergo osteoblast maturation within two weeks, we tested whether sequential treatments by the hit siRNAs (initial 2-4 days) followed by the OS (additional 5-9 days) could facilitate this process. As demonstrated in Figure 4, such sequential treatments of

hMSCs with siADK and OS media clearly enhanced the intensity of alizarin red staining as compared to the control treatment. When the OS treatment was started three days after siRNA transfection and continued for 9 days, among the 12 hits tested, six (siADK, siGNAS, siP2RY11, siGBDR1, siSLC12a2 and siKCNT1) dramatically enhanced the intensity of alizarin red staining, two (siDUSP6 and siBCL2L2) provided modest enhancement, two (siBIRC4 and siADCY8) provided weak enhancement, and two (siTBX3 and siMJD) had no significant effect (Figure 5a). These observations are consistent with the conclusion that the targeted genes of the ten effective hit siRNAs not only play inhibitory roles in osteogenic specification of hMSCs, but also in bone cell maturation. Combinatorial treatment of two siRNA hits, such as siSLC12A2 with siKCNT1, further enhances the osteogenic differentiation process (Figure 6). It has been suggested that the osteogenic differentiation and adipogenic differentiation of hMSCs are two inverse processes, with one process inhibiting the other (Beresford, J. N. et al., *J Cell Sci* **102** (Pt 2):341-351 (1992)). Furthermore, in osteoporotic patients, the number of adipocytes was increased in the marrow, implying a change of cell fate disposition from osteoblast lineage to adipocyte lineage (Justesen, J. et al., *Biogerontology* **2**:165-171 (2001); Meunier, P. et al., *Clin Orthop Relat Res* **80**:147-154 (1971)). We therefore examined the effect of our hit siRNAs on adipogenic differentiation of hMSCs by combined treatment with an adipogenic inducing cocktail (100 µg/ml IBMX, 1 µM DEX and 10 µg/ml insulin). Consistent with previous studies, our hit siRNAs inhibited adipogenic differentiation of hMSCs at various degrees, except for siGNAS (Figure 5b).

[0127] GNAS was identified in our screen as an osteogenic cell fate suppressor in hMSCs. The major GNAS gene product, G protein α -subunit ($G\alpha$), couples transmembrane receptors to adenylyl cyclase and is required for the receptor-stimulated intracellular cAMP production. Inactivating mutations in this gene have been found in two types of disorders (Progressive osseous hetroplasia and Albright's hereditary osteodystrophy) that are characterized with heterotopic ossification (Chan, I. et al., *Clin Exp Dermatol* **29**:77-80 (2004); Eddy, M. C. et al., *J Bone Miner Res* **15**:2074-2083 (2000); Shore, E. M. et al., *N Engl J Med* **346**:99-106 (2002)), whereas activating mutations in this gene were found in patients with McCune-Albright syndrome that is characterized with fibrous dysplasia of bone (Weinstein, L. S. et al., *N Engl J Med* **325**:1688-1695 (1991)), suggesting that $G\alpha$ is a critical negative regulator of osteogenic commitment. This is consistent with our observation as well as others' that reduced GNAS expression switches on osteogenic cell fate in hMSCs (Lietman, S. A. et al.,

Clin Orthop Relat Res, 231-238 (2005)). Coincidentally, our screen identified not only Gsa but also several proteins that are closely involved in cAMP production, which include ADCY8 (adenylate cyclase 8), ADK (adenosine kinase) and P2RY11 (purinergic receptor P2Y, G-protein coupled, 11), suggesting a close linkage between intracellular cAMP signaling and osteogenic differentiation in hMSCs.

[0128] Since the targeted genes are all positively involved in intracellular cAMP signaling, we tested the effect of two compounds of opposing function on cAMP production on osteogenic differentiation of hMSCs, ADK inhibitor 5-iodotubercidin and ADCY activator forskolin (Cottam, H. B. et al., *J Med Chem* 36:3424-3430 (1993); de Souza, N. J. et al., *Med Res Rev* 3:201-219 (1983)). Treatment with 5-iodotubercidin mimicked the effect of the hit siADK and significantly enhanced the osteogenic differentiation of hMSCs when combined with the OS treatment, while treatment with forskolin inhibited this process (Figure 7a). Furthermore, combined treatment of cell permeable cAMP analog such as 8-CPT-cAMP or N6,2'-dibutyryl-cAMP (DB-cAMP) with dexamethasone was sufficient to differentiate hMSCs into mature adipocytes. Moreover, 8-CPT-cAMP could switch cell fate commitment from osteogenic to adipogenic when combined with the OS media (Figure 7b & 7c), consistent with the previous observations that osteogenesis and adipogenesis are mutually exclusive of each other. ATP has been shown as a ligand for P2RY11 receptor (Communi et al., 1997). Treatment of ATP inhibited the OS-induced osteogenesis of hMSCs and slightly increases the number of cells undergoing adipogenic differentiation in a dosage dependent manner, consistent with the notion that P2RY11 acts as an osteogenic suppressor in hMSCs. The present observations are consistent with the conclusion that reducing intracellular cAMP level promotes osteogenic specification but inhibits adipogenic cell fate in hMSCs, and *vice versa*.

