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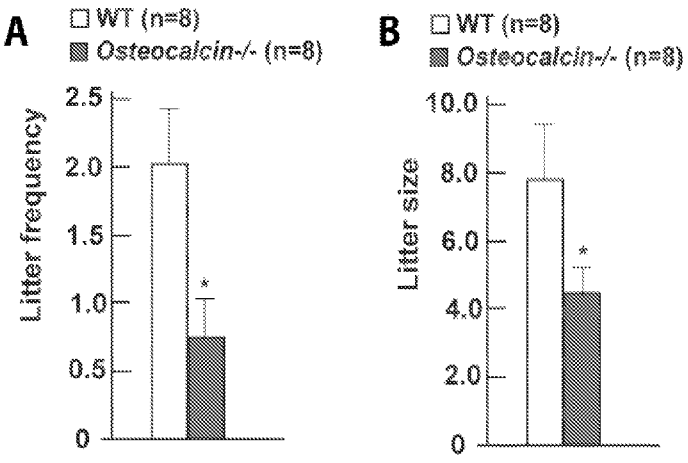
(54) Title: OSTEOCALCIN AS A TREATMENT FOR MALE REPRODUCTIVE DISORDERS

(57) Abstract: Methods and compositions for treating, preventing, or diagnosing disorders related to reproduction in male mammals, preferably humans, are provided. The methods generally involve modulation of the OST-PTP signaling pathway or the PTP-IB signaling pathway involving gamma-carboxylase and osteocalcin. Disorders amenable to treatment by the methods include, but are not limited to, male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes.

FIGURE 1



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OSTEOCALCIN AS A TREATMENT FOR MALE REPRODUCTIVE DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Provisional Patent Application Serial No. 61/296,339, filed January 19, 2010, and U.S. Provisional Patent Application Serial No. 61/296,415, filed January 19, 2010, the disclosures of which are incorporated herein by reference in their entirety.

[0002] This invention was made with Government support under Grant No. PHS 398/2590 (Rev. 09/04, Reissued 4/2006). The Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention is directed to methods and compositions for treating, preventing, and diagnosing disorders related to reproduction in male mammals. Such disorders include, but are not limited to, male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes. The present invention also provides methods of contraception for use in male mammals.

BACKGROUND OF THE INVENTION

[0004] Osteocalcin, one of the very few osteoblast-specific proteins, has several features of a hormone. For instance, it is synthesized as a pre-pro-molecule and is secreted in the general

circulation (Hauschka et al., 1989, Physiol. Review 69:990-1047; Price, 1989, Connect. Tissue Res. 21:51-57 (discussion 57-60)). Because of their exquisite cell-specific expression, the *osteocalcin* genes have been intensively studied to identify osteoblast-specific transcription factors and to define the molecular bases of bone physiology (Ducy et al., 2000, Science 289:1501-1504; Harada & Rodan, 2003, Nature 423:349-355).

[0005] Osteocalcin is the most abundant non-collagenous protein found associated with the mineralized bone matrix and it is currently being used as a biological marker for clinical assessment of bone turnover. Osteocalcin is a small (46-50 residue) bone specific protein that contains 3 gamma-carboxylated glutamic acid residues in its primary structure. The name osteocalcin (*osteo*, Greek for bone; *calc*, Latin for lime salts; *in*, protein) derives from the protein's ability to bind Ca^{2+} and its abundance in bone. Osteocalcin undergoes a peculiar post-translational modification whereby glutamic acid residues are carboxylated to form gamma-carboxyglutamic acid (Gla) residues; hence osteocalcin's other name, bone Gla protein (Hauschka et al., 1989, Physiol. Review 69:990-1047).

[0006] Mature human osteocalcin contains 49 amino acids with a predicted molecular mass of 5,800 kDa (Poser et al., 1980, J. Biol. Chem. 255:8685-8691). Osteocalcin is synthesized primarily by osteoblasts and ondontoblasts and comprises 15 to 20% of the non-collagenous protein of bone. Poser et al., 1980, J. Biol. Chem. 255:8685-8691 showed that mature osteocalcin contains three carboxyglutamic acid residues which are formed by post-translational vitamin K-dependent modification of glutamic acid residues. The carboxylated Gla residues are

at positions 17, 21 and 24 of human mature osteocalcin. Some human osteocalcin has been shown to contain only 2 Gla residues (Poser & Price, 1979, J. Biol. Chem. 254:431-436).

[0007] Osteocalcin has several features of a hormone. Ducy et al.,1996, Nature 382:448-452 demonstrated that mineralized bone from aging osteocalcin-deficient mice was two times thicker than that of wild-type. It was shown that the absence of osteocalcin led to an increase in bone formation without impairing bone resorption and did not affect mineralization. Multiple immunoreactive forms of human osteocalcin have been discovered in circulation (Garnero et al., 1994, J. Bone Miner. Res. 9:255-264) and also in urine (Taylor et al., 1990, J. Clin. Endocrin. Metab. 70:467-472). Fragments of human osteocalcin can be produced either during osteoclastic degradation of bone matrix or as the result of the catabolic breakdown of the circulating protein after synthesis by osteoblasts.

[0008] OST-PTP is the protein encoded by the Esp gene. The Esp gene was originally named for embryonic stem (ES) cell phosphatase and it has also been called the Ptprv gene in mice. (Lee et al, 1996, Mech. Dev. 59:153-164). Because of its bone and testicular localization, the gene product of Esp is often referred to as osteoblast testicular protein tyrosine phosphatase (OST-PTP). OST-PTP is a large, 1711 amino-acid long protein that includes three distinct domains. OST-PTP has a 1068 amino-acid long extracellular domain containing multiple fibronectin type III repeats.

[0009] Esp expression is restricted to ES cells, the gonads and the skeleton. In the gonads, Esp is specifically expressed in Sertoli cells of the testis and coelomic epithelial cells of the ovaries. During development, Esp is initially expressed in the apical ectodermal ridge of the

limbs. Later during embryonic development and after birth, its expression becomes restricted to pre-osteoblasts and osteoblasts (i.e., Runx2-positive cells) of the perichondrium and periosteum.

[0010] Protein tyrosine phosphatase-1B (PTP-1B) is an ~50 kd intracellular protein present in abundant amounts in various human tissues (Charbonneau et al., 1989, Proc. Natl. Acad. Sci. USA 86:5252-5256; Goldstein, 1993, Receptor 3:1-15).

[0011] GPRC6A is an orphan receptor that belongs to the C family of GPCRs (Wellendorph and Brauner-Osborne, 2004, Gene 335:37-46) and has been proposed to be a receptor for amino acids or for calcium in the presence of osteocalcin as a cofactor, and for androgens (Pi et al., 2008, PLoS One.3:e3858; Pi et al., 2005, J. Biol. Chem. 280:40201-40209; Pi et al., 2010, J. Biol. Chem. 285:39953-39964).

SUMMARY OF THE INVENTION

[0012] The present invention provides methods of treating disorders related to reproduction in male mammals comprising administering to a male mammal in need of treatment for a disorder related to reproduction a pharmaceutical composition comprising a therapeutically effective amount of undercarboxylated/uncarboxylated osteocalcin and a pharmaceutically acceptable carrier or excipient. In certain embodiments, the osteocalcin is human osteocalcin. In certain embodiments, the disorder is male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes.

[0013] The present invention also provides methods of treating disorders related to reproduction in male mammals comprising administering to a male mammal in need of treatment for a disorder related to reproduction a pharmaceutical composition comprising an agent that modulates the OST-PTP signaling pathway or the PTP-1B signaling pathway, wherein the agent reduces OST-PTP phosphorylase expression or activity or reduces PTP-1B phosphorylase expression or activity, reduces gamma-carboxylase expression or activity, or increases the level of undercarboxylated/uncarboxylated osteocalcin, wherein the pharmaceutical composition comprises the agent in an amount that produces an effect in a male mammal selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing sperm viability, increasing serum testosterone levels, increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, and reducing excess apoptosis in testes.

[0014] In certain embodiments, the male mammal is a human.

[0015] In certain embodiments, the agent is undercarboxylated/uncarboxylated osteocalcin. In certain embodiments, the agent is human undercarboxylated/uncarboxylated osteocalcin.

[0016] In certain embodiments, the agent inhibits the expression or activity of OST-PTP, inhibits the expression or activity of PTP-1B, inhibits the expression or activity of gamma-carboxylase, inhibits phosphorylation of gamma-carboxylase, inhibits carboxylation of osteocalcin, or decarboxylates osteocalcin. In certain embodiments, the agent is selected from the group consisting of a small molecule, an antibody, or a nucleic acid.

[0017] In certain embodiments where the agent is undercarboxylated/uncarboxylated osteocalcin, at least one of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin is not carboxylated. In certain embodiments, all three of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin are not carboxylated.

[0018] In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin is a preparation of undercarboxylated/uncarboxylated osteocalcin in which more than about 20% of the total Glu residues at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin in the preparation are not carboxylated. In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin shares at least 80% amino acid sequence identity with mature human osteocalcin when the undercarboxylated/uncarboxylated osteocalcin and mature human osteocalcin are aligned for maximum sequence homology. In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin shares about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, or about 98% amino acid sequence identity with mature human osteocalcin when the undercarboxylated/uncarboxylated osteocalcin and mature human osteocalcin are aligned for maximum sequence homology. In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin differs at 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues from mature human osteocalcin.

[0019] In certain embodiments, at least one of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin is not carboxylated. In certain embodiments, all three of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin are not carboxylated. In certain embodiments, the present invention provides methods of administering undercarboxylated/uncarboxylated osteocalcin to a mammal to increase fertility, raise sperm count, increase sperm motility, increase sperm viability, increase serum testosterone levels, increase libido, or ameliorate erectile dysfunction.

[0020] In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin is a polypeptide selected from the group consisting of:

(a) a fragment comprising mature human osteocalcin missing the last 10 amino acids from the C-terminal end;

(b) a fragment comprising mature human osteocalcin missing the first 10 amino acids from the N-terminal end;

(c) a fragment comprising amino acids 62-90 of SEQ ID NO:2;

(d) a fragment comprising amino acids 1-36 of mature human osteocalcin; and

(e) variants of the above.

[0021] In certain embodiments, the pharmaceutical composition comprises a small molecule selected from the group consisting of warfarin, vitamin K inhibitors, and biologically active fragments or variants thereof. In a preferred embodiment, the small molecule is warfarin.

In another preferred embodiment, the agent is a small molecule that increases the activity or expression of osteocalcin.

[0022] In certain embodiments, the pharmaceutical composition comprises an antibody or antibody fragment that binds to and inhibits the activity of OST-PTP, PTP-1B, or gamma-carboxylase. Preferably, the antibody or antibody fragment is a monoclonal antibody. In certain embodiments, the antibody or antibody fragment binds to the extracellular domain of OST-PTP or PTP-1B. In preferred embodiments, the OST-PTP is human OST-PTP or the PTP-1B is human PTP-1B. In certain embodiments, the OST-PTP is the mouse OST-PTP of SEQ ID NO:11 or an OST-PTP having an amino acid sequence that is substantially homologous or identical to SEQ ID NO:11. In certain embodiments, the OST-PTP is an OST-PTP having an amino acid sequence that is at least 70% homologous or identical to SEQ ID NO:11. In certain embodiments, the PTP-1B is human PTP-1B of SEQ ID NO:17 or a PTP-1B having an amino acid sequence that is substantially homologous or identical to SEQ ID NO:17. In certain embodiments, the PTP-1B is a PTP-1B having an amino acid sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% homologous or identical to SEQ ID NO:17.

[0023] In certain embodiments, the pharmaceutical composition comprises a nucleic acid that inhibits the expression or activity of OST-PTP, PTP-1B, or gamma-carboxylase. In certain embodiments, the nucleic acid is an antisense oligonucleotide or a small interfering RNA (siRNA). In certain embodiments, the nucleic acid is an isolated nucleic acid that is selected from the group consisting of an antisense DNA, antisense RNA, and siRNA, which nucleic acid is sufficiently complementary to SEQ ID NO:10 or a sequence that is substantially homologous

or identical to SEQ ID NO:10 to permit specific hybridization to SEQ ID NO:10 or a sequence that is substantially homologous or identical to SEQ ID NO:10, and wherein the hybridization prevents or reduces expression of OST-PTP in osteoblasts. In certain embodiments, the nucleic acid is an isolated nucleic acid that is selected from the group consisting of an antisense DNA, antisense RNA, and siRNA, which nucleic acid is sufficiently complementary to SEQ ID NO:16 or a sequence that is substantially homologous or identical to SEQ ID NO:16 to permit specific hybridization to SEQ ID NO:16 or a sequence that is substantially homologous or identical to SEQ ID NO:16, and wherein the hybridization prevents or reduces expression of PTP-1B in osteoblasts.

[0024] In certain embodiments, the pharmaceutical composition comprises about 0.5 mg to about 5 g, about 1 mg to about 1 g, about 5 mg to about 750 mg, about 10 mg to about 500 mg, about 20 mg to about 250 mg, or about 25 mg to about 200 mg, of the agent. In certain embodiments, the pharmaceutical composition comprises an agent that is formulated into a controlled release preparation. In certain embodiments, the pharmaceutical composition comprises an agent that is chemically modified to prolong its half life in the human body.

[0025] In certain embodiments, the pharmaceutical composition for treating a disorder related to reproduction in male mammals comprises an undercarboxylated/uncarboxylated osteocalcin polypeptide comprising an amino acid sequence

YLYQWLGAPVPYPDPLX$_1$PRRX$_2$VCX$_3$LNPDCDELADHIGFQEAYRRFYGPV (SEQ ID NO:13)

wherein

X_1, X_2 and X_3 are each independently selected from an amino acid or amino acid analog, with the proviso that if X_1, X_2 and X_3 are each glutamic acid, then X_1 is not carboxylated, or less than 50 percent of X_2 is carboxylated, and/or less than 50 percent of X_3 is carboxylated,

or said osteocalcin polypeptide comprises an amino acid sequence that is different from SEQ. ID. NO:13 at 1 to 7 positions other than X_1, X_2 and X_3; and/or

wherein said amino acid sequence can include one or more amide backbone substitutions.

[0026] In certain embodiments, the osteocalcin polypeptide of SEQ. ID. NO:13 is a fusion protein. In certain embodiments, the arginine at position 43 of SEQ. ID. NO:13 is replaced with an amino acid or amino acid analog that reduces susceptibility of the osteocalcin polypeptide to proteolytic degradation. In certain embodiments, the arginine at position 44 of SEQ. ID. NO:13 is replaced with β-dimethyl-arginine. In certain embodiments, the osteocalcin polypeptide is a retroenantiomer of uncarboxylated human osteocalcin (1-49).

[0027] The present invention also provides a method of treating a disorder related to reproduction in male mammals by modulating the OST-PTP signaling pathway or the PTP-1B signaling pathway, the method comprising administering an agent that reduces OST-PTP phosphorylase activity or reduces PTP-1B phosphorylase activity, reduces gamma-carboxylase activity, or increases undercarboxylated/uncarboxylated osteocalcin, wherein the agent is administered in an amount that produces an effect in a male mammal selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing

sperm viability, increasing serum testosterone levels, increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, and reducing excess apoptosis in testes.

[0028] The present invention also provides a method of diagnosing a patient as having or being at risk of developing a disorder related to reproduction in male mammals comprising (i) determining the ratio of undercarboxylated/uncarboxylated osteocalcin to total osteocalcin in a biological sample from the patient; and (ii) comparing the ratio to a standard ratio; wherein, if the patient ratio is lower than the standard ratio, the patient is diagnosed as having or being at risk of developing a disorder related to reproduction in male mammals. In certain embodiments, the method comprises the further step of administering a therapeutic agent as described herein to the patient diagnosed as having or being at risk of developing a disorder related to reproduction in male mammals.

[0029] In certain embodiments, the patient has or is at risk for a disorder related to reproduction in male mammals selected from the group consisting of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes.

[0030] In certain embodiments, the biological sample is blood.

[0031] In certain embodiments of the diagnostic method described above, the standard ratio is 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, or 35%-40%.

[0032] The present invention provides a use of an agent that reduces OST-PTP phosphorylase activity, reduces PTP-1B phosphorylase activity, reduces gamma-carboxylase

activity, and/or increases undercarboxylated/uncarboxylated osteocalcin as a medicament for treating or preventing a disorder related to reproduction in male mammals.

[0033] In certain embodiments, the agent inhibits phosphorylation of gamma-carboxylase. In certain embodiments, the agent increases the level of undercarboxylated/uncarboxylated osteocalcin. In certain embodiments, the agent increases the ratio of undercarboxylated/uncarboxylated osteocalcin compared to carboxylated osteocalcin. In certain embodiments, the agent inhibits carboxylation of osteocalcin. In certain embodiments, the agent decarboxylates osteocalcin.

[0034] In certain embodiments of the use described above, the agent is undercarboxylated/uncarboxylated osteocalcin. In certain embodiments of the use described above, the undercarboxylated/uncarboxylated osteocalcin increases fertility, raises sperm count, increases sperm motility, increases sperm viability, increases serum testosterone levels, increases libido, ameliorates erectile dysfunction, reduces underdevelopment of testes, or reduces excess apoptosis in testes. In certain embodiments, at least one of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin is not carboxylated. In certain embodiments, all three of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin are not carboxylated. In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin is a preparation of undercarboxylated/uncarboxylated osteocalcin in which more than about 20% of the total Glu residues at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin in

the preparation are not carboxylated. In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin shares about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, or about 98% amino acid sequence identity with mature human osteocalcin when the undercarboxylated/uncarboxylated osteocalcin and mature human osteocalcin are aligned for maximum sequence homology. In certain embodiments, the undercarboxylated/uncarboxylated osteocalcin differs at 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues from mature human osteocalcin.

[0035] In certain embodiments of the use described above, the agent is selected from the group consisting of a small molecule, an antibody, or a nucleic acid.

[0036] In certain embodiments of the use described above, the agent is a small molecule that inhibits the expression or activity of OST-PTP, PTP-1B, or gamma-carboxylase. In certain embodiments, the agent is a small molecule selected from the group consisting of warfarin, vitamin K inhibitors, and biologically active fragments or variants thereof. In a preferred embodiment, the small molecule is warfarin. In another preferred embodiment, the agent is a small molecule that increases the activity or expression of osteocalcin.

[0037] The present invention provides the use of an undercarboxylated osteocalcin polypeptide, or mimetic thereof, for the manufacture of a medicament for treatment of a disorder related to reproduction in male mammals. In certain embodiments, the disorder is selected from the group consisting of male infertility, low sperm count, impaired sperm motility, impaired

sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes.

[0038] The present invention also provides the use of an agent that reduces OST-PTP phosphorylase activity, reduces PTP-1B phosphorylase activity, reduces gamma-carboxylase activity, and/or increases undercarboxylated/uncarboxylated osteocalcin for the manufacture of a medicament for treatment of a disorder related to reproduction in male mammals. In certain embodiments, the disorder is selected from the group consisting of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes.

[0039] The present invention provides methods of contraception for use in male mammals. comprising administering to a male mammal in need of contraception a pharmaceutical composition comprising a therapeutically effective amount of an antagonist of undercarboxylated/uncarboxylated osteocalcin and a pharmaceutically acceptable carrier or excipient. In certain embodiments, the antagonist is an antagonist of human undercarboxylated/uncarboxylated osteocalcin.

BRIEF DESCRIPTION OF THE FIGURES

[0040] **Figure 1.** Comparison between the average litter frequency (A) and size (B) generated by *Osteocalcin* -/- and wild type (WT) male mice crossed with WT females (breedings were tested from 6 weeks to 4 months of age).

[0041] **Figure 2.** Analysis of testicular weight (A), size (lower panel in A) and sperm count (B) of *Osteocalcin-/-* and wild-type (+/+) littermate mice at 6 weeks, 3 months, and 6 months of age.

[0042] **Figure 3.** Analysis of testicular weight (A), size (lower panel in A) and sperm count (B) of *Esp-/-* and wild-type (+/+) littermate mice at 6 weeks, 3 months and 6 months of age.

[0043] **Figure 4.** Analysis of cell proliferation in 2-week-old testes from WT and *Osteocalcin* mutant mice 1 day after bromodeoxyuridine (BrdU) injection (A). TUNEL analysis of *Osteocalcin-/-, Esp-/-* testes at 6 weeks and 3 months of age (B-C). Western-blot analysis of extracts from 2-week and 6 week-old Osteocalcin-/- testes using an anti Cleaved Caspase-3 (Asp175) (5A1) Rabbit antibody (D). β-Actin was used as a loading control.

[0044] **Figure 5.** Analysis of testosterone serum levels in *Osteocalcin-/-* and WT littermate breeder mice at 3 months of age (A). Analysis of testosterone serum level of *Esp -/-* and WT littermate non-breeder mice (B) at 3 months of age.

[0045] **Figure 6.** Quantitative PCR (qPCR) analysis of *StAR, Cyp17,* and *Cyp11a* expression in TM3 Leydig cells cultured in the presence of different concentrations of osteocalcin (from 0.3 to 300 ng/ml) for 2 hours.

[0046] **Figure 7.** Analysis of testis weight and size (lower panel) (A), sperm count (B), apoptosis (C) and testosterone serum levels (D) in WT mice injected once daily with vehicle (veh) or a dose of osteocalcin (0, 3 or 30 ng/g) from 2 to 4 months of age.

[0047] **Figure 8**. qPCR analysis of *Osteocalcin* expression in bone, testis, and ovary of 3 month-old WT mice (A). In situ hybridization analysis of *Osteocalcin* expression in bone and testis of 3 month-old WT mice (B). Analysis of mCherry fluorescent protein in bone and testis of *Osteocalcin-mCherry* Knockin mice (C).

[0048] **Figure 9.** Targeting strategy to generate conditional Osteocalcin-/- mice through homologous recombination in embryonic stem (ES) cells (A). Identification of an ES cell clone targeted for the Osteocalcin floxed allele (red arrows) by Southern blot analysis. A 400-bp fragment and a 540-bp fragment respectively located at the 5'end and 3'end of the genomic locus was used as a probe external to the targeting vector. Hybridization of these probes with Hind III or NcoI-digested genomic DNA yielded both a WT and a lower targeted band in the targeted ES cells (B).

[0049] **Figure 10.** Measurement of uncarboxylated (Glu OC) and carboxylated (Gla OC) osteocalcin using uncarboxylated osteocalcin ELISA.

[0050] **Figure 11.** Osteoblasts enhance testosterone biosynthesis by Leydig cells. (A) Schematic representation of the cell-based assay used to determine the role of various mesenchymal cells in steroid sex hormone production. Various primary mesenchymal cells from mice were cultured in Leydig cell medium and supernatants were collected after 24 hours. Then, testis or ovary explants or primary Leydig cells were cultured for 1 hour with these supernatants and radioimmunoassays (RIAs) were performed to measure levels of testosterone, estradiol, or progesterone. (B-D) Testis explants cultured in the presence of supernatants of different mesenchymal cell cultures: RIA measurement of (B) testosterone, (C) estradiol, and (D)

progesterone levels. (E-G) Ovary explants cultured in the presence of supernatants of different mesenchymal cell cultures: RIA measurement of (E) testosterone, (F) estradiol, and (G) progesterone levels. (H) Testosterone production by primary Leydig cells cultured in the presence of osteocalcin (3 ng/ml of culture medium) or vehicle. Error bars represent SEM. Student's t test (*) $P<0.05$.

[0051] **Figure 12.** Osteocalcin favors male fertility by increasing testosterone production by Leydig cells. (A-B) Testosterone production by testis explants (A) or primary Leydig cells (B) cultured in the presence of supernatants of wild type (WT) or *Ocn -/-* osteoblast cultures. (C-D) Testosterone production by testis explants (C) or primary Leydig cells (D) following stimulation with increasing doses of osteocalcin (0, 0.3, 1, 3, 10, 100 ng/ml of culture medium). (E) Circulating testosterone levels in WT mice 1 hour, 4 hours, and 8 hours after vehicle or osteocalcin (3 ng/g of body weight) injection. (F-G) Comparison between the average litter size (F) and frequency (G) generated by WT, *Ocn-/-,* or *Esp-/-* male littermate mice crossed with WT females (breeding was tested from 6 to 16 weeks of age). (H-L) Testis size (H), testis weight (I), epididymides weight (J), seminal vesicles weight (K), and sperm count (L) in *Ocn-/-* and *Esp-/-* compared to WT littermate mice. (M) Circulating steroid sex hormone levels in *Ocn-/-* and *Esp-/-* compared to WT littermate mice. The analyses were performed on breeder and non-breeder mice. Error bars represent SEM. Student's t test (*) $P<0.05$, (**) $P<0.001$.

[0052] **Figure 13.** Osteocalcin promotes male fertility through its expression in osteoblasts. (A) qPCR analysis of *Osteocalcin* expression in bone, testes, and ovaries of 3 month-old WT mice. (B) Western blot analysis of osteocalcin in femur, calvaria, and testis. (C)

In situ hybridization analysis of *Osteocalcin* expression in bone and testis of 3 month-old WT mice. (D). Analysis of mCherry fluorescent protein in bone and testis of *Osteocalcin-mCherry* Knock-in mice. (E-I) Fertility in mice lacking *Ocn* specifically in osteoblasts (Ocn_{osb}-/-) or Leydig cells (Ocn_{Leydig}-/-) compared to WT littermates: (E) Testes weihts, (F) sperm count, (G) epididymides and seminal vesicle (H) weights, and (I) ratio of circulating testosterone levels measured in WT and Ocn_{osb}-/- or in WT and Ocn_{Leydig}-/- littermate mice. (J) Linear regression representation of circulating testosterone levels versus circulating osteocalcin levels in Ocn_{osb}-/- mice (n=11). Each dot represents one Ocn_{osb}-/- mouse. (K-M) Fertility in mice lacking *Esp* specifically in osteoblasts (Esp_{osb}-/-) or Leydig cells (Esp_{Leydig}-/-) compared to WT littermates: (K) Testes weight, (L) sperm count and (M) seminal vesicle weight. (N) Ratio of circulating testosterone levels measured in WT and Esp_{osb}-/- or in WT and Esp_{Leydig}-/- littermate mice. Error bars represent SEM. Student's t test (*) $P<0.05$, (**) $P<0.001$.

[0053] **Figure 14.** Cellular and molecular events triggered by osteocalcin in Leydig cells. (A-C) Histological analyses of Leydig cells in *Ocn* -/- and *Esp*-/- mice: (A) Absolute number of Leydig cells per testis was quantified by the number of 3β-HSD positive cells. (B) Ratio between Leydig cells (immunopositive for 3β-HSD) versus testis interstitial areas in WT, *Ocn*-/-, and *Esp*-/- mice. (C) 3β-HSD immunohistochemistry staining of WT, *Ocn*-/-, and *Esp*-/- testes. (D) Quantification of tubule, lumen, and epithelium areas in WT and *Ocn*-/- mice. (E) Germ cell apoptosis analysis by TUNEL assay in WT, *Ocn*-/-, and *Esp*-/- testes. (F-H) qPCR analysis of the expression of steroidogenic acute regulatory protein (*StAR*), cholesterol side-chain cleavage enzyme (*Cyp11a*), cytochrome P-450 17 alpha (*Cyp17*), 3-β-hydroxysteroid dehydrogenase (*3β-HSD*), aromatase enzyme (*Cyp19*), and 17β-hydroxysteroid dehydrogenase

(*HSD-17*) in testis after treatment with vehicle or 3 ng/ml of osteocalcin (F) in *Ocn-/-* compared to WT littermate testes (G) and in *Esp-/-* compared to WT littermate testes (H). (I) qPCR analysis of *Grth/Ddx25* expression in WT, *Ocn-/-*, *Esp-/-,* and WT mice treated with vehicle or osteocalcin (3 ng/g of body weight). (J) Western blot analysis of cleaved caspase 3 and tACE in WT and *Ocn-/-* testes. Error bars represent SEM. Student's t test (*) $P<0.05$, (**) $P<0.001$.

[0054] **Figure 15.** G-protein coupled receptor OstR is a receptor for osteocalcin. (A) Anti-phospho-tyrosine antibody Western blot analysis of TM3 Leydig cells treated with increasing concentration of osteocalcin, or 10% FBS, or insulin as positive controls, for 1 minute (upper panel). Proteins phosphorylated on tyrosine residues appear in positive controls (asterisks) but not in osteocalcin treated cells. Equal loading was assessed using an anti-actin antibody (lower panel). (B) Western blot analysis of TM3 Leydig cells showing the absence of ERK1/2 phosphorylation upon stimulation with vehicle or osteocalcin. (C) Calcium fluxes in primary Leydig cells upon stimulation with increasing doses of osteocalcin, 10% FBS, and ionophore (A23187) were used as positive controls. (D) cAMP production upon osteocalcin stimulation is increased in TM3 Leydig cells. (E) Schematic representation of the results obtained by the differential expression search for OstR. Among the 103 orphan GPCRs expressed in testis and ovary, 22 were predominantly expressed in testis and only four were enriched in primary Leydig cells compared to the expression in whole testis. (F) Relative expression of *Gprc6a*, *Gpr45*, *Gpr112,* and *Gpr139* in Leydig cells compared to whole testis. (G) Immunofluorescence analysis of OstR expression in WT testes coronal section. Anti -IgG was used as negative control. (H) qPCR analysis of *OstR* expression in 1, 4, 6, and 12 week-old WT testes. (I) Cross sections of testes from WT and *OstR*-deficient mice stained with

biotinylated osteocalcin (b-osteocalcin). Upper left panel: WT testis stained with avidin-biotin complex only; upper middle panel: WT testis stained with 10 nM of b-osteocalcin; upper right panel: testis from *OstR*-deficient mice stained with 10 nM of b-osteocalcin; lower left panel: WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM hCG; lower middle panel: WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM lysine; lower right panel: WT testis stained with 10 nM of b-osteocalcin in the presence of 1000 nM of unlabeled osteocalcin. Error bars represent SEM. Student's t test (*) $P<0.05$, (**) $P<0.001$.

[0055] **Figure 16.** Specific deletion of *OstR* in Leydig cells decreases male fertility. (A-E) Fertility in mice lacking *OstR* in Leydig cells only ($OstR_{Leydig}$-/-) or lacking one allele of *Ocn* or one allele of *OstR* in Leydig cells only (*Ocn*+/- or $OstR_{Leydig}$+/-), or in compound heterozygous mice (*Ocn*+/- *and* $OstR_{Leydig}$+/-) compared to control littermates. (A) Testis size, (B) testis weight, (C) sperm count, (D-E) epididymides and seminal vesicles weights. (F) qPCR analysis of *Grth* expression in mice of indicated genotypes. (G) Ratio between Leydig cells (stained by immunohistochemistry of 3β-HSD) versus testis interstitial areas. (H) Ratio of circulating testosterone levels measured in WT and $OstR_{Leydig}$-/-. (I) qPCR analysis of *StAR*, *Cyp11a*, and *3β-HSD* in $OstR_{Leydig}$-/- and *Ocn*+/-; $OstR_{Leydig}$+/- compared to WT littermate testes. (J) Germ cell apoptosis analysis by TUNEL assay. Error bars represent SEM. Student's t test (*) $P<0.05$.

[0056] **Figure 17.** CREB is a transcription factor mediating osteocalcin-evoked gene expression in Leydig cells. (A) Western blot analysis of CREB activation upon stimulation with osteocalcin. (B-F) Fertility in mice lacking *Creb* in Leydig cells ($Creb_{Leydig}$-/-) or lacking one

allele of *Creb* or one allele of *OstR* in Leydig cells only ($Creb_{Leydig}$+/- or $OstR_{Leydig}$+/-), or of compound heterozygous mice ($Creb_{Leydig}$+/-; $OstR_{Leydig}$+/-) compared to control littermates. (B) Testis size, (C) testis weight, (D) sperm count, (E-F) epididymides and seminal vesicle weights. (G) Quantification of circulating testosterone levels represented as fold change compared to WT. (H) qPCR analysis of *Grth* expression in mice of indicated genotypes. (I) qPCR analysis of *StAR*, *Cyp11a*, *Cyp17*, *3β-HSD*, *Cyp19,* and *HSD-17* in $Creb_{Leydig}$-/- compared to control littermate testes. (J) Chromatin immunoprecipitation (ChIP) using anti-CREB antibody and unspecific isotype IgG antibody. **(K)** Model representing the current knowledge about the regulation of male fertility by the skeleton. Error bars represent SEM. Student's t test (*) $P<0.05$.

[0057] **Figure 18.** (A-C) Sperm motility and morphology analyses. (A) Percentage (%) of motile sperm, (B) abnormal sperm, and (C) dead sperm in WT and *Ocn-/-* male littermate mice analyzed immediately and 2 hours after dissection. (D-E) Fertility analysis of *Ocn-/-* females. (D) Comparison between the average litter frequency and (E) size generated by wild type (WT) and *Ocn-/-* female littermates crossed with WT males (breeding was tested from 6 to 16 weeks of age). (F) Average number of cycles within 2 weeks in WT and *Ocn-/-* female littermate mice. (G) Percentage of WT and *Ocn-/-* female littermate mice to cycle. (H) Uteri and ovaries of WT and *Ocn-/-* female littermate mice. (I) Ovary weight of WT and *Ocn-/-* female littermate mice. (J) Histological analyses of ovary sections and number of follicles from WT and *Ocn-/-* female littermate mice. Follicles are indicated by asterisks (*). (K) Number of apoptotic cells per WT and *Ocn-/-* ovaries. (L) Circulating steroid sex hormone levels in WT and *Ocn-/-* female littermate mice. Error bars represent SEM.

[0058] **Figure 19.** (A-B) Generation of *Ocn-mCherry* knock-in allele. (A) Schematic representation of the targeting strategy. The open reading frames of Exons Bglap1 and Bglap2 are represented by dark rectangles with white lettering; thin lines represent untranslated regions of the *Ocn* locus. The neomycin resistance gene (for positive selection) flanked by two LoxP sites (triangles) (FRT-neoPGK-FRT) and the HSV-tk cassette (for negative selection) are indicated. (B) Southern blot performed with 5' and 3' probes; the position of each probe is shown in (A). (C-D) Generation of *Ocn* conditional allele. (C) Schematic representation of the targeting strategy. The open reading frames of Exons Bglap1 and Bglap2 are represented by dark rectangles with white lettering. Thin lines represent untranslated regions of the *Ocn* locus. The neomycin resistance gene (for positive selection) flanked by two FRT sites (FRT-neoPGK-FRT), the HSV-tk cassette (for negative selection), and LoxP are indicated. (D) Southern blot performed with 5' and 3' probes; the position of each probe is shown in (C).

[0059] **Figure 20.** (A) Germ cell proliferation analyzed by BrdU staining in 2-week-old WT, *Ocn-/-,* and *Esp-/-* male littermates. (B-E) qPCR analysis of *StAR*, *Cyp11a*, *Cyp17*, *3β-HSD*, *Cyp19,* and *HSD-17* in (B) *Ocn-/-* and WT ovaries, (C) *Ocn-/-* and WT adrenals, (D) *Esp-/-* and WT ovaries, and (E) *Esp-/-* and WT adrenals. Error bars represent SEM.

[0060] **Figure 21.** (A) Schematic representation of the results obtained by the differential expression search for the osteocalcin receptor (OstR). Among the 103 orphan GPCRs expressed in testis and ovary, 22 were predominantly expressed in testis and only four (Gprc6a, Gpr139, Gpr112, and Gpr45) were enriched in primary Leydig cells compared to their

expression in whole testis. (B) Immunofluorescence of OstR and IgG control in coronal sections of WT ovaries.

[0061] **Figure 22.** (A-B) Generation of *OstR* conditional allele**.** (A) Schematic representation of the targeting strategy. The open reading frames of ExonI, ExonII, and ExonIII are represented by grey rectangles with white lettering and thin lines represent untranslated regions of the *OstR* locus. The neomycin resistance gene (for positive selection) flanked by two FRT sites (grey triangles) (FRT-neoPGK-FRT), the HSV-tk cassettes (for negative selection) and LoxP sites (light triangles) are indicated. (B) Southern blot performed with 5' and 3' probes; the position of each probe is shown in (A). (C) Specificity of *OstR* deletion was tested by PCR in the indicated tissues. Primer positions are shown in (A).

[0062] **Figure 23.** Nucleotide sequence encoding human GPRC6A from GenBank accession no. AF502962.

[0063] **Figure 24.** Amino acid sequence of human GPRC6A from GenBank accession no. AF502962.

DETAILED DESCRIPTION OF THE INVENTION

[0064] The present invention is based in part on the discovery of a previously unknown biochemical pathway linking osteocalcin and reproductive biology in male mammals by which increased activity of osteocalcin leads to increased activity of enzymes involved in the synthesis of testosterone. This in turn leads to beneficial effects on male reproduction.

[0065] The present invention is also based in part on the observation that *Osteocalcin*-deficient male mice have significantly smaller testes than their wild type (WT) littermates. These mutant mice are also subfertile and display low sperm counts. Histological and molecular studies revealed that in the absence of osteocalcin the entire spermatogenic process seems to be affected because of an increase in apoptosis. Circulating levels of testosterone are also quite low in *Osteocalcin*-deficient mice. These observations indicate that osteocalcin is a critical regulator of male fertility in these mice.

[0066] In view of the observations described above, certain aspects of the invention are directed to the therapeutic use of undercarboxylated/uncarboxylated osteocalcin, as well as fragments and variants thereof, to treat or prevent disorders related to reproduction in male mammals.

[0067] Preventing a disorder related to reproduction in male mammals means actively intervening as described herein prior to overt onset of the disorder to prevent or minimize the extent of the disorder or slow its course of development.

[0068] Treating a disorder related to reproduction in male mammals means actively intervening after onset of the disorder to slow down, ameliorate symptoms of, minimize the extent of, or reverse the disorder in a patient who is known or suspected of having the disorder.

[0069] The present invention also provides methods of increasing testosterone levels, particularly serum testosterone levels, in male mammals by administering the therapeutic agents described herein to male mammals known to be in need of treatment to increase testosterone levels. In some embodiments of the methods of the present invention, the therapeutic agent

increases serum testosterone levels by about 10%-25%, 20%-35%, 30%-50%, 50%-500%, 70%-400%, 100%-300%, or 100%-500% compared to pre-treatment serum testosterone levels.

[0070] A "patient" is a mammal, preferably a human, but can also be a companion animal such as dogs or cats, or farm animals such as horses, cattle, pigs, or sheep.

[0071] A patient in need of treatment or prevention for a disorder related to reproduction in male mammals includes a patient known or suspected of having or being at risk of developing a disorder related to reproduction in male mammals. Such a patient in need of treatment could be, e.g., a male mammal known to have low testosterone levels. Patients in need of treatment or prevention by the methods of the present invention include patients who are known to be in need of therapy to increase serum testosterone levels in order to treat or prevent a disorder related to reproduction in male mammals. In some embodiments, such patients might include male mammals who have been identified as having a serum testosterone level that is about 25%, about 50%, or about 75% lower than the serum testosterone level in normal subjects.

[0072] A patient in need of treatment or prevention for a disorder related to reproduction in male mammals by the methods of the present invention does not include a patient being administered the therapeutic agents described herein where the patient is being administered the therapeutic agents described herein only for a purpose other than to treat or prevent a disorder related to reproduction in male mammals. Thus, e.g., a patient in need of treatment or prevention for a disorder related to reproduction in male mammals by the methods of the present invention does not include a patient being treated with osteocalcin only for the purpose of treating a bone mass disease or a metabolic disorder such as diabetes. A patient in need of treatment or

prevention for a disorder related to reproduction in male mammals by the methods of the present invention also does not include a patient being treated with osteocalcin that is not undercarboxylated/uncarboxylated osteocalcin.

[0073] In certain embodiments, the methods of the present invention comprise the step of identifying a patient in need of therapy for a disorder related to reproduction in male mammals. Thus, the present invention provides a method comprising:

(a) identifying a patient in need of therapy for a disorder related to reproduction in male mammals;

(b) administering to the patient a therapeutically effective amount of undercarboxylated/uncarboxylated osteocalcin or an agent that modulates the OST-PTP signaling pathway or the PTP-1B signaling pathway, wherein the agent reduces OST-PTP phosphorylase expression or activity, reduces PTP-1B phosphorylase expression or activity, or reduces gamma-carboxylase expression or activity.

[0074] The present invention is also based on the observation that gamma-carboxylase carboxylates osteocalcin, thereby producing carboxylated osteocalcin. This provides the opportunity to modulate the degree of carboxylation of the osteocalcin used in the methods of the present invention by modulating the activity of gamma-carboxylase. In particular, this provides the opportunity to lower the degree of carboxylation of the osteocalcin used in the methods of the present invention, thus providing undercarboxylated/uncarboxylated osteocalcin for use in the methods of the present invention. Therefore, certain aspects of the invention are directed to the

therapeutic use of agents that inhibit the activity of gamma-carboxylase to treat or prevent a disorder related to reproduction in male mammals.

[0075] The present invention is further based on the observation that OST-PTP activates gamma-carboxylase through dephosphorylation. As indicated above, activation of gamma-carboxylase leads to carboxylation of osteocalcin. This provides the opportunity to indirectly modulate the degree of carboxylation of the osteocalcin used in the methods of the present invention by modulating the activity of OST-PTP (which will then modulate the activity of gamma-carboxylase). Therefore certain aspects of the invention are directed to the therapeutic use of agents that inhibit the activity of OST-PTP to treat or prevent a disorder related to reproduction in male mammals.

[0076] The present invention is further based on the observation that, in humans, PTP-1B activates gamma-carboxylase through dephosphorylation. As indicated above, activation of gamma-carboxylase leads to carboxylation of osteocalcin. This provides the opportunity to indirectly modulate the degree of carboxylation of the osteocalcin used in the methods of the present invention by modulating the activity of PTP-1B in male humans (which will then modulate the activity of gamma-carboxylase). Therefore certain aspects of the invention are directed to the therapeutic use in male humans of agents that inhibit the activity of PTP-1B to treat or prevent a disorder related to reproduction in male humans.

[0077] Other aspects of the invention are directed to diagnostic methods based on detection of the level of undercarboxylated/uncarboxylated osteocalcin in a patient, which level is associated with disorders related to reproduction in male mammals.

[0078] In one aspect, the method of diagnosing a disorder related to reproduction in male mammals in a patient comprises (i) determining a patient level of undercarboxylated/uncarboxylated osteocalcin in a biological sample taken from the patient (ii) comparing the patient level of undercarboxylated/uncarboxylated osteocalcin and a control level of undercarboxylated/uncarboxylated osteocalcin, and (iii) if the patient level is significantly lower than the control level, then diagnosing the patient as having, or being at risk for, the disorder related to reproduction in male mammals.

[0079] Other aspects of the invention are directed to diagnostic methods based on detection of decreased ratios of undercarboxylated/uncarboxylated vs carboxylated osteocalcin. Such ratios may be associated with disorders related to reproduction in male mammals. In one aspect, the method of diagnosing a disorder related to reproduction in male mammals in a patient comprises (i) determining a patient ratio of undercarboxylated/uncarboxylated vs. carboxylated osteocalcin in a biological sample taken from the patient (ii) comparing the patient ratio of undercarboxylated/uncarboxylated vs carboxylated osteocalcin and a control ratio of undercarboxylated/uncarboxylated vs carboxylated osteocalcin, and (iii) if the patient ratio is significantly lower than the control ratio, then the patient is diagnosed has having, or being at risk for, the disorder related to reproduction in male mammals.