[0129] To find out whether CREB (cAMP Responding Element Binding protein), a common downstream effector of cAMP signaling pathway, is involved in mediating cAMP signaling controlled differentiation of hMSCs, we checked the expression level of the active form of CREB proteins in cells 3 days after siP2RY11, siADK, siGNAS, siSLC12a2 or siCon transfection. In these hit siRNAs or OS treated samples, the level of pCREB protein was increased compared to the siCon treated or untreated samples (Figure 8a), suggesting that CREB activity is required for osteogenic differentiation in hMSCs. To further examine the role of CREB in osteogenesis and adipogenesis, a synthetic 24-mer CRE decoy oligonucleotide that can compete with endogenous CRE enhancers for binding proteins was

transfected into hMSCs (Park, Y. G. et al., *J Biol Chem* 274:1573-1580 (1999)), which were then subjected to the treatment of osteogenic or adipogenic induction media. Compared to the 24-mer CRE mismatch control oligonucleotide, CRE decoy not only inhibited cells from undergoing adipogenic differentiation, but also osteogenic differentiation (Figure 8b), suggesting that CREB activity is required for both osteogenesis and adipogenesis and the inhibitory effect of cAMP signaling on osteogenic differentiation can be mediated by effectors other than CREB in hMSCs.

[0130] While the screen revealed multiple players involved in cAMP signaling pathway that controls osteogenic differentiation of hMSCs, combinatorial siRNA treatment study also suggested that different targeted genes employed different mechanisms to control the same osteogenic process, one of which is to turn on positive regulators of osteogenesis. Several proteins, including BMP-2, PLZF, and TAZ, have been shown to promote osteogenic differentiation in multipotent mesenchymal precursor cells (Hong, J. H. et al., *Science* 309:1074-1078 (2005); Ikeda, R. et al., *J Biol Chem* 280:8523-8530 (2005); Katagiri, T. et al., *J Cell Biol* 127:1755-1766 (1994)). To test their roles in our siRNA induced osteogenic differentiation, RT-PCR was performed on these genes. While the level of BMP-2 and TAZ expression was not significantly altered among the different samples, expression of PLZF was detected in the siMJD or OS treated samples, but not in the rest hit siRNA treated samples (Figure 9a), suggesting that MJD normally suppresses the expression of PLZF in hMSCs. In addition, MAPK signaling pathways have been shown activated in the OS treated hMSCs, and inhibition of these pathways suppresses OS induced osteogenesis (Figure 9b) (Jaiswal, R. K. et al., *J Biol Chem* 275:9645-9652 (2000)). DUSP6 is a dual specificity phosphatase that has been shown to directly dephosphorylate ERK1/2 or p38 kinase (Muda, M. et al., *J Biol Chem* 273:9323-9329 (1998)).

[0131] We therefore examined the activation status of ERK1/2 and p38 in the siDUSP6 treated hMSCs along with other siRNA treated samples by western blot using antibodies against the phosphorylated form of the protein at 72 hours after transfection (Figure 9c). The increased level of active p38 but not pERK1/2 in the siDUSP6 treated sample suggests that DUSP6 normally inhibits osteogenic differentiation of hMSCs at least partly by inhibiting the activation of p38 signaling pathway.

[0132] Our probing of the molecular mechanisms underlying the osteogenic induction effect of identified suppressors is consistent with the conclusion that different suppressors function through discrete pathways to suppress osteogenic differentiation in normal hMSCs.

5 [0133] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above can be used in various combinations. All publications, patents, patent applications, or other documents cited in this application are incorporated by
10 reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

WHAT IS CLAIMED IS:

- 1 1. A method for identifying agents that promote osteogenesis, the
2 methods comprising,
3 (a) contacting a plurality of agents to at least one of the polypeptides
4 selected from Table 1;
5 (b) measuring the activity of at least one of the polypeptides;
6 (c) selecting at least one of the plurality of agents, wherein the selected
7 agent(s) inhibit the activity of at least one of the polypeptides; and
8 (d) measuring the ability of the selected agent(s) to promote osteogenesis,
9 thereby identifying agents that promote osteogenesis.
- 1 2. The method of claim 1, wherein the polypeptides are expressed in a
2 host cell.
- 1 3. The method of claim 2, wherein the measuring step (b) comprises
2 measuring the expression of the at least one polypeptides.
- 1 4. The method of claim 3, wherein measuring the expression comprises
2 measuring the level of transcription.
- 1 5. The method of claim 2, wherein the cells are mammalian cells.
- 1 6. The method of claim 1, wherein the measuring step (d) is carried out
2 *in vitro*.
- 1 7. The method of claim 1, wherein the measuring step (d) is carried out
2 *in vivo*.
- 1 8. A method for promoting osteogenesis in a mammalian cell, the method
2 comprising contacting the cell with an siRNA that inhibits expression of at least one of the
3 gene products selected from Table 1.
- 1 9. The method of claim 8, wherein the cell is a stem cell.
- 1 10. The method of claim 9, wherein the stem cell is a mesenchymal stem
2 cell.

- 1 11. A method for promoting osteogenesis in a mammalian cell, the method
2 comprising contacting a cell with an agent that inhibits the activity of a polypeptide selected
3 from Table 1.
- 1 12. The method of claim 11, wherein the cell is a stem cell.
- 1 13. The method of claim 12, wherein the stem cell is a mesenchymal stem
2 cell.