PHARMACEUTICAL COMPOSITIONS FOR USE IN THE METHODS OF THE INVENTION

[0080] The present invention provides pharmaceutical compositions for use in the treatment of a disorder related to reproduction in male mammals comprising an agent for

modulating the OST-PTP signaling pathway or for modulating the PTP-1B signaling pathway, which pathways involve gamma-carboxylase and osteocalcin. In particular embodiments, the agent inhibits OST-PTP phosphorylase activity, inhibits PTP-1B phosphorylase activity reduces gamma-carboxylase activity, and/or increases undercarboxylated/uncarboxylated osteocalcin. In particular embodiments, the agent decarboxylates osteocalcin. The agent may be selected from the group consisting of small molecules, polypeptides, antibodies, and nucleic acids. The pharmaceutical compositions of the invention provide an amount of the agent effective to treat or prevent a disorder related to reproduction in male mammals. In certain embodiments, the pharmaceutical composition provides an amount of the agent effective to treat or prevent male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes.

[0081] In certain embodiments, the pharmaceutical compositions for use in the methods of the invention may function to increase serum undercarboxylated/uncarboxylated osteocalcin serum levels.

[0082] In particular embodiments of the invention, therapeutic agents that may be administered in the methods of the present invention include undercarboxylated osteocalcin; uncarboxylated osteocalcin; or inhibitors that reduce the expression or activity of gamma-carboxylase, PTP-1B, or OST-PTP (e.g., antibodies, small molecules, antisense nucleic acids or siRNA). The pharmaceutical agents may also include agents that decarboxylate osteocalcin.

[0083] The therapeutic agents are generally administered in an amount sufficient to treat or prevent male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes.

[0084] Biologically active fragments or variants of the therapeutic agents are also within the scope of the present invention. By "biologically active" is meant capable of modulating the OST-PTP signaling pathway or the PTP-1B signaling pathway involving gamma-carboxylase and osteocalcin. "Biologically active" may also mean reducing the expression of OST-PTP or its ability to dephosphorylate gamma-carboxylase and reducing the expression of gamma-carboxylase or its ability to carboxylate osteocalcin, or decarboxylating carboxylated osteocalcin thereby leading to increased levels of undercarboxylated/uncarboxylated osteocalcin.

[0085] "Biologically active" also means reducing the expression of PTP-1B or its ability to dephosphorylate gamma-carboxylase and reducing the expression of gamma-carboxylase or its ability to carboxylate osteocalcin, or decarboxylating carboxylated osteocalcin thereby leading to increased levels of undercarboxylated/uncarboxylated osteocalcin.

[0086] "Biologically active" also refers to fragments or variants of osteocalcin that retain the ability of undercarboxylated/uncarboxylated osteocalcin to treat or prevent a disorder related to reproduction in male mammals.

[0087] "Biologically active" also means capable of producing at least one effect in a male mammal selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing sperm viability, increasing serum testosterone levels,

increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, and reducing excess apoptosis in testes.

PHARMACEUTICAL COMPOSITIONS COMPRISING UNDERCARBOXYLATED/UNCARBOXYLATED OSTEOCALCIN

[0088] In a specific embodiment of the invention, pharmaceutical compositions comprising undercarboxylated/uncarboxylated osteocalcin are provided for use in treating or preventing a disorder related to reproduction in a male mammal.

[0089] "Undercarboxylated osteocalcin" means osteocalcin in which one or more of the Glu residues at positions Glu17, Glu21 and Glu24 of the amino acid sequence of the mature human osteocalcin having 49 amino acids, or at the positions corresponding to Glu17, Glu21 and Glu24 in other forms of osteocalcin, are not carboxylated. Undercarboxylated osteocalcin includes "uncarboxylated osteocalcin," i.e., osteocalcin in which all three of the glutamic acid residues at positions 17, 21, and 24 are not carboxylated. Preparations of osteocalcin are considered to be "undercarboxylated osteocalcin" if more than about 10% of the total Glu residues at positions Glu17, Glu21 and Glu24 (taken together) in mature osteocalcin (or the corresponding Glu residues in other forms) of the preparation are not carboxylated. In particular preparations of undercarboxylated osteocalcin, more than about 20%, more than about 30%, more than about 40%, more than about 50%, more than about 60%, more than about 70%, more than about 80%, more than about 90%, more than about 95%, or more than about 99% of the total Glu residues at positions Glu17, Glu21 and Glu24 in mature osteocalcin (or the corresponding Glu residues in other forms) of the preparation are not carboxylated. In particularly preferred embodiments, essentially all of the Glu residues at positions Glu17, Glu21

and Glu24 in mature osteocalcin (or the corresponding Glu residues in other forms) of the preparation are not carboxylated.

[0090] "Undercarboxylated/uncarboxylated osteocalcin" is used herein to refer collectively to undercarboxylated and uncarboxylated osteocalcin.

[0091] Human osteocalcin cDNA (SEQ ID NO:1) encodes a mature osteocalcin protein represented by the last 49 amino acids of SEQ ID NO:2 (i.e., positions 52-100) with a predicted molecular mass of 5,800 kDa (Poser et al., 1980, J. Biol. Chem. 255:8685-8691). SEQ ID NO:2 is the pre-pro-sequence of human osteocalcin encoded by SEQ ID NO:1 and mature human osteocalcin (SEQ ID NO:12) is the processed product of SEQ ID NO:2. In this application, the amino acid positions of mature human osteocalcin are referred to. It will be understood that the amino acid positions of mature human osteocalcin correspond to those of SEQ ID NO:2 as follows: position 1 of mature human osteocalcin corresponds to position 52 of SEQ ID NO:2; position 2 of mature human osteocalcin corresponds to position 53 of SEQ ID NO:2, etc. In particular, positions 17, 21, and 24 of mature human osteocalcin correspond to positions 68, 72, and 75, respectively, of SEQ ID NO:2.

[0092] When positions in two amino acid sequences correspond, it is meant that the two positions align with each other when the two amino acid sequences are aligned with one another to provide maximum homology between them. This same concept of correspondence also applies to nucleic acids.

[0093] For example, in the two amino acid sequences AGLYSTVLMGRPS and GLVSTVLMGN, positions 2-11 of the first sequence correspond to positions 1-10 of the second

sequence, respectively. Thus, position 2 of the first sequence corresponds to position 1 of the second sequence; position 4 of the first sequence corresponds to position 3 of the second sequence; etc. It should be noted that a position in one sequence may correspond to a position in another sequence, even if the positions in the two sequence are not occupied by the same amino acid.

[0094] "Osteocalcin" includes the mature protein and further includes biologically active fragments derived from full-length osteocalcin (SEQ ID NO:2) or the mature protein, including various domains, as well as variants as described herein.

[0095] In one embodiment of the present invention, the pharmaceutical compositions for use in the methods of the invention comprise a mammalian uncarboxylated osteocalcin. In a preferred embodiment of the invention, the compositions for use in the methods of the invention comprise human uncarboxylated osteocalcin having the amino acid sequence of SEQ ID NO:2, or portions thereof, and encoded for by the nucleic acid of SEQ ID NO:1, or portions thereof. In some embodiments, the compositions for use in the methods of the invention may comprise one or more of the human osteocalcin fragments described herein.

[0096] In a preferred embodiment of the invention, the compositions for use in the methods of the invention comprise human uncarboxylated osteocalcin having the amino acid sequence of SEQ ID NO:12.

[0097] In a specific embodiment, the present invention provides pharmaceutical compositions comprising human undercarboxylated osteocalcin which does not contain a carboxylated glutamic acid at one or more of positions corresponding to positions 17, 21 and 24

of mature human osteocalcin. A preferred form of osteocalcin for use in the methods of the present invention is mature human osteocalcin wherein at least one of the glutamic acid residues at positions 17, 21, and 24 is not carboxylated. In certain embodiments, the glutamic acid residue at position 17 is not carboxylated. Preferably, all three of the glutamic acid residues at positions 17, 21, and 24 are not carboxylated. The amino acid sequence of mature human osteocalcin is shown in SEQ. ID. NO:12.

[0098] The primary sequence of osteocalcin is highly conserved among species and it is one of the ten most abundant proteins in the human body, suggesting that its function is preserved throughout evolution. Conserved features include 3 Gla residues at positions 17, 21, and 24 and a disulfide bridge between Cys23 and Cys29. In addition, most species contain a hydroxyproline at position 9. The N-terminus of osteocalcin shows highest sequence variation in comparison to other parts of the molecule. The high degree of conservation of human and mouse osteocalcin underscores the relevance of the mouse as an animal model for the human, in both healthy and diseased states, and validates the therapeutic and diagnostic use of osteocalcin to treat or prevent disorders related to reproduction in male humans based on the experimental data derived from the mouse model disclosed herein.

[0099] The present invention also includes the use of polypeptide fragments of osteocalcin. Fragments can be derived from the full-length, naturally occurring amino acid sequence of osteocalcin (e.g., SEQ. ID. NO:2). Fragments may also be derived from mature osteocalcin (e.g., SEQ. ID. NO:12). The invention also encompasses fragments of the variants of

osteocalcin described herein. A fragment can comprise an amino acid sequence of any length that is biologically active.

[00100] Preferred fragments of osteocalcin include fragments containing Glu17, Glu21 and Glu24 of the mature protein. Also preferred are fragments of the mature protein missing the last 10 amino acids from the C-terminal end of the mature protein. Also preferred are fragments missing the first 10 amino acids from the N-terminal end of the mature protein. Also preferred is a fragment of the mature protein missing both the last 10 amino acids from the C-terminal end and the first 10 amino acids from the N-terminal end. Such a fragment comprises amino acids 62-90 of SEQ ID NO:2.

[00101] Other preferred fragments of osteocalcin for the pharmaceutical compositions of the invention described herein include polypeptides comprising, consisting of, or consisting essentially of, the following sequences of amino acids:

[00102] - positions 1-19 of mature human osteocalcin

[00103] - positions 20-43 of mature human osteocalcin

[00104] - positions 20-49 of mature human osteocalcin

[00105] - positions 1-43 of mature human osteocalcin

[00106] - positions 1-42 of mature human osteocalcin

[00107] - positions 1-41 of mature human osteocalcin

[00108] - positions 1-40 of mature human osteocalcin

[00109] - positions 1-39 of mature human osteocalcin

[00110] - positions 1-38 of mature human osteocalcin

[00111] - positions 1-37 of mature human osteocalcin

[00112] - positions 1-36 of mature human osteocalcin

[00113] - positions 1-35 of mature human osteocalcin

[00114] - positions 1-34 of mature human osteocalcin

[00115] - positions 1-33 of mature human osteocalcin

[00116] - positions 1-32 of mature human osteocalcin

[00117] - positions 1-31 of mature human osteocalcin

[00118] - positions 1-30 of mature human osteocalcin

[00119] - positions 1-29 of mature human osteocalcin

[00120] - positions 2-49 of mature human osteocalcin

[00121] - positions 2-45 of mature human osteocalcin

[00122] - positions 2-40 of mature human osteocalcin

[00123] - positions 2-35 of mature human osteocalcin

[00124] - positions 2-30 of mature human osteocalcin

[00125] - positions 2-25 of mature human osteocalcin

[00126] - positions 2-20 of mature human osteocalcin

[00127] - positions 4-49 of mature human osteocalcin

[00128] - positions 4-45 of mature human osteocalcin

[00129] - positions 4-40 of mature human osteocalcin

[00130] - positions 4-35 of mature human osteocalcin

[00131] - positions 4-30 of mature human osteocalcin

[00132] - positions 4-25 of mature human osteocalcin

[00133] - positions 4-20 of mature human osteocalcin

[00134] - positions 8-49 of mature human osteocalcin

[00135] - positions 8-45 of mature human osteocalcin

[00136] - positions 8-40 of mature human osteocalcin

[00137] - positions 8-35 of mature human osteocalcin

[00138] - positions 8-30 of mature human osteocalcin

[00139] - positions 8-25 of mature human osteocalcin

[00140] - positions 8-20 of mature human osteocalcin

[00141] - positions 10-49 of mature human osteocalcin

[00142] - positions 10-45 of mature human osteocalcin

[00143] - positions 10-40 of mature human osteocalcin

[00144] - positions 10-35 of mature human osteocalcin

[00145] - positions 10-30 of mature human osteocalcin

[00146] - positions 10-25 of mature human osteocalcin

[00147] - positions 10-20 of mature human osteocalcin

[00148] - positions 6-34 of mature human osteocalcin

[00149] - positions 6-35 of mature human osteocalcin

[00150] - positions 6-36 of mature human osteocalcin

[00151] - positions 6-37 of mature human osteocalcin

[00152] - positions 6-38 of mature human osteocalcin

[00153] - positions 7-34 of mature human osteocalcin

[00154] - positions 7-35 of mature human osteocalcin

[00155] - positions 7-36 of mature human osteocalcin

[00156] - positions 7-37 of mature human osteocalcin

[00157] - positions 7-38 of mature human osteocalcin

[00158] - positions 7-30 of mature human osteocalcin

[00159] - positions 7-25 of mature human osteocalcin

[00160] - positions 7-23 of mature human osteocalcin

[00161] - positions 7-21 of mature human osteocalcin

[00162] - positions 7-19 of mature human osteocalcin

[00163] - positions 7-17 of mature human osteocalcin

[00164] - positions 8-30 of mature human osteocalcin

[00165] - positions 8-25 of mature human osteocalcin

[00166] - positions 8-23 of mature human osteocalcin

[00167] - positions 8-21 of mature human osteocalcin

[00168] - positions 8-19 of mature human osteocalcin

[00169] - positions 8-17 of mature human osteocalcin

[00170] - positions 9-30 of mature human osteocalcin

[00171] - positions 9-25 of mature human osteocalcin

[00172] - positions 9-23 of mature human osteocalcin

[00173] - positions 9-21 of mature human osteocalcin

[00174] - positions 9-19 of mature human osteocalcin

[00175] - positions 9-17 of mature human osteocalcin

[00176] Especially preferred is a fragment comprising positions 1-36 of mature human osteocalcin. Another preferred fragment is a fragment comprising positions 20-49 of mature human osteocalcin. Other fragments can be designed to contain Pro13 to Tyr76 or Pro 13 to Asn26 of mature human osteocalcin. Additionally, fragments containing the cysteine residues at positions 23 and 29 of mature human osteocalcin, and capable of forming a disulfide bond between those two cysteines, are useful.

[00177] Fragments can be discrete (not fused to other amino acids or polypeptides) or can be within a larger polypeptide. Further, several fragments can be comprised within a single larger polypeptide. In one embodiment, a fragment designed for expression in a host can have heterologous pre- and pro-polypeptide regions fused to the amino terminus of the osteocalcin fragment and/or an additional region fused to the carboxyl terminus of the fragment.

[00178] Also provided for use in the compositions and methods of the present invention are variants of osteocalcin and the osteocalcin fragments described above. "Variants" refers to osteocalcin peptides that contain modifications in their amino acid sequences such as one or more amino acid substitutions, additions, deletions and/or insertions but that are still biologically active. In some instances, the antigenic and/or immunogenic properties of the variants are not

substantially altered, relative to the corresponding peptide from which the variant was derived. Such modifications may be readily introduced using standard mutagenesis techniques, such as oligonucleotide directed site-specific mutagenesis as taught, for example, by Adelman et al., 1983, DNA 2:183, or by chemical synthesis. Variants and fragments are not mutually exclusive terms. Fragments also include peptides that may contain one or more amino acid substitutions, additions, deletions and/or insertions such that the fragments are still biologically active.

[00179] One particular type of variant that is within the scope of the present invention is a variant in which one of more of the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin is occupied by an amino acid that is not glutamic acid. In some embodiments, the amino acid that is not glutamic acid is also not aspartic acid. Such variants are versions of undercarboxylated osteocalcin because at least one of the three positions corresponding to positions 17, 21 and 24 of mature human osteocalcin is not carboxylated glutamic acid, since at least one of those positions is not occupied by glutamic acid.

[00180] In particular embodiments, the present invention provides osteocalcin variants comprising the amino acid sequence

YLYQWLGAPV PYPDPLX$_1$PRR X$_2$VCX$_3$LNPDCD ELADHIGFQE AYRRFYGPV (SEQ. ID. NO:13)

wherein

X_1, X_2 and X_3 are each independently selected from an amino acid or amino acid analog, with the proviso that if X_1, X_2 and X_3 are each glutamic acid, then X_1 is not carboxylated, or less than 50 percent of X_2 is carboxylated, and/or less than 50 percent of X_3 is carboxylated.

[00181] In certain embodiments, the osteocalcin variants comprise an amino acid sequence that is different from SEQ. ID. NO:13 at 1 to 7 positions other than X_1, X_2 and X_3.

[00182] In other embodiments, the osteocalcin variants comprise an amino acid sequence that includes one or more amide backbone substitutions.

[00183] Fully functional variants typically contain only conservative variation or variation in non-critical residues or in non-critical regions. Functional variants can also contain substitutions of similar amino acids, which results in no change, or an insignificant change, in function. Alternatively, such substitutions may positively or negatively affect function to some degree. The activity of such functional osteocalcin variants can be determined using assays such as those described herein.

[00184] Variants can be naturally-occurring or can be made by recombinant means, or chemical synthesis, to provide useful and novel characteristics for undercarboxylated/uncarboxylated osteocalcin. For example, the variant osteocalcin polypeptides may have reduced immunogenicity, increased serum half-life, increased bioavailability and/or increased potency. In particular embodiments, serum half-life is increased by substituting one or more of the native Arg residues at positions 19, 20, 43, and 44 of mature osteocalcin with another amino acid or an amino acid analog, e.g., β-dimethyl-arginine. Such substitutions can be combined with the other changes in the native amino acid sequence of osteocalcin described herein.

[00185] Provided for use in the pharmaceutical compositions and methods of the present invention are variants that are also derivatives of the osteocalcin and osteocalcin fragments

described above. Derivatization is a technique used in chemistry which transforms a chemical compound into a product of similar chemical structure, called derivative. Generally, a specific functional group of the compound participates in the derivatization reaction and transforms the compound to a derivate of different reactivity, solubility, boiling point, melting point, aggregate state, functional activity, or chemical composition. Resulting new chemical properties can be used for quantification or separation of the derivatized compound or can be used to optimize the derivatized compound as a therapeutic agent. The well-known techniques for derivatization can be applied to the above-described osteocalcin and osteocalcin fragments. Thus, derivatives of the osteocalcin and osteocalcin fragments described above will contain amino acids that have been chemically modified in some way so that they differ from the natural amino acids.

[00186] Provided also are osteocalcin mimetics. "Mimetic" refers to a synthetic chemical compound that has substantially the same structural and functional characteristics of a naturally or non-naturally occurring osteocalcin polypeptide, and includes, for instance, polypeptide- and polynucleotide-like polymers having modified backbones, side chains, and/or bases. Peptide mimetics are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. Generally, mimetics are structurally similar (i.e., have the same shape) to a paradigm polypeptide that has a biological or pharmacological activity, but one or more polypeptide linkages are replaced. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity.

[00187] By way of examples that can be adapted to osteocalcin by those skilled in the art: Cho et al., 1993, Science 261:1303-1305 discloses an "unnatural biopolymer" consisting of chiral aminocarbonate monomers substituted with a variety of side chains, synthesis of a library of such polymers, and screening for binding affinity to a monoclonal antibody. Simon et al., 1992, Proc. Natl. Acad. Sci. 89:9367-9371 discloses a polymer consisting of N-substituted glycines ("peptoids") with diverse side chains. Schumacher et al, 1996, Science 271:1854-1857 discloses D-peptide ligands identified by screening phage libraries of L-peptides against proteins synthesized with D-amino acids and then synthesizing a selected L-peptide using D-amino acids. Brody et al., 1999, Mol. Diagn. 4:381-8 describes generation and screening of hundreds to thousands of aptamers.

[00188] A particular type of osteocalcin variant within the scope of the invention is an osteocalcin mimetic in which one or more backbone amides is replaced by a different chemical structure or in which one or more amino acids are replaced by an amino acid analog. In a particular embodiment, the osteocalcin mimetic is a retroenantiomer of uncarboxylated human osteocalcin.

[00189] Osteocalcin, as well as its fragments and variants, is optionally produced by chemical synthesis or recombinant methods and may be produced as a modified osteocalcin molecule (i.e., osteocalcin fragments or variants) as described herein. Osteocalcin polypeptides can be produced by any conventional means (Houghten, 1985, Proc. Natl. Acad. Sci. USA 82:5131-5135). Simultaneous multiple peptide synthesis is described in U.S. Pat. No. 4,631,211

and can also be used. When produced recombinantly, osteocalcin may be produced as a fusion protein, e.g., a GST-osteocalcin fusion protein.

[00190] Undercarboxylated/uncarboxylated osteocalcin molecules that can be used in the methods of the invention include proteins substantially homologous to human osteocalcin, including proteins derived from another organism, i.e., an ortholog of human osteocalcin. One particular ortholog is mouse osteocalcin. Mouse osteocalcin gene 1 cDNA is SEQ ID NO:3; mouse osteocalcin gene 2 cDNA is SEQ ID NO:4; the amino acid sequence of mouse osteocalcin gene 1 and gene 2 is SEQ ID NO:5.

[00191] As used herein, two proteins are substantially homologous when their amino acid sequences are at least about 70-75% homologous. Typically the degree of homology is at least about 80-85%, and most typically at least about 90-95%, 97%, 98% or 99% or more. "Homology" between two amino acid sequences or nucleic acid sequences can be determined by using the algorithms disclosed herein. These algorithms can also be used to determine percent identity between two amino acid sequences or nucleic acid sequences.