Figure 1

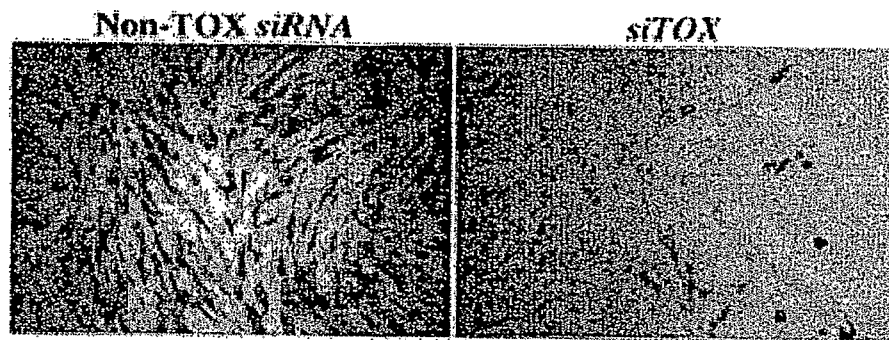


Figure 2

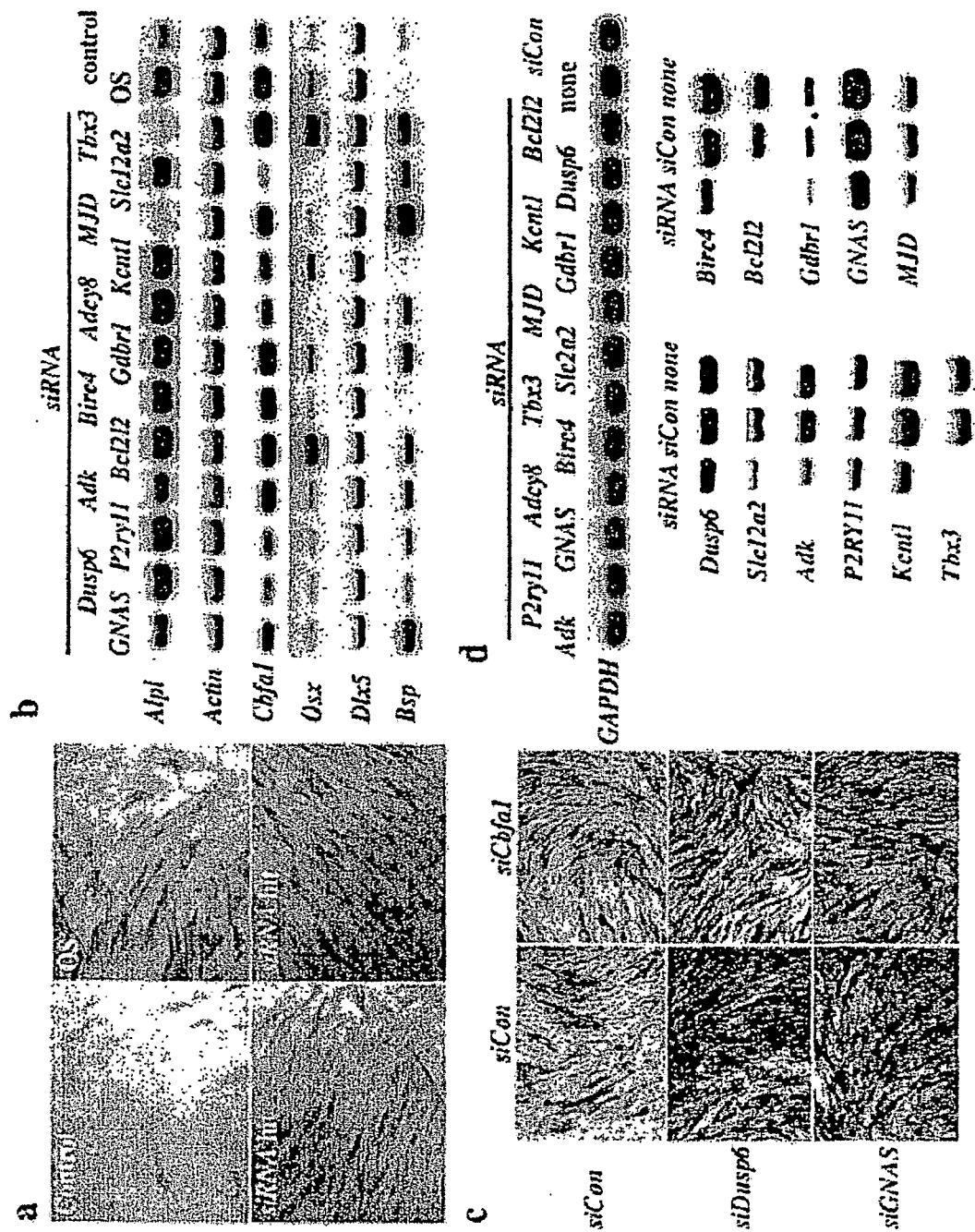


Figure 3

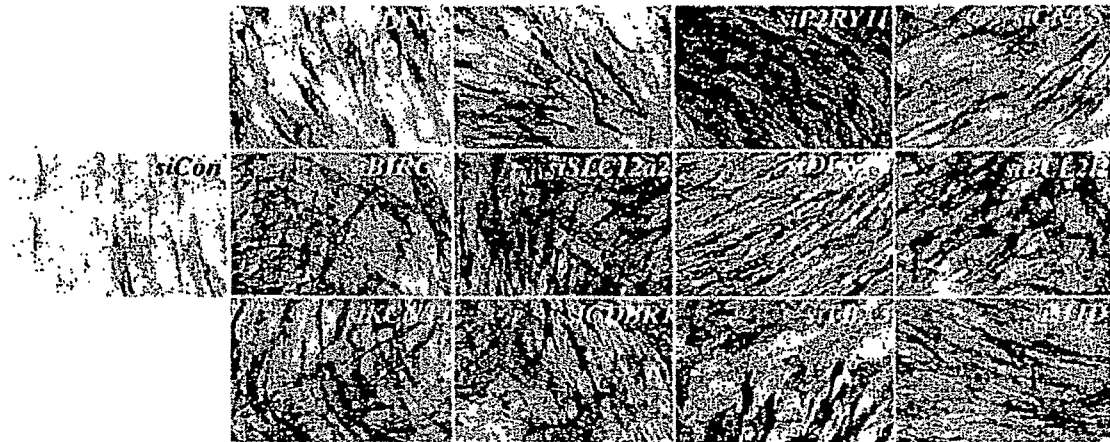