[00192] In a specific embodiment of the invention, the undercarboxylated/uncarboxylated osteocalcin is an osteocalcin molecule sharing at least 80% homology with the human osteocalcin of SEQ ID:2 or a portion of SEQ ID:2 that is at least 8 amino acids long. In another embodiment, the undercarboxylated/uncarboxylated osteocalcin is an osteocalcin molecule sharing at least 80%, at least 90%, at least 95%, or at least 97% amino acid sequence identity with the human osteocalcin of SEQ ID:2 or a portion of SEQ ID:2 that is at least 8 amino acids long. Homologous sequences include those sequences that are substantially identical. In

preferred embodiments, the homology or identity is over the entire length of mature human osteocalcin.

[00193] To determine the percent homology or percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). Preferably, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% or more of the length of the sequence that the reference sequence is compared to. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[00194] The invention also encompasses polypeptides having a lower degree of identity but which have sufficient similarity so as to perform one or more of the same functions performed by undercarboxylated/uncarboxylated osteocalcin. Similarity is determined by considering conserved amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Conservative

substitutions are likely to be phenotypically silent. Guidance concerning which amino acid changes are likely to be phenotypically silent may be found in Bowie et al., 1990, Science 247:1306-1310.

[00195] Examples of conservative substitutions are the replacements, one for another, among the hydrophobic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between the amide residues Asn and Gln; exchange of the basic residues Lys, His and Arg; replacements among the aromatic residues Phe, Trp and Tyr; exchange of the polar residues Gln and Asn; and exchange of the small residues Ala, Ser, Thr, Met, and Gly.

[00196] The comparison of sequences and determination of percent identity and homology between two osteocalcin polypeptides can be accomplished using a mathematical algorithm. See, for example, Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, HG., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, van Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991. A non-limiting example of such a mathematical algorithm is described in Karlin et al., 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877.

[00197] The percent identity or homology between two osteocalcin amino acid sequences may be determined using the Needleman et al., 1970, J. Mol. Biol. 48:444-453 algorithm.

[00198] A substantially homologous osteocalcin, according to the present invention, may also be a polypeptide encoded by a nucleic acid sequence capable of hybridizing to the human osteocalcin nucleic acid sequence under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encoding a functionally equivalent gene product; or under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989 supra), yet which still encodes a biologically active undercarboxylated/uncarboxylated osteocalcin.

[00199] A substantially homologous osteocalcin according to the present invention may also be a polypeptide encoded by a nucleic acid sequence capable of hybridizing to a sequence having at least 70-75%, typically at least about 80-85%, and most typically at least about 90-95%, 97%, 98% or 99% identity to the human osteocalcin nucleic acid sequence, under stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encoding a functionally equivalent gene product; or under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989 supra), yet which still encodes a biologically active undercarboxylated/uncarboxylated osteocalcin.

[00200] It will be understood that a biologically active fragment or variant of human osteocalcin may contain a different number of amino acids than native human osteocalcin. Accordingly, the position number of the amino acid residues corresponding to positions 17, 21 and 24 of mature human osteocalcin may differ in the fragment or variant. One skilled in the art would easily recognize such corresponding positions from a comparison of the amino acid sequence of the fragment or variant with the amino acid sequence of mature human osteocalcin.

[00201] Peptides corresponding to fusion proteins in which full length osteocalcin, mature osteocalcin, or an osteocalcin fragment or variant is fused to an unrelated protein or polypeptide are also within the scope of the invention and can be designed on the basis of the osteocalcin nucleotide and amino acid sequences disclosed herein. Such fusion proteins include fusions to an enzyme, fluorescent protein, or luminescent protein which provides a marker function. In a preferred embodiment of the invention, the fusion protein comprises fusion to a polypeptide capable of targeting the osteocalcin to a particular target cell or location in the body. For example, osteocalcin polypeptide sequences may be fused to a ligand molecule capable of targeting the fusion protein to a cell expressing the receptor for said ligand. In a particular embodiment, osteocalcin polypeptide sequences may be fused to a ligand capable of targeting the fusion protein to cells of the testes, e.g., Leydig cells.

[00202] Osteocalcin can also be made as part of a chimeric protein for drug screening or use in making recombinant protein. These chimeric proteins comprise an osteocalcin peptide sequence linked to a heterologous peptide having an amino acid sequence not substantially homologous to the osteocalcin. The heterologous peptide can be fused to the N-terminus or C-

terminus of osteocalcin or can be internally located. In one embodiment, the fusion protein does not affect osteocalcin function. For example, the fusion protein can be a GST-fusion protein in which the osteocalcin sequences are fused to the N- or C-terminus of the GST sequences. Other types of fusion proteins include, but are not limited to, enzymatic fusion proteins, for example beta-galactosidase fusions, yeast two-hybrid GAL-4 fusions, poly-His fusions and Ig fusions. Such fusion proteins, particularly poly-His fusions, can facilitate the purification of recombinant osteocalcin. In certain host cells (e.g., mammalian host cells), expression and/or secretion of a protein can be increased by using a heterologous signal sequence. Therefore, the fusion protein may contain a heterologous signal sequence at its N-terminus.

[00203] Those skilled in art would understand how to adapt well-known techniques for use with osteocalcin. For example, EP 0 464 533 discloses fusion proteins comprising various portions of immunoglobulin constant regions (Fc regions). The Fc region is useful in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (see, e.g., EP 0 232 262). In drug discovery, for example, human proteins have been fused with Fc regions for the purpose of high-throughput screening assays to identify antagonists (Bennett et al., 1995, J. Mol. Recog. 8:52-58 and Johanson et al., 1995, J. Biol. Chem. 270:9459-9471). Thus, various embodiments of this invention also utilize soluble fusion proteins containing an osteocalcin polypeptide and various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (e.g., IgG, IgM, lgA, IgE, lgB). Preferred as immunoglobulin is the constant part of the heavy chain of human IgG, particularly IgG1, where fusion takes place at the hinge region. For some uses, it is desirable to remove the Fc region after the fusion protein has been used for its intended purpose. In a particular embodiment, the

Fc part can be removed in a simple way by a cleavage sequence, which is also incorporated and can be cleaved, e.g., with factor Xa.

[00204] A chimeric or fusion protein can be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different protein sequences can be ligated together in-frame in accordance with conventional techniques. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence (see Ausubel et al., 1992, Current Protocols in Molecular Biology). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST protein). An osteocalcin-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to osteocalcin.

[00205] Chimeric osteocalcin proteins can be produced in which one or more functional sites are derived from a different isoform, or from another osteocalcin molecule from another species. Sites also could be derived from osteocalcin-related proteins that occur in the mammalian genome but which have not yet been discovered or characterized.

[00206] Polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally-occurring amino acids. Further, many amino acids, including the terminal amino acids, may be modified by natural processes, such as processing and other post-translational modifications, or by chemical modification techniques well known in the art.

[00207] Accordingly, the osteocalcin polypeptides useful in the methods of the present invention also encompass derivatives which contain a substituted non-naturally occurring amino acid residue that is not one encoded by the genetic code, in which a substituent group is included, in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or in which the additional amino acids are fused to the osteocalcin polypeptide, such as a leader or secretory sequence or a sequence for purification of the osteocalcin polypeptide or a pro-protein sequence.

[00208] Undercarboxylated/uncarboxylated osteocalcin can be modified according to known methods in medicinal chemistry to increase its stability, half-life, uptake or efficacy. Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[00209] In a specific embodiment of the invention, modifications may be made to the osteocalcin to reduce susceptibility to proteolysis at residue Arg43 as a means for increasing

serum half life. Such modifications include, for example, the use of retroenantio isomers, D-amino acids, or other amino acid analogs.

[00210] Acylation of the N-terminal amino group can be accomplished using a hydrophilic compound, such as hydroorotic acid or the like, or by reaction with a suitable isocyanate, such as methylisocyanate or isopropylisocyanate, to create a urea moiety at the N-terminus. Other agents can also be N-terminally linked that will increase the duration of action of the osteocalcin derivative.

[00211] Reductive amination is the process by which ammonia is condensed with aldehydes or ketones to form imines which are subsequently reduced to amines. Reductive amination is a useful method for conjugating undercarboxylated/uncarboxylated osteocalcin and its fragments or variants to polyethylene glycol (PEG). Covalent linkage of PEG to undercarboxylated/uncarboxylated osteocalcin and its fragments and variants may result in conjugates with increased water solubility, altered bioavailability, pharmacokinetics, immunogenic properties, and biological activities. See, e.g., Bentley et al., 1998, J. Pharm. Sci. 87:1446-1449.

[00212] Several particularly common modifications that may be applied to undercarboxylated/uncarboxylated osteocalcin and its fragments and variants such as glycosylation, lipid attachment, sulfation, hydroxylation and ADP-ribosylation are described in most basic texts, such as Proteins-Structure and Molecular Properties, 2nd ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as by Wold, F., Posttranslational Covalent Modification of Proteins, B. C. Johnson,

Ed., Academic Press, New York 1-12 (1983); Seifter et al., 1990, Meth. Enzymol. 182:626-646 and Rattan et al., 1992, Ann. New York Acad. Sci. 663:48-62.

[00213] As is also well known, polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of post-translation events, including natural processing events and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translational natural processes and by synthetic methods. Well-known techniques for preparing such non-linear polypeptides may be adapted by those skilled in the art to produce non-linear osteocalcin polypeptides.

[00214] Modifications can occur anywhere in the undercarboxylated/uncarboxylated osteocalcin and its fragments and variants, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. Blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally-occurring and synthetic polypeptides and may be applied to the undercarboxylated/uncarboxylated osteocalcin or its fragments and variants used in the present invention. For instance, the amino terminal residue of polypeptides made in *E. coli*, prior to proteolytic processing, almost invariably will be N-formylmethionine. Thus, the use of undercarboxylated/uncarboxylated osteocalcin and its fragments and variants with N-formylmethionine as the amino terminal residue are within the scope of the present invention.

[00215] A brief description of various protein modifications that come within the scope of this invention are set forth in the table below:

Table 1

Protein Modification	Description
Acetylation	Acetylation of N-terminus or ε-lysines. Introducing an acetyl group into a protein, specifically, the substitution of an acetyl group for an active hydrogen atom. A reaction involving the replacement of the hydrogen atom of a hydroxyl group with an acetyl group (CH_3CO) yields a specific ester, the acetate. Acetic anhydride is commonly used as an acetylating agent, which reacts with free hydroxyl groups. Acylation may facilitate addition of other functional groups. A common reaction is acylation of e.g., conserved lysine residues with a biotin appendage.
ADP-ribosylation	Covalently linking proteins or other compounds via an arginine-specific reaction.
Alkylation	Alkylation is the transfer of an alkyl group from one molecule to another. The alkyl group may be transferred as an alkyl carbocation, a free radical or a carbanion (or their equivalents). Alkylation is accomplished by using certain functional groups such as alkyl electrophiles, alkyl nucleophiles or sometimes alkyl radicals or carbene acceptors. A common example is methylation (usually at a lysine or arginine residue).
Amidation	Reductive animation of the N-terminus. Methods for amidation of insulin are described in U.S. 4,489,159.

Protein Modification	Description
Carbamylation	Nigen et al. describes a method of carbamylating hemoglobin.
Citrullination	Citrullination involves the addition of citrulline amino acids to the arginine residues of a protein, which is catalyzed by peptidylarginine deaminase enzymes (PADs). This generally converts a positively charged arginine into a neutral citrulline residue, which may affect the hydrophobicity of the protein (and can lead to unfolding).
Condensation of amines with aspartate or glutamate	Such reactions, may be used, e.g., to attach a peptide to other proteins labels.
Covalent attachment of flavin	Flavin mononucleotide (FAD) may be covalently attached to serine and/or threonine residues. May be used, e.g., as a light-activated tag.
Covalent attachment of heme moiety	A heme moiety is generally a prosthetic group that consists of an iron atom contained in the center of a large heterocyclic organic ring, which is referred to as a porphyrin. The heme moiety may be used, e.g., as a tag for the peptide.
Attachment of a nucleotide or nucleotide derivative	May be used as a tag or as a basis for further derivatising a peptide.
Cross-linking	Cross-linking is a method of covalently joining two proteins. Cross-linkers contain reactive ends to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other molecules. Several chemical groups may be targets for reactions in proteins and peptides. For example, Ethylene glycol bis[succinimidylsuccinate, Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, and Bis[sulfosuccinimidyl] suberate link amines to amines.
Cyclization	For example, cyclization of amino acids to create optimized delivery forms that are resistant to, e.g., aminopeptidases (e.g., formation of pyroglutamate, a cyclized form of glutamic acid).

Protein Modification	Description
Disulfide bond formation	Disulfide bonds in proteins are formed by thiol-disulfide exchange reactions, particularly between cysteine residues (e.g., formation of cystine).
Demethylation	See, e.g., U.S. 4,250,088 (Process for demethylating lignin).
Formylation	The addition of a formyl group to, e.g., the N-terminus of a protein. See, e.g., U.S. Patent Nos. 4,059,589, 4,801,742, and 6,350,902.
Glycylation	The covalent linkage of one to more than 40 glycine residues to the tubulin C-terminal tail.
Glycosylation	Glycosylation may be used to add saccharides (or polysaccharides) to the hydroxy oxygen atoms of serine and threonine side chains (which is also known as O-linked Glycosylation). Glycosylation may also be used to add saccharides (or polysaccharides) to the amide nitrogen of asparagine side chains (which is also known as N-linked Glycosylation), e.g., via oligosaccharyl transferase.
GPI anchor formation	The addition of glycosylphosphatidylinositol to the C-terminus of a protein. GPI anchor formation involves the addition of a hydrophobic phosphatidylinositol group - linked through a carbohydrate containing linker (e.g., glucosamine and mannose linked to phosphoryl ethanolamine residue) - to the C-terminal amino acid of a protein.

Protein Modification	Description
Hydroxylation	Chemical process that introduces one or more hydroxyl groups (-OH) into a protein (or radical). Hydroxylation reactions are typically catalyzed by hydroxylases. Proline is the principal residue to be hydroxylated in proteins, which occurs at the C^{γ} atom, forming hydroxyproline (Hyp). In some cases, proline may be hydroxylated at its C^{β} atom. Lysine may also be hydroxylated on its C^{δ} atom, forming hydroxylysine (Hyl). These three reactions are catalyzed by large, multi-subunit enzymes known as prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl 5-hydroxylase, respectively. These reactions require iron (as well as molecular oxygen and α-ketoglutarate) to carry out the oxidation, and use ascorbic acid to return the iron to its reduced state.
Iodination	See, e.g., U.S. 6,303,326 for a disclosure of an enzyme that is capable of iodinating proteins. U.S. 4,448,764 discloses, e.g., a reagent that may be used to iodinate proteins.
ISGylation	Covalently linking a peptide to the ISG15 (Interferon-Stimulated Gene 15) protein, for, e.g., modulating immune response.
Methylation	Reductive methylation of protein amino acids with formaldehyde and sodium cyanoborohydride has been shown to provide up to 25% yield of N-cyanomethyl ($-CH_2CN$) product. The addition of metal ions, such as Ni^{2+}, which complex with free cyanide ions, improves reductive methylation yields by suppressing by-product formation. The N-cyanomethyl group itself, produced in good yield when cyanide ion replaces cyanoborohydride, may have some value as a reversible modifier of amino groups in proteins. (Gidley et al.) Methylation may occur at the arginine and lysine residues of a protein, as well as the N- and C-terminus thereof.

Protein Modification	Description
Myristoylation	Myristoylation involves the covalent attachment of a myristoyl group (a derivative of myristic acid), via an amide bond, to the alpha-amino group of an N-terminal glycine residue. This addition is catalyzed by the N-myristoyltransferase enzyme.
Oxidation	-Oxidation of cysteines. -Oxidation of N-terminal Serine or Threonine residues (followed by hydrazine or aminooxy condensations). -Oxidation of glycosylations (followed by hydrazine or aminooxy condensations).
Palmitoylation	Palmitoylation is the attachment of fatty acids, such as palmitic acid, to cysteine residues of proteins. Palmitoylation increases the hydrophobicity of a protein.
(Poly)glutamylation	Polyglutamylation occurs at the glutamate residues of a protein. Specifically, the gamma-carboxy group of a glutamate will form a peptide-like bond with the amino group of a free glutamate whose alpha-carboxy group may be extended into a polyglutamate chain. The glutamylation reaction is catalyzed by a glutamylase enzyme (or removed by a deglutamylase enzyme). Polyglutamylation has been carried out at the C-terminus of proteins to add up to about six glutamate residues. Using such a reaction, Tubulin and other proteins can be covalently linked to glutamic acid residues.
Phosphopantetheinylation	The addition of a 4'-phosphopantetheinyl group.

Protein Modification	Description
Phosphorylation	A process for phosphorylation of a protein or peptide by contacting a protein or peptide with phosphoric acid in the presence of a non-aqueous apolar organic solvent and contacting the resultant solution with a dehydrating agent is disclosed e.g., in U.S. 4,534,894. Insulin products are described to be amenable to this process. See, e.g., U.S. 4,534,894. Typically, phosphorylation occurs at the serine, threonine, and tyrosine residues of a protein.
Prenylation	Prenylation (or isoprenylation or lipidation) is the addition of hydrophobic molecules to a protein. Protein prenylation involves the transfer of either a farnesyl (linear grouping of three isoprene units) or a geranyl-geranyl moiety to C-terminal cysteine(s) of the target protein.
Proteolytic Processing	Processing, e.g., cleavage of a protein at a peptide bond.
Selenoylation	The exchange of, e.g., a sulfur atom in the peptide for selenium, using a selenium donor, such as selenophosphate.
Sulfation	Processes for sulfating hydroxyl moieties, particularly tertiary amines, are described in, e.g., U.S. 6,452,035. A process for sulphation of a protein or peptide by contacting the protein or peptide with sulphuric acid in the presence of a non-aqueous apolar organic solvent and contacting the resultant solution with a dehydrating agent is disclosed. Insulin products are described to be amenable to this process. See, e.g., U.S. 4,534,894.
SUMOylation	Covalently linking a peptide a SUMO (small ubiquitin-related Modifier) protein, for, e.g., stabilizing the peptide.
Transglutamination	Covalently linking other protein(s) or chemical groups (e.g., PEG) via a bridge at glutamine residues
tRNA-mediated addition of amino acids (e.g., arginylation)	For example, the site-specific modification (insertion) of an amino acid analog into a peptide.

Protein Modification	Description
Ubiquitination	The small peptide ubiquitin is covalently linked to, e.g., lysine residues of a protein. The ubiquitin-proteasome system can be used to carryout such reaction. See, e.g., U.S. 2007-0059731.

[00216] The present invention also encompasses the use of prodrugs of undercarboxylated/uncarboxylated osteocalcin or derivative or variant thereof that can be produced by esterifying the carboxylic acid functions of the undercarboxylated/uncarboxylated osteocalcin or derivative or variant thereof with a lower alcohol, e.g., methanol, ethanol, propanol, isopropanol, butanol, etc. The use of prodrugs of the undercarboxylated/uncarboxylated osteocalcin or derivative or variant thereof that are not esters is also contemplated. For example, pharmaceutically acceptable carbonates, thiocarbonates, N-acyl derivatives, N-acyloxyalkyl derivatives, quaternary derivatives of tertiary amines, N-Mannich bases, Schiff bases, amino acid conjugates, phosphate esters, metal salts and sulfonate esters of the undercarboxylated/uncarboxylated osteocalcin or derivative or variant thereof are also contemplated. In some embodiments, the prodrugs will contain a biohydrolyzable moiety (e.g., a biohydrolyzable amide, biohydrolyzable carbamate, biohydrolyzable carbonate, biohydrolyzable ester, biohydrolyzable phosphate, or biohydrolyzable ureide analog). Guidance for the preparation of prodrugs of the undercarboxylated/uncarboxylated osteocalcin or derivative or variant thereof disclosed herein can be found in publications such as Design of Prodrugs, Bundgaard, A. Ed., Elsevier, 1985; Design and Application of Prodrugs, A Textbook of

Drug Design and Development, Krosgaard-Larsen and H. Bundgaard, Ed., 1991, Chapter 5, pages 113-191; and Bundgaard, H., Advanced Drug Delivery Review, 1992, 8, pages 1-38.

[00217] To practice the methods of the present invention, it may be desirable to recombinantly express osteocalcin, e.g., by recombinantly expressing a cDNA sequence encoding osteocalcin. The cDNA sequence and deduced amino acid sequence of human osteocalcin is represented in SEQ ID NO:1 and SEQ ID NO:2. Osteocalcin nucleotide sequences may be isolated using a variety of different methods known to those skilled in the art. For example, a cDNA library constructed using RNA from a tissue known to express osteocalcin can be screened using a labeled osteocalcin probe. Alternatively, a genomic library may be screened to derive nucleic acid molecules encoding osteocalcin. Further, osteocalcin nucleic acid sequences may be derived by performing a polymerase chain reaction (PCR) using two oligonucleotide primers designed on the basis of known osteocalcin nucleotide sequences. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express osteocalcin.

[00218] While the osteocalcin polypeptides and peptides can be chemically synthesized (e.g., see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y.), large polypeptides derived from osteocalcin and the full length osteocalcin itself may be advantageously produced by recombinant DNA technology using techniques well known in the art for expressing a nucleic acid. Such methods can be used to construct expression vectors containing the osteocalcin nucleotide sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques,

synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Ausubel et al., 1989, supra.

[00219] A variety of host-expression vector systems may be utilized to express the osteocalcin nucleotide sequences. In a preferred embodiment, the osteocalcin peptide or polypeptide is secreted and may be recovered from the culture media.

[00220] Appropriate expression systems can be chosen to ensure that the correct modification, processing and subcellular localization of the osteocalcin protein occurs. To this end, bacterial host cells are useful for expression of osteocalcin, as such cells are unable to carboxylate osteocalcin.

[00221] The isolated osteocalcin can be purified from cells that naturally express it, e.g., osteoblasts, or purified from cells that naturally express osteocalcin but have been recombinantly modified to overproduce osteocalcin, or purified from cells that that do not naturally express osteocalcin but have been recombinantly modified to express osteocalcin. In a particular embodiment, a recombinant cell has been manipulated to activate expression of the endogenous osteocalcin gene. For example, International Patent Publications WO 99/15650 and WO 00/49162 describe a method of expressing endogenous genes termed random activation of gene expression (RAGE), which can be used to activate or increase expression of endogenous osteocalcin. The RAGE methodology involves non-homologous recombination of a regulatory sequence to activate expression of a downstream endogenous gene. Alternatively, International Patent Publications WO 94/12650, WO 95/31560, and WO 96/29411, as well as U.S. Patent No. 5,733,761 and U.S. Patent No. 6,270,985, describe a method of increasing expression of an

endogenous gene that involves homologous recombination of a DNA construct that includes a targeting sequence, a regulatory sequence, an exon, and a splice-donor site. Upon homologous recombination, a downstream endogenous gene is expressed. The methods of expressing endogenous genes described in the foregoing patents are hereby expressly incorporated by reference.

[00222] In certain embodiments of methods of the present invention where the therapeutic agent is undercarboxylated/uncarboxylated osteocalcin or a derivative or variant thereof, the undercarboxylated/uncarboxylated osteocalcin or a derivative or variant thereof is administered to a patient in a dosage range of from about 0.5 μg/kg/day to about 100 mg/kg/day, from about 1 μg/kg/day to about 90 mg/kg/day, from about 5 μg/kg/day to about 85 mg/kg/day, from about 10 μg/kg/day to about 80 mg/kg/day, from about 20 μg/kg/day to about 75 mg/kg/day, from about 50 μg/kg/day to about 70 mg/kg/day, from about 150 μg/kg/day to about 65 mg/kg/day, from about 250 μg/kg/day to about 50 mg/kg/day, from about 500 μg/kg/day to about 50 mg/kg/day, from about 1 mg/kg/day to about 50 mg/kg/day, from about 5 mg/kg/day to about 40 mg/kg/day, from about 10 mg/kg/day to about 35 mg/kg/day, from about 15 mg/kg/day to about 30 mg/kg/day, from about 5 mg/kg/day to about 16 mg/kg/day, or from about 5 mg/kg/day to about 15 mg/kg/day.

[00223] In certain embodiments of methods of the present invention where the therapeutic agent is undercarboxylated/uncarboxylated osteocalcin or a derivative or variant thereof, the undercarboxylated/uncarboxylated osteocalcin or a derivative or variant thereof is administered to a patient in a dosage range of from about 0.5 μg/kg/day to about 100 μg/kg/day, from about 1

μg/kg/day to about 80 μg/kg/day, from about 3 μg/kg/day to about 50 μg/kg/day, or from about 3 μg/kg/day to about 30 μg/kg/day.

[00224] In certain embodiments of methods of the present invention where the therapeutic agent is undercarboxylated/uncarboxylated osteocalcin or a derivative or variant thereof, the undercarboxylated/uncarboxylated osteocalcin or a derivative or variant thereof is administered to a patient in a dosage range of from about 0.5 ng/kg/day to about 100 ng/kg/day, from about 1 ng/kg/day to about 80 ng/kg/day, from about 3 ng/kg/day to about 50 ng/kg/day, or from about 3 ng/kg/day to about 30 ng/kg/day.

COMPOSITIONS COMPRISING INHIBITORS OF GAMMA-CARBOXYLASE, PTP-1B, AND/OR OST-PTP

[00225] In certain embodiments of the invention, the pharmaceutical compositions useful in the method of the invention comprise an inhibitor that reduces the expression or activity of gamma-carboxylase, PTP-1B, or OST-PTP. Preferably, the biological activity of gamma-carboxylase, PTP-1B, or OST-PTP is inhibited. The inhibitors may be antibodies (monoclonal or polyclonal) or fragments of antibodies, small molecules, polypeptides or proteins, or nucleic acids (e.g., antisense DNA or RNA, siRNA).

[00226] In certain embodiments, the inhibitors reduce the activity of OST-PTP having the amino acid sequence of SEQ ID NO:11. In other embodiments, the inhibitors reduce the activity of an OST-PTP having an amino acid sequence that is substantially homologous or substantially identical, as previously described, to the amino acid sequence of SEQ ID NO:11.

[00227] In certain embodiments, the inhibitors reduce the activity of human PTP-1B having the amino acid sequence of SEQ ID NO:17. In other embodiments, the inhibitors reduce the activity of a PTP-1B having an amino acid sequence that is substantially homologous or substantially identical, as previously described, to the amino acid sequence of SEQ ID NO:17.

[00228] In certain embodiments, the inhibitors reduce the activity of human gamma-carboxylase having the amino acid sequence of SEQ ID NO:7. In other embodiments, the inhibitors reduce the activity of a gamma-carboxylase having an amino acid sequence that is substantially homologous or identical to SEQ ID NO:7.

SMALL MOLECULE INHIBITORS OF OST-PTP, PTP-1B, AND GAMMA-CARBOXYLASE

[00229] In certain embodiments, the agent is a small molecule. By "small molecule" is meant organic compounds of molecular weight of more than 100 and less than about 2,500 daltons, and preferably less than 500 daltons. Such small molecules inhibit the biological activity of OST-PTP, PTP-1B, or gamma-carboxylase.

[00230] The small molecule inhibitors may comprise agents that act as inhibitors of vitamin K. Warfarin and other vitamin K inhibitors, including Coumadin and other derivatives, may be administered to patients who would benefit from inhibition of gamma-carboxylase in order to treat or prevent a disorder related to reproduction in male mammals. In a specific embodiment of the invention, the small molecule warfarin may be used to inhibit the activity of gamma-carboxylase. Warfarin derivatives are exemplified by acenocoumarol, phenprocoumon and phenindione. Warfarin and other Coumadin derivatives block vitamin K-dependent gamma-

carboxylation of osteocalcin, thus increasing the level of undercarboxylated/uncarboxylated osteocalcin.

[00231] Other inhibitors include thiol specific inhibitors of gamma-carboxylase. Cys and His residues of gamma-carboxylase are implicated in the carboxylase mechanism of gamma-carboxylase and it is observed that the enzyme is inhibited by thiol-specific inhibitors, such as *N*-ethylmaleimide (NEM) and mercurials such as *p*-hydroxymurcuribenzoate (pHMB). Additional non-limiting examples of these inhibitors include 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 2-nitro-5-thiocyanobenzoic acid (NTCB), iodoacetamide (IA), N-phenylmaleimide (PheM), N-(1-pyrenyl) maleimide (PyrM), naphthalene-1,5-dimaleimide (NDM), N,N'-(1,2-phenylene) dimaleimide (oPDM), N,N'-1,4-phenylene dimaleimide (pPDM), N,N'-1,3-phenylene dimaleimide (mPDM), 1,1-(methylenedi-4,1-phenylene)bismaleimide (BM), 4-(N-maleimido)phenyltrimethylammonium (MPTM), N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (BMP), N-succinimidyl 3-(2-pyridyldithio)propionate, diethyl pyrocarbonate, p-chloromercuribenzene sulphonic acid and thiosulfinates. These inhibitors may also be provided as conjugate or derivative, such as with, e.g., BSA or aminodextran.

ANTIBODY INHIBITORS OF OST-PTP, PTP-1B, AND GAMMA-CARBOXYLASE

[00232] The present invention also provides compositions comprising an antibody or antibodies, as well as biologically active fragments or variants thereof, that are capable of binding to an epitope of OST-PTP, PTP-1B, or gamma-carboxylase polypeptides and inhibiting the activity of OST-PTP, PTP-1B, or gamma-carboxylase.

[00233] An antibody against OST-PTP that decreases its activity can be used therapeutically. In certain embodiments, the antibody against OST-PTP binds to the extracellular domain of OST-PTP.

[00234] In certain embodiments, the antibody against OST-PTP binds to an epitope in the mouse OST-PTP of SEQ ID NO:11 or an OST-PTP having an amino acid sequence that is substantially homologous or identical to SEQ ID NO:11. In other embodiments, the antibody against OST-PTP binds to an epitope in an OST-PTP having an amino acid sequence that is at least 70% homologous or identical to SEQ ID NO:11.

[00235] Human OST-PTP can be obtained by isolating the human ortholog of mouse OST-PTP (SEQ ID NO:10) or rat OST-PTP (SEQ ID NO:14) by methods known in the art. For example, one could prepare a cDNA library from human osteoblasts and identify human OST-PTP cDNA by hybridizing the cDNA clones from the library to a mouse probe. The mouse probe could be based on a portion of mouse OST-PTP (SEQ ID NO:10). Alternatively, PCR, using primers based on the mouse sequence, can be used to obtain the human OST-PTP gene.

[00236] An antibody against human PTP-1B that decreases its activity can be used therapeutically in the methods of the present invention. In certain embodiments, the antibody against human PTP-1B binds to the extracellular domain of human PTP-1B.

[00237] In certain embodiments, the antibody against human PTP-1B binds to an epitope in the human PTP-1B of SEQ ID NO:17 or an OST-PTP having an amino acid sequence that is substantially homologous or identical to SEQ ID NO:17. In other embodiments, the antibody

against human PTP-1B binds to an epitope in a human PTP-1B having an amino acid sequence that is at least 70% homologous or identical to SEQ ID NO:17.

[00238] Gamma-carboxylase is an intracellular protein, so antibodies or fragments of antibodies against it are preferably used therapeutically when combined with technologies for delivering the antibodies, fragments or variants into the interior of target cells expressing gamma-carboxylase, e.g., osteoblasts. Antibodies or antibody fragments or variants against osteocalcin similarly can be used with technologies for delivering the antibodies or fragments into the interior of target cells and can also be used in diagnostics and drug screening assays.

[00239] In a particular embodiment, the present invention provides antibodies, fragments or variants of antibodies that recognize an epitope in OST-PTP that includes the amino acid at position 1316 of mouse OST-PTP or the corresponding position of human OST-PTP. In certain embodiments, these antibodies, fragments or variants of antibodies block or inhibit the ability of OST-PTP to activate gamma-carboxylase. In certain embodiments, use of these antibodies or fragments results in OST-PTP losing 50%, 60%, 70%, 80%, 90%, 95%, or essentially all of its ability to activate gamma-carboxylase.

[00240] The term "epitope" refers to an antigenic determinant on an antigen to which an antibody binds. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains, and typically have specific three-dimensional structural characteristics, as well as specific charge characteristics. Epitopes generally have at least five contiguous amino acids but some epitopes are formed by discontiguous amino acids that are brought together by the folding of the protein that contains them.

[00241] The terms "antibody" and "antibodies" include polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, single chain Fv antibody fragments, Fab fragments, and $F(ab')_2$ fragments. Polyclonal antibodies are heterogeneous populations of antibody molecules that are specific for a particular antigen, while monoclonal antibodies are homogeneous populations of antibodies to a particular epitope contained within an antigen. Monoclonal antibodies are particularly useful in the present invention.

[00242] Antibody fragments that have specific binding affinity for the polypeptide of interest (e.g., OST-PTP, PTP-1B, or gamma-carboxylase) can be generated by known techniques. Such antibody fragments include, but are not limited to, $F(ab')_2$ fragments that can be produced by pepsin digestion of an antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed. See, for example, Huse et al., 1989, Science 246:1275-1281. Single chain Fv antibody fragments are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge (e.g., 15 to 18 amino acids), resulting in a single chain polypeptide. Single chain Fv antibody fragments can be produced through standard techniques, such as those disclosed in U.S. Patent No. 4,946,778.

[00243] Once produced, antibodies or fragments thereof can be tested for recognition of the target polypeptide by standard immunoassay methods including, for example, enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay assay (RIA). See, Short Protocols in Molecular Biology eds. Ausubel et al., Green Publishing Associates and John Wiley & Sons (1992).

[00244] The immunoassays, immunohistochemistry, RIA, IRMAs used herein are based on the generation of various antibodies, including those that specifically bind to osteocalcin, OST-PTP, PTP-1B, gamma-carboxylase, vitamin K, or their fragments or variants. Antibodies and methods of using antibodies to quantitate the amount of osteocalcin, in particular, in a sample are also described in U.S. Patent No. 5,681,707. U.S. Patent No. 5,681,707 discloses antibodies that bind to the N-terminal 20 amino acids, or the C-terminal 14 amino acids of osteocalcin. Anti-OST-PTP antibodies are commercially available.

[00245] In one embodiment, antibodies against OST-PTP, PTP-1B, or gamma-carboxylase that reduce its activity are useful in the treatment of a patient having a disorder related to reproduction in male mammals.

NUCLEIC ACID INHIBITORS OF OST-PTP, PTP-1B, AND GAMMA-CARBOXYLASE

[00246] Other embodiments of the present invention are directed to the use of antisense nucleic acids or small interfering RNA (siRNA) to reduce or inhibit expression and hence the biological activity of osteocalcin, OST-PTP, PTP-1B, and/or gamma-carboxylase. cDNA sequences encoding osteocalcin, OST-PTP, PTP-1B, and/or gamma-carboxylase are set forth herein. Based on these sequences, antisense DNA or RNA that hybridize sufficiently to the respective gene or mRNA encoding osteocalcin, OST-PTP, PTP-1B, and/or gamma-carboxylase to turn off or reduce expression can be readily designed and engineered, using methods known in the art.

[00247] In a specific embodiment of the invention, antisense or siRNA molecules for use in the methods of the present invention include those that bind under stringent conditions to the

human gamma-carboxylase nucleic acid sequence of SEQ ID NO:6. In yet another embodiment of the invention, the antisense or siRNA molecules are those that that bind under stringent conditions to the OST-PTP nucleic acid sequence of SEQ ID NO:10, or sequences that are substantially homologous to SEQ ID NO:10.

[00248] In a specific embodiment of the invention, antisense or siRNA molecules for use in the methods of the present invention include those that bind under stringent conditions to the human PTP-1B nucleic acid sequence of SEQ ID NO:16, or sequences that are substantially homologous to SEQ ID NO:16.

[00249] Antisense-RNA and anti-sense DNA have been used therapeutically in mammals to treat various diseases. See for example Agrawal & Zhao, 1998, Curr. Opin. Chemical Biol. 2: 519-528; Agrawal & Zhao, 1997, CIBA Found. Symp. 209:60-78; and Zhao et al., 1998, Antisense Nucleic Acid Drug Dev. 8:451-458; the entire contents of which are hereby incorporated by reference as if fully set forth herein. Antisense oligodeoxyribonucleotides (antisense-DNA), oligoribonucleotides (antisense-RNA), and other polymeric antisense compounds (e.g., oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside linkages and non-naturally-occurring portions which function similarly) can base pair with a gene or its transcript. Anderson et al., 1996, Antimicrobiol. Agents Chemother. 40:2004-2011 and U.S. Patent No. 6, 828,151 describe methods for making and using antisense nucleic acids and their formulation, the entire contents of which are hereby incorporated by reference as if fully set forth herein. The disclosures of the foregoing

publications can adapted by those skilled in the art for use in the methods of the present invention.

[00250] Methods of making antisense nucleic acids are well known in the art. Further provided by the present invention are methods of modulating the expression of OST-PTP, PTP1B, and gamma-carboxylase genes and mRNA in cells or tissues by contacting the cells or tissues with one or more antisense compounds or compositions in order to treat or prevent a disorder related to reproduction in male mammals. As used herein, the term "target nucleic acid" encompasses DNA encoding osteocalcin, OST-PTP, PTP-1B, or gamma-carboxylase and RNA (including pre-mRNA and mRNA) transcribed from such DNA. The specific hybridization of a nucleic acid oligomeric compound with its target nucleic acid interferes with the normal function of the target nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense." The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of the protein encoded by the DNA or RNA. In the context of the present invention, "modulation" means reducing or inhibiting in the expression of the gene or mRNA for osteocalcin, OST-PTP and/or gamma-carboxylase. DNA is the preferred antisense nucleic acid.

[00251] The targeting process includes determination of a site or sites within the target DNA or RNA encoding the osteocalcin, OST-PTP, PTP-1B, and/or gamma-carboxylase for the antisense interaction to occur such that the desired inhibitory effect is achieved. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the mRNA for osteocalcin, OST-PTP, PTP-1B, or gamma-carboxylase, preferably human osteocalcin, OST-PTP, PTP-1B, or gamma-carboxylase. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine in eukaryotes. It is also known in the art that eukaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene. Routine experimentation will determine the optimal sequence of the antisense or siRNA.

[00252] It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively).

[00253] The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

[00254] The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene.

[00255] It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop

variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.

[00256] Once one or more target sites have been identified, nucleic acids are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect of inhibiting gene expression and transcription or mRNA translation.

[00257] In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of a nucleic acid is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the nucleic acid and the DNA or RNA are considered to be complementary to each other at that position. The nucleic acid and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the nucleic acid and the

DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of function, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

[00258] Antisense nucleic acids have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense nucleic acid drugs, including ribozymes, have been safely and effectively administered to humans in numerous clinical trials. It is thus established that nucleic acids can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans, for example to regulate expression of osteocalcin, OST-PTP, PTP-1B, and/or gamma-carboxylase.

[00259] Nucleic acids in the context of this invention includes "oligonucleotides," which refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as,

for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

[00260] While antisense nucleic acids are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e., from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense nucleic acids comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) nucleic acids (oligozymes), and other short catalytic RNAs or catalytic nucleic acids which hybridize to the target nucleic acid and modulate its expression.

[00261] The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare nucleic acids such as the phosphorothioates and alkylated derivatives.

[00262] The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, and prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder such male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes, which can be

treated by modulating the expression of osteocalcin, gamma-carboxylase, PTP-1B, or OST-PTP, is treated by administering antisense compounds in accordance with this invention. The compounds useful in the methods of the invention can be formulated into pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. The antisense compounds and methods of the present invention are useful prophylactically, e.g., to prevent or delay the appearance of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes. The antisense compounds and methods of the invention are also useful to retard the progression of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes.

[00263] The present invention also encompasses the use of siRNA to treat or prevent a disorder related to reproduction in male mammals. U.S. Patent Application Publication No. 2004/0023390 (the entire contents of which are hereby incorporated by reference as if fully set forth herein) teaches that double-stranded RNA (dsRNA) can induce sequence-specific posttranscriptional gene silencing in many organisms by a process known as RNA interference (RNAi). However, in mammalian cells, dsRNA that is 30 base pairs or longer can induce sequence-nonspecific responses that trigger a shut-down of protein synthesis and even cell death through apoptosis. Recent work shows that RNA fragments are the sequence-specific mediators of RNAi (Elbashir et al., 2001, Nature 411:494-498). Interference of gene expression by these small interfering RNA (siRNA) is now recognized as a naturally occurring strategy for silencing

genes in *C. elegans*, Drosophila, plants, and in mouse embryonic stem cells, oocytes and early embryos (Baulcombe, 1996, Plant Mol Biol. 32:79-88; Timmons & Fire, 1998, Nature 395:854; Wianny and Zernicka-Goetz, 2000, Nat Cell Biol. 2:70-75; Svoboda et al., 2000, Development 127:4147-4156).

[00264] In mammalian cell culture, a siRNA-mediated reduction in gene expression has been accomplished by transfecting cells with synthetic RNA nucleic acids (Elbashir et al., 2001, Nature 411:494-498). U.S. Patent Application Publication No. 2004/0023390, the entire contents of which are hereby incorporated by reference as if fully set forth herein, provides exemplary methods using a viral vector containing an expression cassette containing a pol II promoter operably-linked to a nucleic acid sequence encoding a small interfering RNA molecule (siRNA) targeted against a gene of interest.

[00265] As used herein, RNAi is the process of RNA interference. A typical mRNA produces approximately 5,000 copies of a protein. RNAi is a process that interferes with or significantly reduces the number of protein copies made by an mRNA, preferably encoding osteocalcin, OST-PTP, PTP-1B, or gamma-carboxylase. For example, a double-stranded short interfering RNA (siRNA) molecule is engineered to complement and match the protein-encoding nucleotide sequence of the target mRNA to be interfered with. In certain embodiments of the present invention, following intracellular delivery, the siRNA molecule associates with an RNA-induced silencing complex (RISC) and binds the target mRNA (such as mRNA encoding osteocalcin, gamma-carboxylase, PTP-1B or OST-PTP) through a base-pairing interaction and degrades it. The RISC remains capable of degrading additional copies of the targeted mRNA.

Other forms of RNA such as short hairpin RNA and longer RNA molecules can be used in the methods of the present invention. Longer molecules cause cell death, for example by instigating apoptosis and inducing an interferon response. Cell death was the major hurdle to achieving RNAi in mammals because dsRNAs longer than 30 nucleotides activated defense mechanisms that resulted in non-specific degradation of RNA transcripts and a general shutdown of the host cell. Using from about 20 to about 29 nucleotide siRNAs to mediate gene-specific suppression in mammalian cells has apparently overcome this obstacle. These siRNAs are long enough to cause gene suppression but not of a length that induces an interferon response. In a specific embodiment of the present invention, the targets for suppression are osteocalcin mRNA, OST-PTP mRNA, PTP-1B mRNA, or gamma-carboxylase mRNA. siRNA molecules useful in the methods of the present invention include those sequences that bind under stringent conditions to the human PTP-1B sequence of SEQ ID:16, the human gamma-carboxylase sequence of SEQ ID:6, or the mouse OST-PTP sequence of SEQ ID NO:10. siRNA molecules useful in the methods of the present invention also include those sequences that bind under stringent conditions to nucleic acids that are 80%, 85%, 90%, or 95% homologous to SEQ ID NO:16, SEQ ID NO:6 or SEQ ID NO:10.