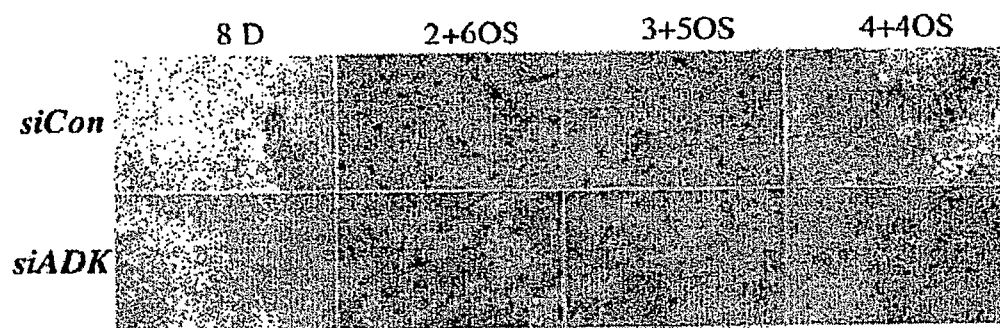
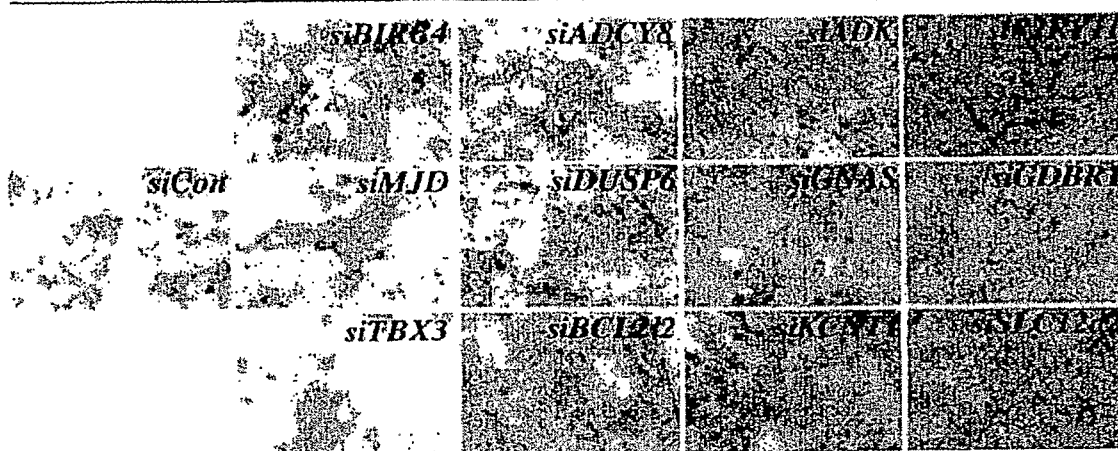
Figure 4

Figure 5

a.

3+9OS



b.

adipogenic inducing media

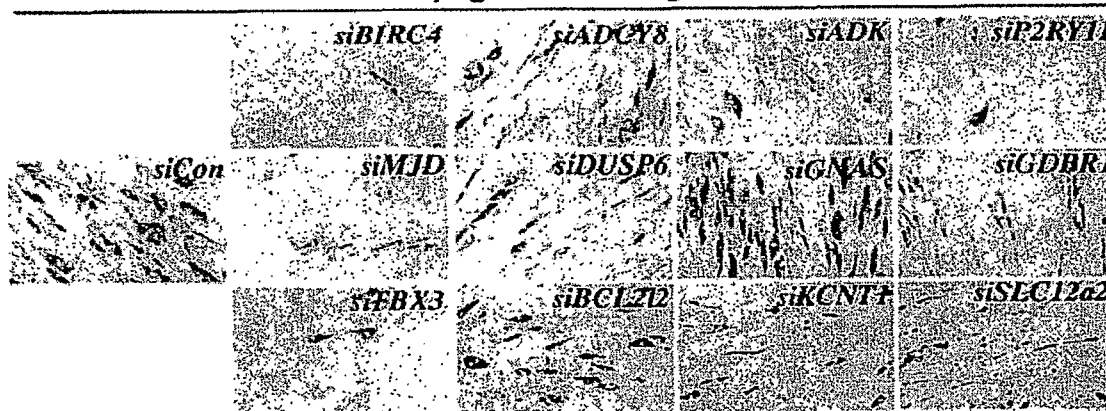


Figure 6

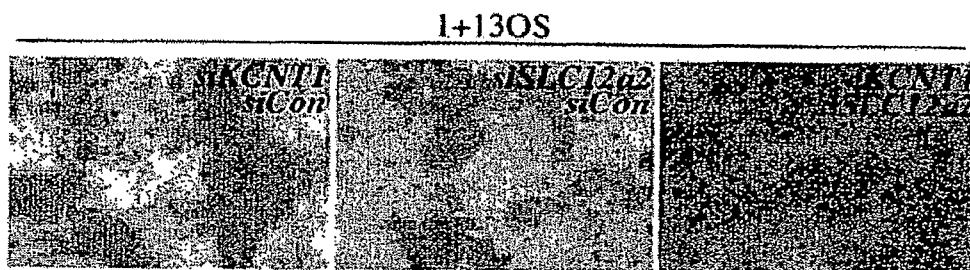