FORMULATION AND ADMINISTRATION OF PHARMACEUTICAL COMPOSITIONS

[00266] The present invention encompasses the use of the polypeptides, nucleic acids, antibodies, small molecules and other therapeutic agents described herein formulated in pharmaceutical compositions to administer to a subject. The therapeutic agents (also referred to as "active compounds") can be incorporated into pharmaceutical compositions suitable for

administration to a subject, e.g., a human. Such compositions typically comprise the polypeptides, nucleic acids, antibodies, small molecules and a pharmaceutically acceptable carrier. Preferably, such compositions are non-pyrogenic when administered to humans.

[00267] The pharmaceutical compositions of the invention are administered in an amount sufficient to modulate the OST-PTP signaling pathway or the PTP-1B signaling pathway involving gamma-carboxylase and osteocalcin.

[00268] As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, binders, diluents, disintegrants, lubricants, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. As long as any conventional media or agent is compatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds or therapeutic agents can also be incorporated into the compositions. A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, intranasal, subcutaneous, oral, inhalation, transdermal (topical), transmucosal, and rectal administration.

[00269] The term "administer" is used in its broadest sense and includes any method of introducing the compositions of the present invention into a subject. This includes producing polypeptides or polynucleotides *in vivo* as by transcription or translation of polynucleotides that

have been exogenously introduced into a subject. Thus, polypeptides or nucleic acids produced in the subject from the exogenous compositions are encompassed in the term "administer."

[00270] Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylene diamine tetra acetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00271] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where the therapeutic agents are water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL® (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof.

The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00272] Sterile injectable solutions can be prepared by incorporating the active compound (e.g., undercarboxylated/uncarboxylated osteocalcin protein or anti-OST-PTP antibody) in the required amount in an appropriate solvent with one or a combination of the ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00273] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. Depending on the specific conditions being treated, pharmaceutical compositions of the present invention for treatment of disorders relating to reproduction in male mammals can be formulated and administered

systemically or locally. Techniques for formulation and administration can be found in "Remington: The Science and Practice of Pharmacy" (20th edition, Gennaro (ed.) and Gennaro, Lippincott, Williams & Wilkins, 2000). For oral administration, the agent can be contained in enteric forms to survive the stomach or further coated or mixed to be released in a particular region of the GI tract by known methods. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, PRIMOGEL®, or corn starch; a lubricant such as magnesium stearate or STEROTES®; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[00274] For administration by inhalation, the compounds may be delivered in the form of an aerosol spray from pressured container or dispenser, which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

[00275] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated

are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[00276] If appropriate, the compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[00277] In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to particular cells with, e.g., monoclonal antibodies) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

[00278] It is especially advantageous to formulate oral or parenteral compositions in unit dosage form for ease of administration and uniformity of dosage. "Unit dosage form" as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated;

each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the unit dosage forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[00279] As previously noted, the agent may be administered continuously by pump or frequently during the day for extended periods of time. In certain embodiments, the agent may be administered at a rate of from about 0.3-100 ng/hour, preferably about 1-75 ng/hour, more preferably about 5-50 ng/hour, and even more preferably about 10-30 ng/hour. The agent may be administered at a rate of from about 0.1-100 μg/hr, preferably about 1-75 μg/hr, more preferably about 5-50 μg/hr, and even more preferably about 10-30 μg/hr. It will also be appreciated that the effective dosage of antibody, protein, or polypeptide used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from monitoring the level of undercarboxylated/uncarboxylated osteocalcin in a biological sample, preferably blood or serum.

[00280] In an embodiment of the invention, the agent can be delivered by subcutaneous, long-term, automated drug delivery using an osmotic pump to infuse a desired dose of the agent for a desired time. Insulin pumps are widely available and are used by diabetics to automatically deliver insulin over extended periods of time. Such insulin pumps can be adapted to deliver the agent for use in the methods of the present invention. The delivery rate of the agent can be

readily adjusted through a large range to accommodate changing requirements of an individual (e.g., basal rates and bolus doses). New pumps permit a periodic dosing manner, i.e., liquid is delivered in periodic discrete doses of a small fixed volume rather than in a continuous flow manner. The overall liquid delivery rate for the device is controlled and adjusted by controlling and adjusting the dosing period. The pump can be coupled with a continuous monitoring device and remote unit, such as a system described in U.S. Patent No. 6,560,471, entitled "Analyte Monitoring Device and Methods of Use." In such an arrangement, the hand-held remote unit that controls the continuous blood monitoring device could wirelessly communicate with and control both the blood monitoring unit and the fluid delivery device delivering therapeutic agents for use in the methods of the present invention.

[00281] In some embodiments of the present invention, routine experimentation may be used to determine the appropriate dosage value for each patient by monitoring the effect of the therapeutic agent on serum testosterone levels, which can be frequently and easily monitored. The agent can be administered once or multiple times per day. Serum testosterone levels can be monitored before and during therapy to determine the appropriate amount of therapeutic agent to administer to raise serum testosterone levels or bring serum testosterone levels to normal and to maintain normal levels over extended periods of time. In a preferred embodiment, a patient is tested to determine if his serum testosterone levels are significantly lower than normal levels (about 25% below) before administering treatment with the therapeutic agent. The frequency of administration may vary from a single dose per day to multiple doses per day. Preferred routes of administration include oral, intravenous and intraperitoneal, but other forms of administration may be chosen as well.

[00282] A "therapeutically effective amount" of a protein or polypeptide, small molecule, antibody, or nucleic acid is an amount that achieves the desired therapeutic result. For example, if a therapeutic agent is administered to treat or prevent a disorder relating to reproduction in male mammals, a therapeutically effective amount is an amount that ameliorates one or more symptoms of the disorder, or produces at least one effect selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing sperm viability, increasing serum testosterone levels, increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, or reducing excess apoptosis in testes.

[00283] A therapeutically effective amount of protein or polypeptide, small molecule or nucleic acid for use in the present invention typically varies and can be an amount sufficient to achieve serum therapeutic agent levels typically of between about 1 nanogram per milliliter and about 10 micrograms per milliliter in the subject, or an amount sufficient to achieve serum therapeutic agent levels of between about 1 nanogram per milliliter and about 7 micrograms per milliliter in the subject. Other preferred serum therapeutic agent levels include about 0.1 nanogram per milliliter to about 3 micrograms per milliliter, about 0.5 nanograms per milliliter to about 1 microgram per milliliter, about 1 nanogram per milliliter to about 750 nanograms per milliliter, about 5 nanograms per milliliter to about 500 nanograms per milliliter, and about 5 nanograms per milliliter to about 100 nanograms per milliliter.

[00284] The amount of therapeutic agent disclosed herein to be administered to a patient in the methods of the present invention may range from about 5 mg/kg/day to about 500 mg/kg/day, from about 5 mg/kg/day to about 400 mg/kg/day, from about 5 mg/kg/day to about

300 mg/kg/day, from about 5 mg/kg/day to about 250 mg/kg/day, from about 5 mg/kg/day to about 200 mg/kg/day, from about 5 mg/kg/day to about 150 mg/kg/day, from about 5 mg/kg/day to about 100 mg/kg/day, from about 5 mg/kg/day to about 75 mg/kg/day, from about 5 mg/kg/day to about 50 mg/kg/day, from about 5 mg/kg/day to about 40 mg/kg/day, from about 5 mg/kg/day to about 35 mg/kg/day, from about 5 mg/kg/day to about 30 mg/kg/day, from about 5 mg/kg/day to about 25 mg/kg/day, from about 5 mg/kg/day to about 24 mg/kg/day, from about 5 mg/kg/day to about 23 mg/kg/day, from about 5 mg/kg/day to about 22 mg/kg/day, from about 5 mg/kg/day to about 21 mg/kg/day, from about 5 mg/kg/day to about 20 mg/kg/day, from about 5 mg/kg/day to about 19 mg/kg/day, from about 5 mg/kg/day to about 18 mg/kg/day, from about 5 mg/kg/day to about 17 mg/kg/day, from about 5 mg/kg/day to about 16 mg/kg/day, from about 5 mg/kg/day to about 15 mg/kg/day, from about 5 mg/kg/day to about 14 mg/kg/day, from about 5 mg/kg/day to about 13 mg/kg/day, from about 5 mg/kg/day to about 12 mg/kg/day, from about 5 mg/kg/day to about 11 mg/kg/day, or from about 5 mg/kg/day to about 10 mg/kg/day.

[00285] Other dose ranges that may be used include from about 10 mg/kg/day to about 500 mg/kg/day, from about 10 mg/kg/day to about 400 mg/kg/day, from about 10 mg/kg/day to about 300 mg/kg/day, from about 10 mg/kg/day to about 250 mg/kg/day, from about 10 mg/kg/day to about 200 mg/kg/day, from about 10 mg/kg/day to about 150 mg/kg/day, from about 10 mg/kg/day to about 100 mg/kg/day, from about 10 mg/kg/day to about 75 mg/kg/day, from about 10 mg/kg/day to about 50 mg/kg/day, from about 10 mg/kg/day to about 45 mg/kg/day, from about 10 mg/kg/day to about 40 mg/kg/day, from about 10 mg/kg/day to about 35 mg/kg/day, from about 10 mg/kg/day to about 34 mg/kg/day, from about 10 mg/kg/day to about 33 mg/kg/day, from about 10 mg/kg/day to about 32 mg/kg/day, from about 10 mg/kg/day

to about 31 mg/kg/day, from about 10 mg/kg/day to about 30 mg/kg/day, from about 10 mg/kg/day to about 29 mg/kg/day, from about 10 mg/kg/day to about 28 mg/kg/day, from about 10 mg/kg/day to about 27 mg/kg/day, from about 10 mg/kg/day to about 26 mg/kg/day, from about 10 mg/kg/day to about 25 mg/kg/day, from about 10 mg/kg/day to about 24 mg/kg/day, from about 10 mg/kg/day to about 23 mg/kg/day, from about 10 mg/kg/day to about 22 mg/kg/day, from about 10 mg/kg/day to about 21 mg/kg/day, from about 10 mg/kg/day to about 20 mg/kg/day, from about 10 mg/kg/day to about 19 mg/kg/day, from about 10 mg/kg/day to about 18 mg/kg/day, from about 10 mg/kg/day to about 17 mg/kg/day, from about 10 mg/kg/day to about 16 mg/kg/day, or from about 10 mg/kg/day to about 15 mg/kg/day.

[00286] Other dose ranges that may be used include from about 15 mg/kg/day to about 500 mg/kg/day, from about 15 mg/kg/day to about 400 mg/kg/day, from about 15 mg/kg/day to about 300 mg/kg/day, from about 15 mg/kg/day to about 250 mg/kg/day, from about 15 mg/kg/day to about 200 mg/kg/day, from about 15 mg/kg/day to about 150 mg/kg/day, from about 15 mg/kg/day to about 100 mg/kg/day, from about 15 mg/kg/day to about 75 mg/kg/day, from about 15 mg/kg/day to about 50 mg/kg/day, from about 15 mg/kg/day to about 40 mg/kg/day, from about 15 mg/kg/day to about 30 mg/kg/day, from about 15 mg/kg/day to about 25 mg/kg/day, or from about 15 mg/kg/day to about 20 mg/kg/day.

[00287] Other dose ranges that may be used include from about 20 mg/kg/day to about 500 mg/kg/day, from about 20 mg/kg/day to about 400 mg/kg/day, from about 20 mg/kg/day to about 300 mg/kg/day, from about 20 mg/kg/day to about 250 mg/kg/day, from about 20 mg/kg/day to about 200 mg/kg/day, from about 20 mg/kg/day to about 150 mg/kg/day, from

about 20 mg/kg/day to about 100 mg/kg/day, from about 20 mg/kg/day to about 75 mg/kg/day, from about 20 mg/kg/day to about 50 mg/kg/day, from about 20 mg/kg/day to about 40 mg/kg/day, from about 20 mg/kg/day to about 30 mg/kg/day, or from about 20 mg/kg/day to about 25 mg/kg/day.

[00288] Other dose ranges that may be used include from about 25 mg/kg/day to about 500 mg/kg/day, from about 25 mg/kg/day to about 400 mg/kg/day, from about 25 mg/kg/day to about 300 mg/kg/day, from about 25 mg/kg/day to about 250 mg/kg/day, from about 25 mg/kg/day to about 200 mg/kg/day, from about 25 mg/kg/day to about 150 mg/kg/day, from about 25 mg/kg/day to about 100 mg/kg/day, from about 25 mg/kg/day to about 75 mg/kg/day, from about 25 mg/kg/day to about 50 mg/kg/day, from about 25 mg/kg/day to about 40 mg/kg/day, or from about 25 mg/kg/day to about 30 mg/kg/day.

[00289] Other dose ranges that may be used include from about 30 mg/kg/day to about 500 mg/kg/day, from about 30 mg/kg/day to about 400 mg/kg/day, from about 30 mg/kg/day to about 300 mg/kg/day, from about 30 mg/kg/day to about 250 mg/kg/day, from about 30 mg/kg/day to about 200 mg/kg/day, from about 30 mg/kg/day to about 150 mg/kg/day, from about 30 mg/kg/day to about 100 mg/kg/day, from about 30 mg/kg/day to about 75 mg/kg/day, from about 30 mg/kg/day to about 50 mg/kg/day, or from about 30 mg/kg/day to about 40 mg/kg/day.

[00290] Other dose ranges that may be used include from about 40 mg/kg/day to about 500 mg/kg/day, from about 40 mg/kg/day to about 400 mg/kg/day, from about 40 mg/kg/day to about 300 mg/kg/day, from about 40 mg/kg/day to about 250 mg/kg/day, from about 40

mg/kg/day to about 200 mg/kg/day, from about 40 mg/kg/day to about 150 mg/kg/day, from about 40 mg/kg/day to about 100 mg/kg/day, from about 40 mg/kg/day to about 75 mg/kg/day, from about 40 mg/kg/day to about 60 mg/kg/day, or from about 40 mg/kg/day to about 50 mg/kg/day.

[00291] Other dose ranges that may be used include from about 50 mg/kg/day to about 500 mg/kg/day, from about 50 mg/kg/day to about 400 mg/kg/day, from about 50 mg/kg/day to about 300 mg/kg/day, from about 50 mg/kg/day to about 250 mg/kg/day, from about 50 mg/kg/day to about 200 mg/kg/day, from about 50 mg/kg/day to about 175 mg/kg/day, from about 50 mg/kg/day to about 150 mg/kg/day, from about 50 mg/kg/day to about 125 mg/kg/day, from about 50 mg/kg/day to about 100 mg/kg/day, from about 50 mg/kg/day to about 75 mg/kg/day, or from about 50 mg/kg/day to about 60 mg/kg/day.

[00292] Other dose ranges that may be used include from about 60 mg/kg/day to about 500 mg/kg/day, from about 60 mg/kg/day to about 400 mg/kg/day, from about 60 mg/kg/day to about 300 mg/kg/day, from about 60 mg/kg/day to about 250 mg/kg/day, from about 60 mg/kg/day to about 200 mg/kg/day, from about 60 mg/kg/day to about 175 mg/kg/day, from about 60 mg/kg/day to about 150 mg/kg/day, from about 60 mg/kg/day to about 125 mg/kg/day, from about 60 mg/kg/day to about 100 mg/kg/day, or from about 60 mg/kg/day to about 75 mg/kg/day.

[00293] Other dose ranges that may be used include from about 70 mg/kg/day to about 500 mg/kg/day, from about 70 mg/kg/day to about 400 mg/kg/day, from about 70 mg/kg/day to about 300 mg/kg/day, from about 70 mg/kg/day to about 250 mg/kg/day, from about 70

mg/kg/day to about 200 mg/kg/day, from about 70 mg/kg/day to about 175 mg/kg/day, from about 70 mg/kg/day to about 150 mg/kg/day, from about 70 mg/kg/day to about 125 mg/kg/day, or from about 70 mg/kg/day to about 100 mg/kg/day.

[00294] Other dose ranges that may be used include from about 80 mg/kg/day to about 500 mg/kg/day, from about 80 mg/kg/day to about 400 mg/kg/day, from about 80 mg/kg/day to about 300 mg/kg/day, from about 80 mg/kg/day to about 250 mg/kg/day, from about 80 mg/kg/day to about 200 mg/kg/day, from about 80 mg/kg/day to about 175 mg/kg/day, from about 80 mg/kg/day to about 150 mg/kg/day, from about 80 mg/kg/day to about 125 mg/kg/day, or from about 80 mg/kg/day to about 100 mg/kg/day.

[00295] Other dose ranges that may be used include from about 90 mg/kg/day to about 500 mg/kg/day, from about 90 mg/kg/day to about 400 mg/kg/day, from about 90 mg/kg/day to about 300 mg/kg/day, from about 90 mg/kg/day to about 250 mg/kg/day, from about 90 mg/kg/day to about 200 mg/kg/day, from about 90 mg/kg/day to about 175 mg/kg/day, from about 90 mg/kg/day to about 150 mg/kg/day, from about 90 mg/kg/day to about 125 mg/kg/day, or from about 90 mg/kg/day to about 100 mg/kg/day.

[00296] Other dose ranges that may be used include from about 100 mg/kg/day to about 500 mg/kg/day, from about 100 mg/kg/day to about 400 mg/kg/day, from about 100 mg/kg/day to about 300 mg/kg/day, from about 100 mg/kg/day to about 250 mg/kg/day, from about 100 mg/kg/day to about 200 mg/kg/day, from about 100 mg/kg/day to about 175 mg/kg/day, from about 100 mg/kg/day to about 150 mg/kg/day, or from about 100 mg/kg/day to about 125 mg/kg/day.

[00297] Other dosages that may be used include about 5 mg/kg/day, about 6 mg/kg/day, about 7 mg/kg/day, about 8 mg/kg/day, about 9 mg/kg/day, about 10 mg/kg/day, about 11 mg/kg/day, about 12 mg/kg/day, about 13 mg/kg/day, about 14 mg/kg/day, about 15 mg/kg/day, about 16 mg/kg/day, about 17 mg/kg/day, about 18 mg/kg/day, about 19 mg/kg/day, about 20 mg/kg/day, about 21 mg/kg/day, about 22 mg/kg/day, about 23 mg/kg/day, about 24 mg/kg/day, about 25 mg/kg/day, about 26 mg/kg/day, about 27 mg/kg/day, about 28 mg/kg/day, about 29 mg/kg/day, about 30 mg/kg/day, about 31 mg/kg/day, about 32 mg/kg/day, about 33 mg/kg/day, about 34 mg/kg/day, about 35 mg/kg/day, about 36 mg/kg/day, about 37 mg/kg/day, about 38 mg/kg/day, about 39 mg/kg/day, about 40 mg/kg/day, about 45 mg/kg/day, about 50 mg/kg/day, about 60 mg/kg/day, about 70 mg/kg/day, about 80 mg/kg/day, about 90 mg/kg/day, about 100 mg/kg/day, about 125 mg/kg/day, about 150 mg/kg/day, about 175 mg/kg/day, about 200 mg/kg/day, about 250 mg/kg/day, or about 350 mg/kg/day.

[00298] The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the condition, previous treatments, the general health and/or age of the subject, and other disorders or diseases present.

[00299] Treatment of a subject with a therapeutically effective amount of a protein, polypeptide, nucleotide or antibody can include a single treatment or, preferably, can include a series of treatments.

[00300] In certain embodiments, treatment of a subject with undercarboxylated/uncarboxylated osteocalcin leads to undercarboxylated/uncarboxylated

osteocalcin being about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, or about 50% of the total osteocalcin in the blood of the patient.

[00301] It is understood that the appropriate dose of a small molecule agent depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, and the effect which the practitioner desires the small molecule to have. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one or more of these small molecules is to be administered to an animal (e.g., a human) in order to modulate expression or activity of OST-PTP, PTP-1B, or gamma-carboxylase, a relatively low dose may be prescribed at first, with the dose subsequently increased until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, and diet of the subject, the time of administration, the route of administration, the rate of excretion, whether other drugs are being administered to the patient, and the degree of expression or activity to be modulated.

[00302] For prevention or treatment, a suitable subject can be an individual who is suspected of having, has been diagnosed as having, or is at risk of developing a disorder relating to reproduction in male mammals.

[00303] Suitable routes of administration of the pharmaceutical compositions useful in the methods of the present invention can include oral, intestinal, parenteral, transmucosal, transdermal, intramuscular, subcutaneous, transdermal, rectal, intramedullary, intrathecal, intravenous, intraventricular, intraatrial, intraaortal, intraarterial, or intraperitoneal administration. The pharmaceutical compositions useful in the methods of the present invention can be administered to the subject by a medical device, such as, but not limited to, catheters, balloons, implantable devices, biodegradable implants, prostheses, grafts, sutures, patches, shunts, or stents. In one preferred embodiment, the therapeutic agent (e.g., undercarboxylated/uncarboxylated osteocalcin) can be coated on a stent for localized administration to the target area. In this situation a slow release preparation of undercarboxylated/uncarboxylated osteocalcin, for example, is preferred.

[00304] The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations and that may be consulted by those skilled in the art for techniques useful for practicing the present invention include, but are not limited to, U.S. Patents Nos.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

[00305] In yet another aspect of the invention, undercarboxylated/uncarboxylated osteocalcin is administered as a pharmaceutical composition with a pharmaceutically acceptable excipient. Exemplary pharmaceutical compositions for undercarboxylated/uncarboxylated osteocalcin include injections as solutions or injections as injectable self-setting or self-gelling mineral polymer hybrids. Undercarboxylated/uncarboxylated osteocalcin may be administered using a porous crystalline biomimetic bioactive composition of calcium phosphate. See U.S. Patents Nos. 5,830,682; 6,514,514; and 6,511,958 and U.S. Patent Application Publications Nos. 2006/0063699; 2006/0052327; 2003/199615; 2003/0158302; 2004/0157864; 2006/0292670; 2007/0099831 and 2006/0257492, all of which are incorporated herein in their entirety by reference.