Figure 7

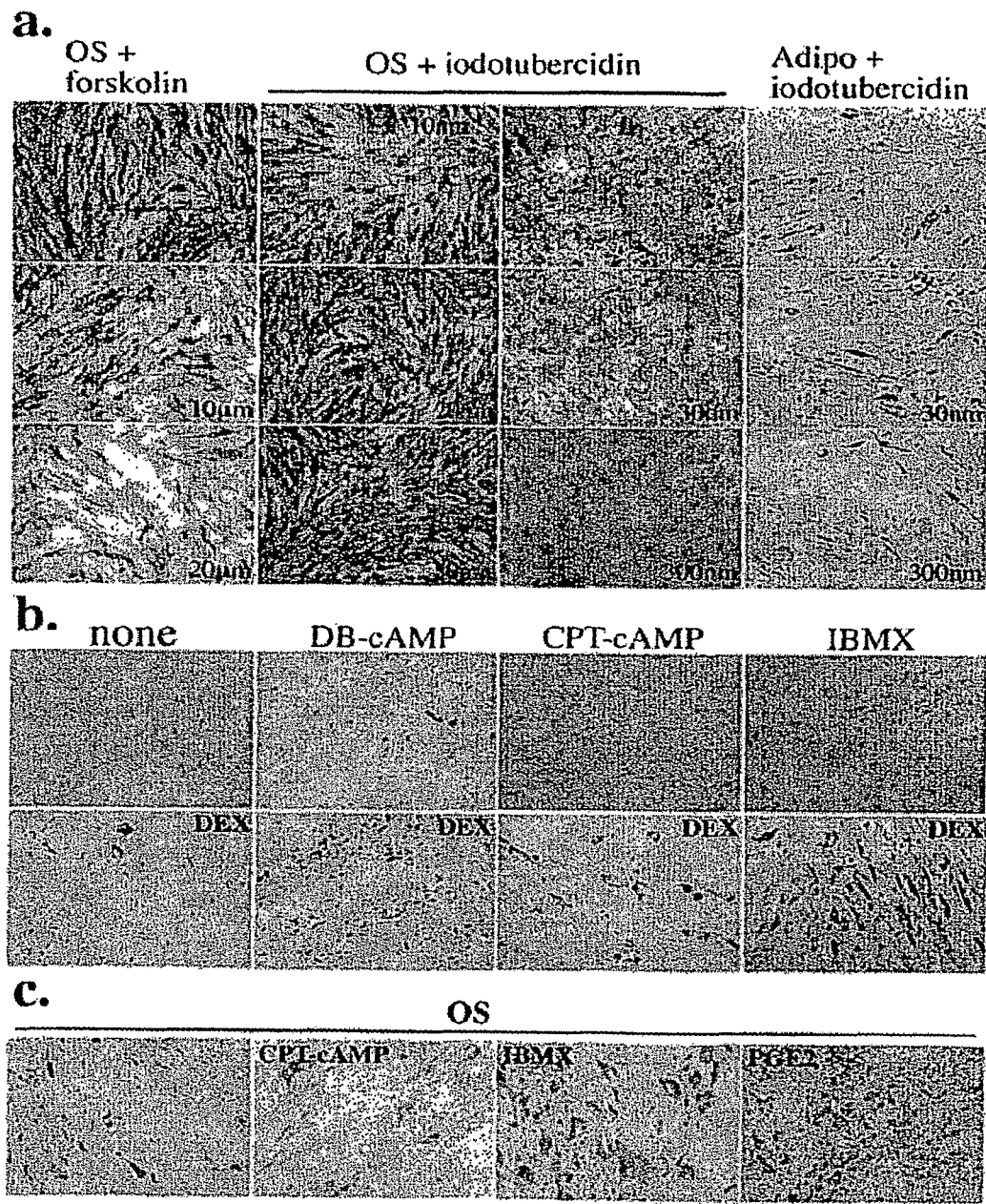


Figure 8

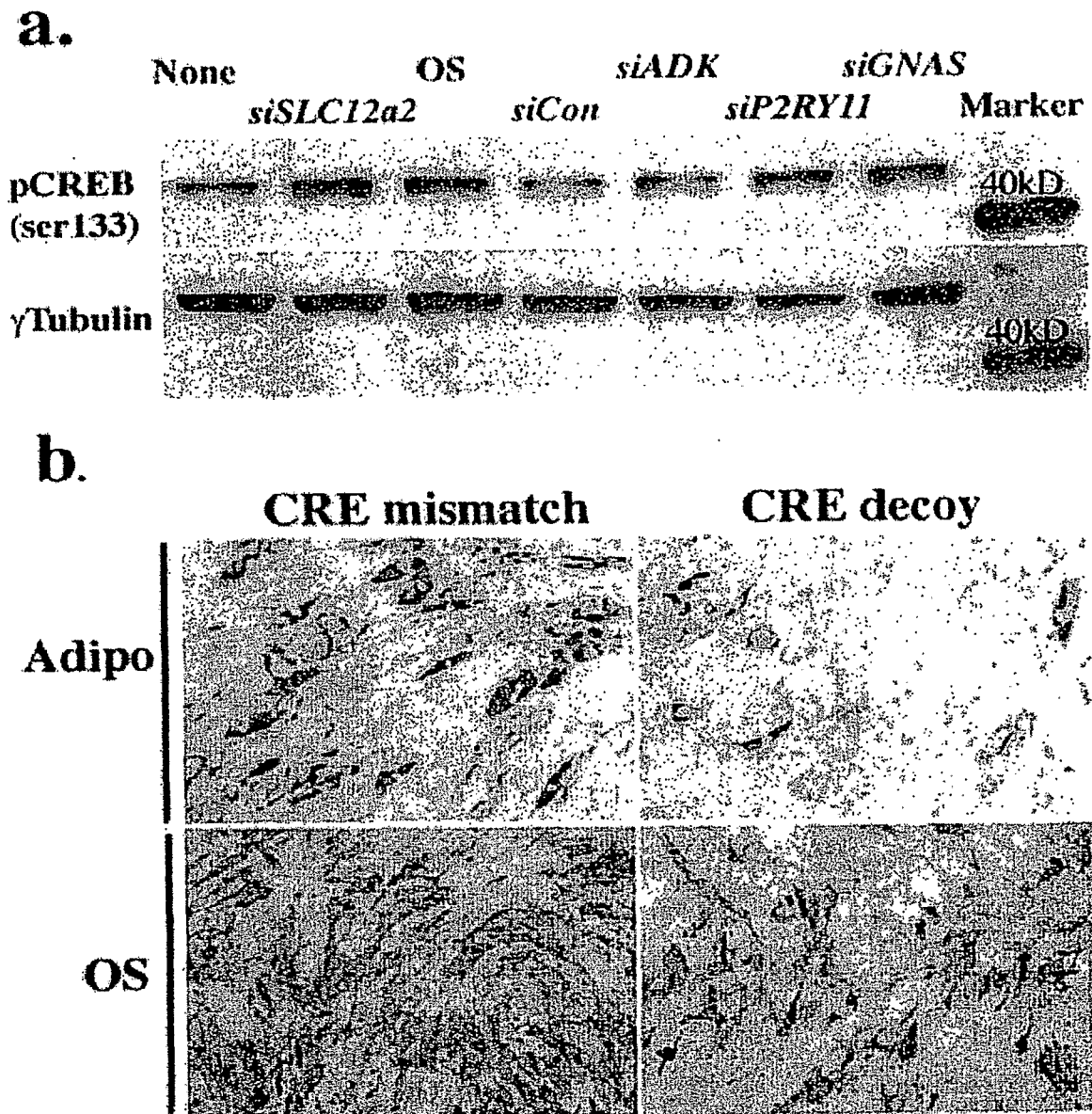


Figure 9

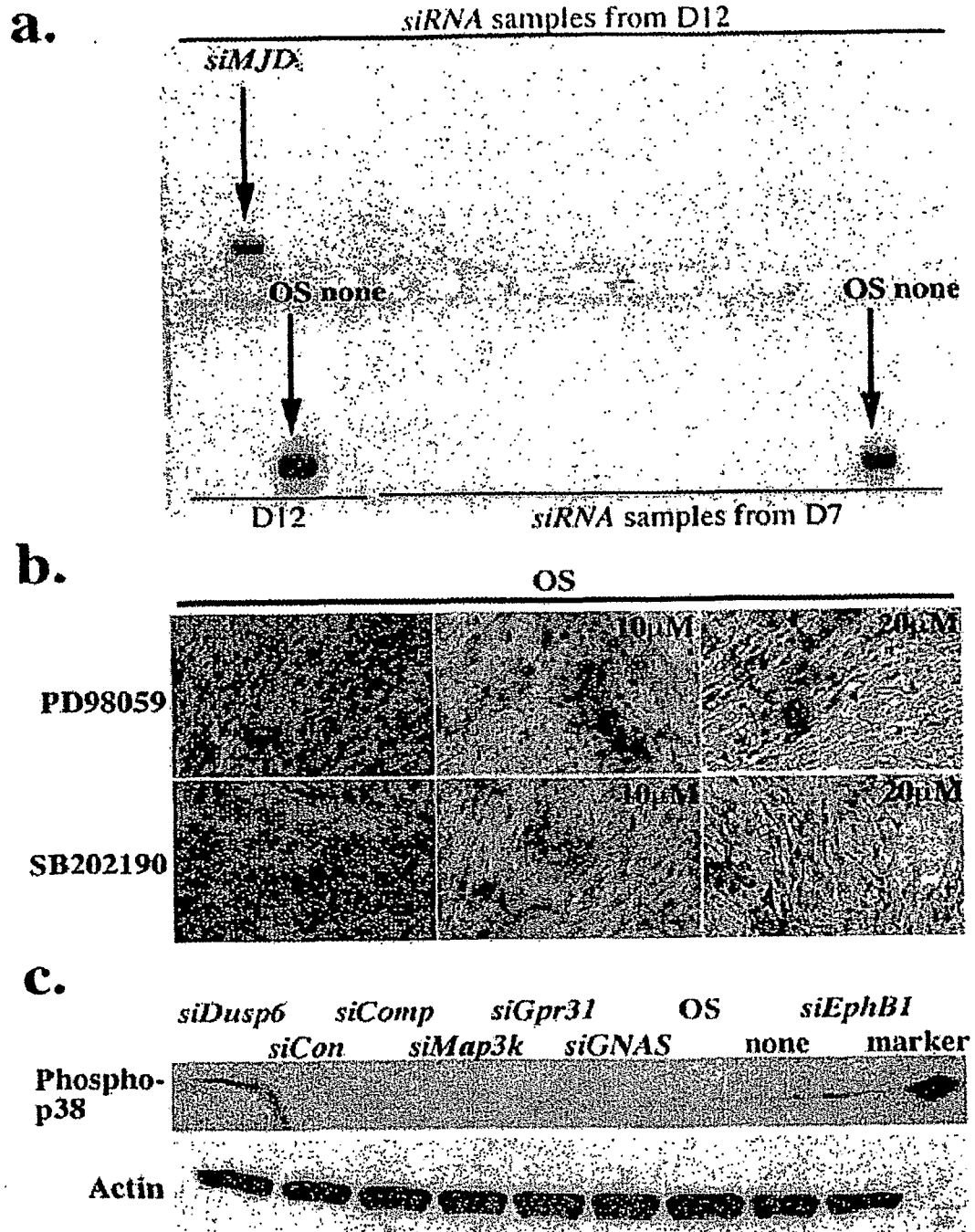


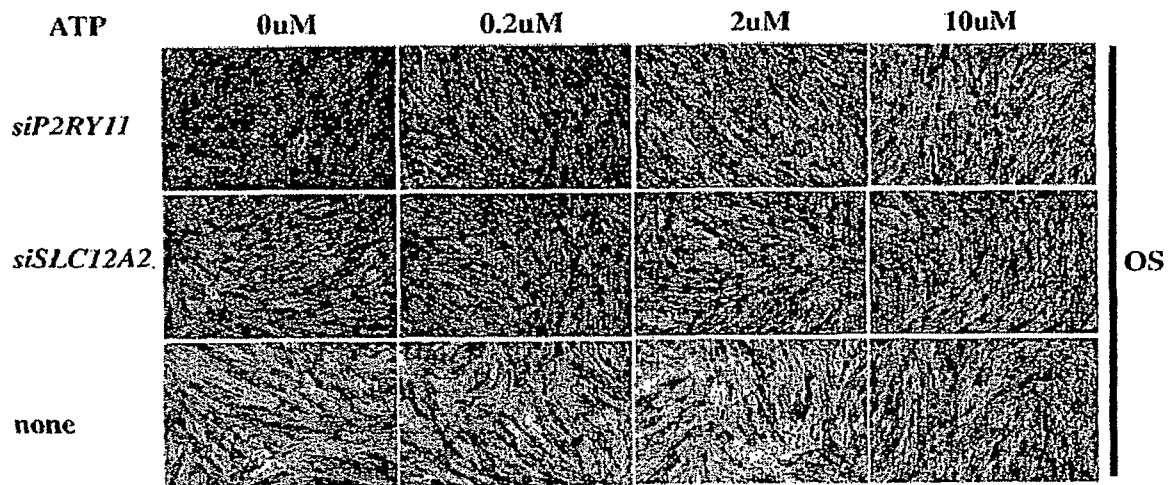
Figure 10

Figure 11