METHODS OF TREATMENT

[00306] The present invention provides methods for modulating the level of undercarboxylated/uncarboxylated osteocalcin in male mammals through modulating the OST-PTP signaling pathway or the PTP-1B signaling pathway for treating or preventing a variety of different disorders relating to reproduction in the male mammals. In particular, the methods are used to inhibit OST-PTP phosphorylase activity, inhibit PTP-1B phosphorylase activity, reduce gamma-carboxylase activity, and/or increase undercarboxylated/uncarboxylated osteocalcin. According to the invention, the methods provide an amount of an agent effective to treat or prevent a disorder associated with the OST-PTP signaling pathway or the PTP-1B signaling pathway. The agent may be selected from the group consisting of small molecules, antibodies and nucleic acids. Such disorders include, but are not limited to, male infertility, low sperm

count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes.

[00307] In certain embodiments, the methods comprise identifying a patient in need of treatment or prevention of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes and then applying the methods disclosed herein to the patient.

[00308] In one embodiment of the invention, the method of treatment comprises administering to a patient in need thereof a therapeutically effective amount of undercarboxylated/uncarboxylated osteocalcin sufficient to raise the patient's blood level of undercarboxylated/uncarboxylated osteocalcin compared to the pretreatment patient level. Preferably, the patient is a male human. In another embodiment, the method of treatment comprises administering to a patient in need thereof a therapeutically effective amount of undercarboxylated/uncarboxylated osteocalcin sufficient to raise the ratio of undercarboxylated/uncarboxylated osteocalcin to total osteocalcin in the patient's blood compared to the pretreatment patient ratio.

[00309] In another aspect of the invention, a method is provided for treating or preventing a disorder relating to reproduction in a male mammal comprising administering to a male mammal in need thereof undercarboxylated/uncarboxylated osteocalcin in a therapeutically effective amount that produces at least one effect selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing sperm viability,

increasing serum testosterone levels, increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, and reducing excess apoptosis in testes, compared to pretreatment levels. Preferably, the male mammal is a human.

[00310] In an embodiment of the invention, a method is provided for treating or preventing a disorder relating to reproduction in a male mammal comprising administering to a male mammal in need of such treatment or prevention a therapeutically effective amount of an agent that reduces OST-PTP expression or activity in osteoblasts, or reduces PTP-1B expression or activity in osteoblasts, sufficient to produce at least one effect selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing sperm viability, increasing serum testosterone levels, increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, and reducing excess apoptosis in testes, compared to pretreatment levels. Preferably, the patient is a human.

[00311] The present invention is directed to methods (i) for treating or preventing a disorder relating to reproduction in a male mammal comprising administering to a male mammal in need of such treatment or prevention in a therapeutically effective amount an agent that reduces gamma-carboxylase expression or activity in osteoblasts sufficient to produce at least one effect selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing sperm viability, increasing serum testosterone levels, increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, and reducing excess apoptosis in testes, compared to pretreatment levels comprising administering to the male mammal in need of such treatment or prevention in a therapeutically effective amount

an agent that reduces gamma-carboxylase expression or activity in osteoblasts sufficient to increase fertility, raise sperm count, increase sperm motility, increase sperm viability, increase serum testosterone levels, increase libido, ameliorate erectile dysfunction, reduce underdevelopment of testes, or reduce excess apoptosis in testes. Preferably, the male mammal is a human. In an embodiment of the invention, the agent is an isolated nucleic acid that is selected from the group consisting of cDNA, antisense DNA, antisense RNA, and small interfering RNA, which nucleic acid is sufficiently complementary to the gene or mRNA encoding gamma-carboxylase to permit specific hybridization to the gene or mRNA, and wherein the hybridization prevents or reduces expression of gamma-carboxylase in osteoblasts. In another embodiment of the invention, the nucleic acid is conjugated to a phosphate group or other targeting ligand to facilitate uptake by osteoblasts.

[00312] In the methods described herein, it will be understood that "treating" a disease or disorder encompasses not only improving the disease or disorder or its symptoms but also retarding the progression of the disease or disorder or ameliorating the deleterious effects of the disease or disorder.

[00313] The present invention also encompasses the use of gene therapy for treatment of disorders relating to reproduction in male mammals. This can be accomplished by introducing a gene encoding osteocalcin or a biologically active fragment or variant thereof into a vector, and transfecting or infecting cells from a patient afflicted with the disorder or at a high risk of developing the disorder with the vector, according to various methods known in the art. The cells may be transfected or infected by *ex vivo* or by *in vivo* methods.

[00314] Methods of gene therapy known in the art can be adapted for use in the methods of the present invention. Adeno-associated virus (AAV) is one of the most promising vectors for gene therapy and may be used in the methods of the present invention. Conventional methods of gene transfer and gene therapy are described in, e.g., Gene Therapy: Principles and Applications, ed. T. Blackenstein, Springer Verlag, 1999; Gene Therapy Protocols (Methods in Molecular Medicine), ed. P. D. Robbins, Humana Press, 1997; and Retro-vectors for Human Gene Therapy, ed. C. P. Hodgson, Springer Verlag, 1996. AAV is an attractive vector system for human gene therapy because it is non-pathogenic for humans, it has a high frequency of integration, and it can infect non-dividing cells, thus making it useful for delivery of genes into mammalian cells both in tissue culture and in whole animals. See, e.g., Muzyczka, 1992, Curr. Top. Microbiol. Immunol., 158:97-129. Recent studies have demonstrated AAV to be a potentially useful vector for gene delivery. LaFace et al., 1998, Virology, 162:483-486; Zhou et al., 1993, Exp. Hematol. (NY), 21:928-933; Flotte et al., 1993, Proc. Natl. Acad. Sci. USA 90:10613-10617; and Walsh et al., 1994, Blood 84:1492-1500. Recombinant AAV vectors have been used successfully for *in vitro* and *in vivo* transduction of marker genes (Kaplitt et al., 1994, Nature Genetics, 8:148-154; Lebkowski et al., 1988, Mol. Cell. Biol. 8:3988-3996; Samulski et al., 1989, J. Virol., 63:3822-3828; Shelling & Smith, 1994, Gene Therapy 1:165-169; Yoder et al., 1994, Blood, 82:suppl. 1:347A; Zhou et al., 1994, J. Exp. Med., 179:1867-1875; Hermonat & Muzyczka, 1984, Proc. Natl. Acad. Sci. USA., 81:6466-6470; Tratschin et al., 1984, Mol. Cell. Biol., 4:2072-2081; McLaughlin et al., 1988, J. Virol., 62:1963-1973) as well as genes involved in human diseases (Flotte et al., 1992, Am. J. Respir. Cell Mol. Biol. 7:349-356; Luo et al., 1994, Blood, 82:suppl.

1,303A; Ohi et al., 1990, Gene, 89:279-282; Walsh et al., 1992, Proc. Natl. Acad. Sci. USA 89:7257-7261; Wei et al., 1994, Gene Therapy, 1:261-268).

[00315] In certain other embodiments, the gene of interest (e.g., osteocalcin) can be transferred into a target cell using a retroviral vector. Retroviruses refer to viruses that belong to the Retroviridae family, and include oncoviruses, foamy viruses (Russell & Miller, 1996, J. Virol. 70:217-222; Wu et al., 1999, J. Virol. 73:4498-4501, and lentiviruses (for example, HIV-1 (Naldini et al., 1996, Science 272:263-267; Poeschla et al., 1996, Proc. Natl. Acad. Sci. USA 93:11395-11399; Srinivasakumar et al., 1997, J. Virol. 71:5841-5848; Zufferey et al., 1997, Nat. Biotechnol. 15:871-875; Kim et al., 1998, J. Virol. 72:811-816) and feline immunodeficiency virus (Johnston et al., 1999, J. Virol. 73:4991-5000; Johnston & Power, 1999, Virol. 73:2491-2498; Poeschla et al., 1998, Nat. Med. 4:354-357). The disclosures of these publications may be adapted for use in the methods of the present invention. Numerous gene therapy methods that take advantage of retroviral vectors for treating a wide variety of diseases are well-known in the art and can be adapted for use in the methods of the present invention (see, e.g., U.S. Patents Nos. 4,405,712 and 4,650,764; Friedmann, 1989, Science, 244:1275-1281; Mulligan, 1993, Science, 260:926-932; Crystal, 1995, Science 270:404-410, and U.S. Patent No. 6,899,871, each of which are incorporated herein by reference in their entirety). An increasing number of these methods are currently being applied in human clinical trials (Morgan, 1993, BioPharm, 6:32-35; see also The Development of Human Gene Therapy, Theodore Friedmann, Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999. ISBN 0-87969-528-5, which is incorporated herein by reference in its entirety).

[00316] Efficacy of the methods of treatment described herein can be monitored by determining whether the methods ameliorate any of the symptoms of the disease or disorder being treated. Alternatively, one can monitor the level of serum undercarboxylated/uncarboxylated osteocalcin (either in absolute terms or as a ratio of undercarboxylated/uncarboxylated osteocalcin/total osteocalcin), which levels should increase in response to therapy.

METHODS OF MALE CONTRACEPTION

[00317] The discovery of the previously unknown biochemical pathway linking osteocalcin and reproductive biology in male mammals provides methods of contraception for use in male mammals. In such methods, a male mammal in need of contraception is administered a pharmaceutical composition comprising a therapeutically effective amount of an agent that antagonizes the effect of undercarboxylated/uncarboxylated osteocalcin. In certain embodiments, the agent has the effect of lowering the serum level of undercarboxylated/uncarboxylated osteocalcin in the male mammal. In certain embodiments, the agent acts as an antagonist of undercarboxylated/uncarboxylated osteocalcin.

[00318] In certain embodiments, the male mammal in need of contraception is a human.

[00319] In certain embodiments, the pharmaceutical compositions useful in the methods of contraception comprise an agent that increases the expression or activity of gamma-carboxylase, PTP-1B, or OST-PTP. This results in a greater amount of osteocalcin being present in the carboxylated state rather than the undercarboxylated/uncarboxylated state. The agents that increase the expression or activity of gamma-carboxylase, PTP-1B, or OST-PTP may be

antibodies (monoclonal or polyclonal) or fragments of antibodies, small molecules, polypeptides or proteins, or nucleic acids (e.g., antisense DNA or RNA, siRNA).

[00320] In other embodiments, the pharmaceutical compositions useful in the methods of contraception comprise an agent that is a "negative mimetic" of undercarboxylated/uncarboxylated osteocalcin. A "negative mimetic" refers to a synthetic chemical compound that has substantially the same structural characteristics of naturally occurring undercarboxylated/uncarboxylated osteocalcin but antagonizes the biological effects of naturally occurring undercarboxylated/uncarboxylated osteocalcin. Such negative mimetics may include, for instance, polypeptide- and polynucleotide-like polymers having modified backbones, side chains, and/or bases.

DIAGNOSTICS

[00321] The present invention provides methods and compositions for diagnosing disorders related to reproduction in male mammals based on decreased levels of undercarboxylated/uncarboxylated osteocalcin. Such disorders include, but are not limited to, male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes.

[00322] In a specific embodiment of the invention, a method is provided for diagnosing a patient having or at risk of developing a disorder selected from the group consisting of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in

testes, comprising: (i) determining a patient level of undercarboxylated/uncarboxylated osteocalcin in a biological sample taken from the patient and a control level of undercarboxylated/uncarboxylated osteocalcin in a biological sample taken from a subject that does not have the disorder, (ii) comparing the patient and control levels, and (iii) diagnosing the patient as having or as being at risk of developing the disorder if the patient level is lower than the control level.

[00323] "Biological samples" include solid and fluid body samples. The biological samples of the present invention may include tissue, organs, cells, protein or membrane extracts of cells, blood or biological fluids such as blood, serum, ascites fluid or brain fluid (e.g., cerebrospinal fluid). Preferably, the biological sample is blood.

[00324] In another embodiment of the invention, a method is provided for diagnosing a patient having or at risk of developing a disorder selected from the group consisting of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes, comprising: (i) determining a patient level of undercarboxylated/uncarboxylated osteocalcin in a biological sample taken from the patient; and (ii) comparing the patient level to a standard level; where, if the patient level is lower than the standard level, diagnosing the patient as having or at risk of developing the disorder. In instances where the method is practiced on male humans, the standard level can be a level of undercarboxylated/uncarboxylated osteocalcin that has been previously determined to be the normal range for men who are not at risk of

developing the disorder. In preferred embodiments, the biological sample is blood, serum, plasma, cerebrospinal fluid, urine, a cell sample, or a tissue sample.

[00325] A "standard level" of undercarboxylated/uncarboxylated osteocalcin in male humans can include values of 0.1 ng/ml to 10 ng/ml, preferably 0.2 ng/ml to 7.5 ng/ml, more preferably 0.5 ng/ml to 5 ng/ml, and even more preferably 1 ng/ml to 5 ng/ml of undercarboxylated/uncarboxylated osteocalcin. A standard level of undercarboxylated/uncarboxylated osteocalcin in humans can also include about 0.1 ng/ml, about 0.5 ng/ml, about 1 ng/ml, about 2 ng/ml, about 3 ng/ml, about 4 ng/ml, about 5 ng/ml, about 6 ng/ml, about 7 ng/ml, or about 10 ng/ml of undercarboxylated/uncarboxylated osteocalcin.

[00326] In another embodiment of the invention, a method is provided for diagnosing a patient having or at risk of developing a disorder selected from the group consisting of male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, and excess apoptosis in testes, comprising: (i) determining the ratio of undercarboxylated/uncarboxylated osteocalcin to total osteocalcin in a biological sample taken from the patient; and (ii) comparing the ratio to a standard ratio; where, if the patient ratio is lower than the standard ratio, diagnosing the patient as having or being at risk of developing the disorder. In certain embodiments, the standard ratio is 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, or 30%-35%. In certain embodiments, the standard ratio is about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%,

34%, or 35%. Preferably, the patient is a male human. In preferred embodiments, the biological sample is blood, serum, plasma, cerebrospinal fluid, urine, a cell sample, or a tissue sample.

[00327] Assays for detecting the levels of protein expression, e.g., osteocalcin expression, are well known to those of skill in the art. Such assays include, for example, antibody-based immunoassays. Methods for using antibodies as disclosed herein are particularly applicable to the cells, tissues and other biological samples from patient with disorders relating to reproduction in male mammals that differentially express osteocalcin, OST-PTP, PTP-1B, or gamma-carboxylase. The methods use antibodies that selectively bind to the protein of interest and its fragments or variants.

[00328] The amount of osteocalcin in a biological sample may be determined by an assay such as a radioimmunoassay, an immunoradiometric assay, and/or an enzyme immunoassay. A "radioimmunoassay" is a technique for detecting and measuring the concentration of an antigen using a labeled (e.g., radioactively labeled) form of the antigen. Examples of radioactive labels for antigens include ^{3}H, ^{14}C, and ^{125}I. The concentration of antigen (e.g., osteocalcin) in a biological sample may be measured by having the antigen in the sample compete with a labeled (e.g., radioactively, fluorescently) antigen for binding to an antibody to the antigen. To ensure competitive binding between the labeled antigen and the unlabeled antigen, the labeled antigen is present in a concentration sufficient to saturate the binding sites of the antibody. The higher the concentration of antigen in the sample, the lower the concentration of labeled antigen that will bind to the antibody.

[00329] In a radioimmunoassay, to determine the concentration of labeled antigen bound to antibody, the antigen-antibody complex must be separated from the free antigen. One method for separating the antigen-antibody complex from the free antigen is by precipitating the antigen-antibody complex with an anti-isotype antiserum. Another method for separating the antigen-antibody complex from the free antigen is by precipitating the antigen-antibody complex with formalin-killed *S. aureus*. Yet another method for separating the antigen-antibody complex from the free antigen is by performing a "solid-phase radioimmunoassay" where the antibody is linked (e.g., covalently) to Sepharose® beads, polystyrene wells, polyvinylchloride wells, or microtiter wells. By comparing the concentration of labeled antigen bound to antibody to a standard curve based on samples having a known concentration of antigen, the concentration of antigen in the biological sample can be determined.

[00330] An "Immunoradiometric Assay" (IRMA) is an immunoassay in which the antibody reagent is radioactively labeled. An IRMA requires the production of a multivalent antigen conjugate, by techniques such as conjugation to a protein, e.g., rabbit serum albumin (RSA). The multivalent antigen conjugate must have at least 2 antigen residues per molecule and the antigen residues must be of sufficient distance apart to allow binding by at least two antibodies to the antigen. For example, in an IRMA the multivalent antigen conjugate can be attached to a solid surface such as a plastic sphere. Unlabeled "sample" antigen and antibody to antigen which is radioactively labeled are added to a test tube containing the multivalent antigen conjugate coated sphere. The antigen in the sample competes with the multivalent antigen conjugate for antigen antibody binding sites. After an appropriate incubation period, the unbound reactants are removed by washing and the amount of radioactivity on the solid phase is

determined. The amount of bound radioactive antibody is inversely proportional to the concentration of antigen in the sample.

[00331] The most common enzyme immunoassay is the "Enzyme-Linked Immunosorbent Assay (ELISA)." The "Enzyme-Linked Immunosorbent Assay (ELISA)" is a technique for detecting and measuring the concentration of an antigen using a labeled (e.g., enzyme linked) form of the antibody. In a "sandwich ELISA," an antibody (e.g., to osteocalcin) is linked to a solid phase (e.g., a microtiter plate) and exposed to a biological sample containing antigen (e.g., osteocalcin). The solid phase is then washed to remove unbound antigen. A labeled (e.g., enzyme linked) antibody is then bound to the bound-antigen (if present) forming an antibody-antigen-antibody sandwich. Examples of enzymes that can be linked to the antibody include alkaline phosphatase, horseradish peroxidase, luciferase, urease, and β-galactosidase. The enzyme linked antibody reacts with a substrate to generate a colored reaction product that can be assayed.

[00332] In a "competitive ELISA," antibody is incubated with a sample containing antigen (e.g., osteocalcin). The antigen-antibody mixture is then contacted with an antigen-coated solid phase (e.g., a microtiter plate). The more antigen present in the sample, the less free antibody that will be available to bind to the solid phase. A labeled (e.g., enzyme linked) secondary antibody is then added to the solid phase to determine the amount of primary antibody bound to the solid phase.

[00333] In an "immunohistochemistry assay," a section of tissue is tested for specific proteins by exposing the tissue to antibodies that are specific for the protein that is being

assayed. The antibodies are then visualized by any of a number of methods to determine the presence and amount of the protein present. Examples of methods used to visualize antibodies are, for example, through enzymes linked to the antibodies (e.g., luciferase, alkaline phosphatase, horseradish peroxidase, or β-galactosidase), or chemical methods (e.g., DAB/Substrate chromagen).

[00334] In addition to detecting levels of protein expression, the diagnostic assays of the invention may employ methods designed to detect the level of RNA expression. Levels of RNA expression may be determined using methods well known to those of skill in the art, including, for example, the use of northern blots, RT-PCR or *in situ* hybridizations.

[00335] Carboxylation of osteocalcin confers a greater affinity for hydroxyapatite. Total osteocalcin may be measured by immunoassay followed by incubation with hydroxyapatite and centrifugation. The supernatant, which contains osteocalcin that has not adsorbed to hydroxyapatite is then measured using the same immunoassay. The results of this procedure can be expressed either as absolute concentrations or as a ratio of undercarboxylated to carboxylated osteocalcin.

[00336] Another procedure uses monoclonal antibodies that distinguish the carboxylation state of all or some of the Glu/Gla residues of osteocalcin. For example, GluOC4-5 (TaKaRa catalog no. M171) reacts with human osteocalcin with glutamic acid residues (decarboxylated) at positions 21 and 24, and does not react with react with Gla-type osteocalcin.

[00337] For a review of osteocalcin measurement methods, see Lee et al., 2000, Ann. Clin. Biochem. 37:432-446.

DRUG SCREENING AND ASSAYS

[00338] Cell-based and non-cell based methods of drug screening are provided to identify candidate agents that reduce OST-PTP, PTP-1B, or gamma-carboxylase activity or expression, and/or increase the level of undercarboxylated/uncarboxylated osteocalcin activity or expression. Such agents find use in treating or preventing disorders related to reproduction in male mammals. Such agents may also be used to treat disorders characterized by decreased testosterone production.

[00339] Non-cell based screening methods are provided to identify compounds that bind to OST-PTP, PTP-1B, gamma-carboxylase or osteocalcin and thereby modulate the activity of these proteins.

[00340] Such non-cell based methods include a method to identify, or assay for, an agent that binds to OST-PTP, the method comprising the steps of: (i) providing a mixture comprising OST-PTP or a fragment or variant thereof, (ii) contacting the mixture with a candidate agent, (iii) determining whether the candidate agent binds to the OST-PTP, wherein if the agent binds to the OST-PTP or a fragment or variant thereof (iv) determining whether the agent reduces the ability of OST-PTP to dephosphorylate gamma-carboxylase and (v) administering the agent to a patient in need of treatment for a disorder related to reproduction in male mammals. In certain embodiments, the mixture comprises membrane fragments comprising OST-PTP or a fragment or variant thereof.

[00341] A screening method is provided to identify or assay for an agent that binds to the phosphatase 1 domain of OST-PTP, the method comprising the steps of: (i) providing a mixture comprising the phosphatase 1 domain of OST-PTP or a fragment or variant thereof, (ii) contacting the mixture with an agent, (iii) determining whether the agent binds to the phosphatase 1 domain of OST-PTP, wherein if the agent binds to the phosphatase 1 domain of OST-PTP or a fragment or variant thereof (iv) determining whether the agent inhibits the phosphatase 1 domain of OST-PTP and, if the agent inhibits the phosphatase 1 domain of OST-PTP (v) administering the agent to a patient in need of treatment for a disorder related to reproduction in male mammals.

[00342] A screening method is provided to identify or assay for an agent that binds to PTP-1B, the method comprising the steps of: (i) providing a mixture comprising PTP-1B or a fragment or variant thereof, (ii) contacting the mixture with a candidate agent, (iii) determining whether the candidate agent binds to the PTP-1B, wherein if the agent binds to the PTP-1B or a fragment or variant thereof (iv) determining whether the agent reduces the ability of PTP-1B to dephosphorylate gamma-carboxylase and (v) administering the agent to a patient in need of treatment for a disorder related to reproduction in male mammals. In certain embodiments, the mixture comprises membrane fragments comprising PTP-1B or a fragment or variant thereof.

[00343] A screening method is provided to identify, or assay for, an agent that binds to gamma-carboxylase, the method comprising the steps of: (i) providing a mixture comprising the gamma-carboxylase or a fragment or variant thereof, (ii) contacting the mixture with an agent, (iii) determining whether the agent binds to the gamma-carboxylase, wherein if the agent binds

to the gamma-carboxylase or a fragment or variant thereof (iv) administering the agent to a patient in need of treatment for a disorder related to reproduction in male mammals. The method may further comprise the step of determining whether the agent reduces gamma-carboxylase activity.

[00344] The binding of the agent to the target molecule in the above-described assays may be determined through the use of competitive binding assays. The competitor is a binding moiety known to bind to the target molecule. Under certain circumstances, there may be competitive binding as between the agent and the binding moiety, with the binding moiety displacing the agent or the agent displacing the binding moiety.

[00345] Either the agent or the competitor may be labeled. Either the agent, or the competitor is added first to the protein for a time sufficient to allow binding. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4°C and 40°C. Incubation periods may also be chosen for optimum binding, but may also optimized to facilitate rapid high throughput screening. Typically, between 0.1 and 1 hour will be sufficient. Excess agent and competitor are generally removed or washed away.

[00346] Using such assays, the competitor may be added first, followed by the agent. Displacement of the competitor is an indication that the agent is binding to the target molecule and thus is capable of binding to, and potentially modulating, the activity of the target molecule. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent.

[00347] In another example, the agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate that the agent is bound to the target molecule with a higher affinity than the competitor. Thus, if the agent is labeled, the presence of the label on the target molecule, coupled with a lack of competitor binding, may indicate that the agent is capable of binding to the target molecule.

[00348] The method may comprise differential screening to identify agents that are capable of modulating the activity of the target molecule. In such an instance, the methods comprise combining the target molecule and a competitor in a first sample. A second sample comprises an agent, the target molecule, and a competitor. Addition of the agent is performed under conditions which allow the modulation of the activity of the target molecule. The binding of the competitor is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of an agent capable of binding to the target molecule and potentially modulating its activity. That is, if the binding of the competitor is different in the second sample relative to the first sample, the agent is capable of binding to the target molecule.

[00349] Positive controls and negative controls may be used in the assays. Preferably, all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the target molecule. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound agent.

[00350] A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

[00351] Thus, in one example, the methods comprise combining a sample comprising OST-PTP, PTP-1B, or gamma-carboxylase and an agent, and evaluating the effect on OST-PTP, PTP-1B, or gamma-carboxylase enzyme activity. By enzyme activity, specifically OST-PTP, PTP-1B, or gamma-carboxylase enzyme activity, is meant one or more of the biological activities associated with the enzyme. For OST-PTP and PTP-1B, this activity is preferably the dephosphorylation of gamma-carboxylase; for gamma-carboxylase, it is the carboxylation of osteocalcin. The screening assays are designed to find agents that reduce OST-PTP, PTP-1B, or gamma-carboxylase activity, and/or increase levels of undercarboxylated/uncarboxylated osteocalcin.

[00352] Specifically, a screening method is provided to identify an agent that reduces OST-PTP activity, the method comprising the steps of: (a) providing a control mixture comprising OST-PTP or a fragment or variant thereof and a test mixture comprising OST-PTP or a fragment or variant thereof, (b) contacting the test mixture with an agent, (c) determining the level of activity of OST-PTP in the test mixture and in the control mixture, (d) identifying the agent as an agent that reduces OST-PTP activity if the level of OST-PTP activity in the test

mixture is lower than the level of OST-PTP activity in the control mixture, and (e) administering the identified agent to a patient in need of treatment for a disorder related to reproduction in male mammals.

[00353] A screening method is provided to identify an agent that reduces PTP-1B activity, the method comprising the steps of: (a) providing a control mixture comprising PTP-1B or a fragment or variant thereof and a test mixture comprising PTP-1B or a fragment or variant thereof, (b) contacting the test mixture with an agent, (c) determining the level of activity of PTP-1B in the test mixture and in the control mixture, (d) identifying the agent as an agent that reduces PTP-1B activity if the level of PTP-1B activity in the test mixture is lower than the level of PTP-1B activity in the control mixture, and (e) administering the identified agent to a patient in need of treatment for a disorder related to reproduction in male mammals.

[00354] A screening method is provided to identify an agent that reduces gamma-carboxylase activity, the method comprising the steps of: (a) providing a control mixture comprising gamma-carboxylase or a fragment or variant thereof and a test mixture comprising gamma-carboxylase or a fragment or variant thereof, (b) contacting the test mixture with an agent, (c) determining the level of activity of gamma-carboxylase in the test mixture and in the control mixture, (d) identifying the agent as an agent that reduces gamma-carboxylase activity if the level of gamma-carboxylase activity in the test mixture is lower than the level of gamma-carboxylase activity in the control mixture, and (e) administering the identified agent to a patient in need of treatment for a disorder related to reproduction in male mammals.

[00355] The present invention also provides a screening method to identify an agent that decarboxylates osteocalcin, the method comprising the steps of: (a) providing a control mixture comprising carboxylated osteocalcin and a test mixture comprising carboxylated osteocalcin, (b) adding to the test mixture an agent, (c) determining the level of carboxylated osteocalcin in the test mixture and in the control mixture, (d) identifying the agent as an agent that decarboxylates osteocalcin if the level of carboxylated osteocalcin in the test mixture is lower than the level of carboxylated osteocalcin in the control mixture, and (e) administering the identified agent to a patient in need of treatment for a disorder related to reproduction in male mammals.

[00356] A cell-based method is provided for identifying an agent that increases osteocalcin gene expression, the method comprising steps: (a) determining a first expression level of osteocalcin in a cell, (b) determining a second expression level of osteocalcin after contact with a test agent; and (c) comparing the first expression level with the second expression level, wherein if the first expression level is lower than the second expression level the agent is identified as an agent that increases osteocalcin gene expression, and (e) administering the identified agent to a patient in need of treatment for a disorder related to reproduction in male mammals. The level of osteocalcin gene expression may be determined by measuring the amount of osteocalcin mRNA made or the amount of osteocalcin protein made. In certain embodiments, the cell is an osteoblast.

[00357] The present invention also provides screening methods to identify agents that activate GPRC6A and are suitable for use in the prevention and treatment of a reproductive disorder in male mammals. In certain embodiments, the method comprises:

[00358] (a) providing a cell that expresses GPRC6A;

[00359] (b) exposing the cell to a candidate substance; and

[00360] (c) determining if the candidate substance binds to and/or activates the GPRC6A expressed by the cell.

[00361] Optionally, the method also comprises: (d) determining if the candidate substance is suitable for use in the prevention and treatment of a reproductive disorder in male mammals.

[00362] In certain embodiments, step (a) comprises providing Leydig cells, testis explants, or cells that recombinantly express GPRC6A. In certain embodiments, the cells that recombinantly express GPRC6A are NIH 3T3 cells, HEK 293 cells, BHK cells, COS cells, CHO cells, *Xenopus* oocytes, or insect cells. In certain embodiments, the GPRC6A is human GPRC6A. In certain embodiments, the GPRC6A is encoded by the nucleotide sequence shown in SEQ ID NO: 30. In certain embodiments, the GPRC6A comprises the amino acid sequence shown in SEQ ID NO: 31.

[00363] In certain embodiments, the candidate substance is from a library of candidate substances. In certain embodiments, the entire library of substances is exposed to the cell. In certain embodiments, a portion of the library is exposed to the cell.

[00364] In certain embodiments, step (b) is carried out by growing the cell in tissue culture and adding the candidate substance to the medium in which the cell is growing or has been grown. Alternatively, the medium in which the cell is growing or has been grown may be

removed and fresh medium containing the candidate substance may be added the tissue culture plate or well in which the cell is growing or has been grown.

[00365] In certain embodiments, step (c) comprises determining if the candidate substance competes with labeled uncarboxlated osteocalcin for binding to the GPRC6A. In certain embodiments, step (c) comprises labeling the candidate substance and determining if the labeled candidate substance binds to the GPRC6A expressed by the cell.

[00366] In certain embodiments, step (c) comprises determining if the candidate substance produces a physiological response in the cell selected from the group consisting of: an increase in the concentration of cAMP in the cell. an increase in testosterone synthesis in the cell, an increase in the expression of S*tAR* in the cell, an increase in the expression of *Cyp11a* in the cell, an increase in the expression of *Cyp17* in the cell, an increase in the expression of *3β-HSD* in the cell, an increase in the expression of *Grth* in the cell, an increase in the expression of *tACE* in the cell, an increase in CREB phosphorylation in the cell, and a decrease in the amount cleaved Caspase 3 in the cell. The physiological response may also be a combination of any of the foregoing physiological responses. In certain embodiments, the physiological response is an increase in the concentration of cAMP in the cell together with a lack of an increase in tyrosine phosphorylation, ERK activation, and intracellular calcium accumulation. In embodiments where a physiological response is determined, it may be advantageous to use a cell that does not naturally express GPRC6A but that has been engineered to recombinantly expresses GPRC6A. In such cases, the cell prior to transformation to a state that recombinantly expresses GPRC6A can serve as a negative control. In such case, the candidate substance should evoke the

physiolical response in the cell that recombinantly expresses GPRC6A but not in the negative control cell.

[00367] In certain embodiments, step (c) comprises determining if the candidate substance affects the binding of a G protein to the GPRC6A. Here, too, it may be advantageous to use cells that recombinantly express GPRC6A and to use those same cells before transformation as negative controls. In certain embodiments, the cell is co-transfected with a construct encoding GPRC6A and a construct encoding a G_{α} protein. See, e.g., Christiansen et al., 2007, Br. J. Pharmacol. 150:798-807 and Pi et al., 2005, J. Biol. Chem. 280:40201-40209.

[00368] In certain embodiments, step (d) comprises administering the candidate substance to a male mammal and determining that the candidate substance produces an effect in the male mammal selected from the group consisting of increased fertility, raised sperm count, increased sperm motility, increased sperm viability, increased serum testosterone levels, increased libido, amelioration of erectile dysfunction, reduction of the underdevelopment of testes, and reduction of excess apoptosis in testes.

[00369] The present invention also provides screening methods to identify agents that activate GPRC6A and are suitable for use in the prevention and treatment of a reproductive disorder in male mammals where the methods comprise:

[00370] (a) providing cell membranes containing GPRC6A protein;

[00371] (b) exposing the cell membranes to a candidate substance;

[00372] (c) determining if the candidate substance binds to the GPRC6A in the cell membranes; and

[00373] (d) determining if the candidate substance is suitable for use in the prevention and treatment of a reproductive disorder in male mammals.

[00374] In certain embodiments, step (a) comprises providing cell membranes from Leydig cells, testis explants, or cells that recombinantly express GPRC6A. In certain embodiments, the cells that recombinantly express GPRC6A are NIH 3T3 cells, HEK 293 cells, BHK cells, COS cells, CHO cells, *Xenopus* oocytes, or insect cells. In certain embodiments, the GPRC6A is human GPRC6A. In certain embodiments, the GPRC6A is encoded by the nucleotide sequence shown in SEQ ID NO: 30. In certain embodiments, the GPRC6A comprises the amino acid sequence shown in SEQ ID NO: 31

[00375] In certain embodiments, the candidate substance is from a library of candidate substances. In certain embodiments, the entire library of substances is exposed to the cell membranes. In certain embodiments, a portion of the library is exposed to the cell membranes.

[00376] In certain embodiments, step (c) comprises determining if the candidate substance competes with labeled uncarboxlated osteocalcin for binding to the GPRC6A. In certain embodiments, step (c) comprises labeling the candidate substance and determining if the labeled candidate substance binds to the GPRC6A in the cell membranes.

[00377] In certain embodiments, step (d) comprises administering the candidate substance to a male mammal and determining that the candidate substance produces an effect in the male

mammal selected from the group consisting of increased fertility, raised sperm count, increased sperm motility, increased sperm viability, increased serum testosterone levels, increased libido, amelioration of erectile dysfunction, reduction of the underdevelopment of testes, and reduction of excess apoptosis in testes.

[00378] In certain embodiments of the methods disclosed above, GPRC6A is the protein disclosed at GenBank accession no. AF502962. The nucleotide and amino acid sequences disclosed at GenBank accession no. AF502962 are shown in Figures 23 and 24 herein, respectively.

[00379] In certain embodiments of the methods disclosed above, GPRC6A is a protein homologous to the protein disclosed at GenBank accession no. AF502962. In certain embodiments of the methods disclosed above, GPRC6A is a protein having about 80-99%, about 85-97%, or about 90-95% amino acid sequence identity to the protein disclosed at GenBank accession no. AF502962.

[00380] In certain embodiments of the methods disclosed above, GPRC6A is the protein disclosed Wellendorph & Bräuner-Osborne, 2004, Gene 335:37-46.

[00381] In certain embodiments of the present invention, the agents identified by the methods of screening against GPRC6A are administered to a male mammal in need of treatment for a disorder related to reproduction. Accordingly, the present invention includes a method of treating disorders related to reproduction in male mammals comprising administering to a male mammal in need of treatment for a disorder related to reproduction a pharmaceutical

composition comprising a therapeutically effective amount of an agent that activates GPRC6A and a pharmaceutically acceptable carrier or excipient.

[00382] In certain embodiments, the an agent that activates GPRC6A is identified by a method comprising:

[00383] (a) providing a cell that expresses GPRC6A;

[00384] (b) exposing the cell to a candidate substance; and

[00385] (c) determining if the candidate substance binds to and/or activates the GPRC6A expressed by the cell.

[00386] Agents that activate GPCR6A include ornithine, lysine, and arginine (Christiansen et al., 2007, Br. J. Pharmacol. 150:798-807).

[00387] Gamma carboxylase catalyzes the posttranslational modification of specific glutamic acid residues within osteocalcin to form γ-carboxyglutamic acid residues. In an embodiment of the assays described herein, the level of gamma carboxylase activity or decarboxylase activity is determined by measuring the level of osteocalcin carboxylation.

[00388] Cells to be used in the screening or assaying methods described herein include cells that naturally express OST-PTP, the phosphatase 1 domain of OST-PTP, PTP-1B, gamma-carboxylase, or osteocalcin as well as cells that have been genetically engineered to express (or overexpress) OST-PTP, the phosphatase 1 domain of OST-PTP, PTP-1B gamma-carboxylase, or

osteocalcin. Such cells include transformed osteoblasts that overexpress OST-PTP, the phosphatase 1 domain of OST-PTP, PTP-1B, or gamma-carboxylase.

[00389] A method is provided for identifying an agent useful for treating or preventing a disorder related to reproduction in male mammals comprising: (a) providing an animal that has a disorder related to reproduction in male mammals, (b) determining the amount of undercarboxylated/uncarboxylated osteocalcin in a pre-administration biological sample taken from the animal, (c) administering an agent to the animal, (d) determining the amount of undercarboxylated/uncarboxylated osteocalcin in a post-administration biological sample taken from the animal, and (e) identifying the agent as useful for treating or preventing the disorder related to reproduction in male mammals if the amount of undercarboxylated/uncarboxylated osteocalcin in the post-administration biological sample is higher than the amount of undercarboxylated/uncarboxylated osteocalcin in the pre-administration biological sample.

[00390] The term "agent" as used herein includes any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, lipid, etc., or mixtures thereof. Some of the agents can be used therapeutically. An agent may be OST-PTP, PTP-1B, gamma-carboxylase, osteocalcin, or fragments thereof.

[00391] Generally, in the assays described herein, a plurality of assay mixtures is run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., is at zero concentration or below the level of detection.

[00392] Agents for use in screening encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons, preferably less than about 500 daltons. Agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of these functional chemical groups. The agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred biomolecules are peptides.

[00393] Libraries of high-purity small organic ligands and peptides that have well-documented pharmacological activities are available from numerous sources for use in the assays herein. One example is an NCI diversity set which contains 1,866 drug-like compounds (small, intermediate hydrophobicity). Another is an Institute of Chemistry and Cell Biology (ICCB; maintained by Harvard Medical School) set of known bioactives (467 compounds) which includes many extended, flexible compounds. Some other examples of the ICCB libraries are: Chem Bridge DiverSet E (16,320 compounds); Bionet 1 (4,800 compounds); CEREP (4,800 compounds); Maybridge 1 (8,800 compounds); Maybridge 2 (704 compounds); Maybridge HitFinder (14,379 compounds); Peakdale 1 (2,816 compounds); Peakdale 2 (352 compounds); ChemDiv Combilab and International (28,864 compounds); Mixed Commercial Plate 1 (352 compounds); Mixed Commercial Plate 2 (320 compounds); Mixed Commercial Plate 3 (251

compounds); Mixed Commercial Plate 4 (331 compounds); ChemBridge Microformat (50,000 compounds); Commercial Diversity Set1 (5,056 compounds). Other NCI Collections are: Structural Diversity Set, version 2 (1,900 compounds); Mechanistic Diversity Set (879 compounds); Open Collection 1 (90,000 compounds); Open Collection 2 (10,240 compounds); Known Bioactives Collections: NINDS Custom Collection (1,040 compounds); ICCB Bioactives 1 (489 compounds); SpecPlus Collection (960 compounds); ICCB Discretes Collections. The following ICCB compounds were collected individually from chemists at the ICCB, Harvard, and other collaborating institutions: ICCB1 (190 compounds); ICCB2 (352 compounds); ICCB3 (352 compounds); ICCB4 (352 compounds). Natural Product Extracts: NCI Marine Extracts (352 wells); Organic fractions--NCI Plant and Fungal Extracts (1,408 wells); Philippines Plant Extracts 1 (200 wells); ICCB-ICG Diversity Oriented Synthesis (DOS) Collections; DDS1 (DOS Diversity Set) (9600 wells). Compound libraries are also available from commercial suppliers, such as ActiMol, Albany Molecular, Bachem, Sigma-Aldrich, TimTec, and others.

[00394] Known and novel pharmacological agents identified in screens may be further subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, or amidification to produce structural analogs.

[00395] When screening, designing, or modifying compounds, other factors to consider include the Lipinski rule-of-five (not more than 5 hydrogen bond donors (OH and NH groups); not more than 10 hydrogen bond acceptors (notably N and O); molecular weight under 500 g/mol; partition coefficient log *P* less than 5), and Veber criteria, which are recognized in the

pharmaceutical art and relate to properties and structural features that make molecules more or less drug-like.

[00396] The agent may be a protein. By "protein" in this context is meant at least two covalently attached amino acids, and includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid," or "peptide residue," as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and norleucine are considered amino acids for the purposes of the invention. "Amino acids" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard *in vivo* degradations.

[00397] The agent may be a naturally occurring protein or fragment or variant of a naturally occurring protein. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way, libraries of prokaryotic and eukaryotic proteins may be made for screening against one of the various proteins. Libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred, may be used.

[00398] Agents may be peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above,

random peptides, or "biased" random peptides. By "random" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized agent bioactive proteinaceous agents.

[00399] The library may be fully randomized, with no sequence preferences or constants at any position. Alternatively, the library may be biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

[00400] The agent may be an isolated nucleic acid, preferably antisense, siRNA, or cDNA that binds to either the gene encoding the protein of interest, or its mRNA, to block gene expression or mRNA translation, respectively. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. Such nucleic acids will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., 1993, Tetrahedron 49:1925 and references therein;

Letsinger, 1970, J. Org. Chem. 35:3800; Sprinzl et al., 1977, Eur. J. Biochem. 81:579; Letsinger et al., 1986, Nucl. Acids Res. 14:3487; Sawai et al, 1984, Chem. Lett. 805; Letsinger et al., 1988, J. Am. Chem. Soc. 110:4470; and Pauwels et al., 1986, Chemica Scripta 26:141); phosphorothioate (Mag et al., 1991, Nucleic Acids Res. 19:1437; and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., 1989, J. Am. Chem. Soc. 111:2321); O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, 1992, J. Am. Chem. Soc. 114:1895; Meier et al., 1992, Chem. Int. Ed. Engl. 31:1008; Nielsen, 1993, Nature, 365:566; Carlsson et al., 1996, Nature 380:207); all of which publications are incorporated by reference and may be consulted by those skilled in the art for guidance in designing nucleic acid agents for use in the methods described herein.

[00401] Other analog nucleic acids include those with positive backbones (Denpcy et al., 1995, Proc. Natl. Acad. Sci. USA 92:6097); non-ionic backbones (U.S. Patent Nos. 5,386,023; 5,637,684; 5,602,240; 5,216,141; and 4,469,863; Kiedrowshi et al., 1991, Angew. Chem. Intl. Ed. English 30:423; Letsinger et al., 1988, J. Am. Chem. Soc. 110:4470; Letsinger et al., 1994, Nucleoside & Nucleoside 13:1597; Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research," Ed. Y. S. Sanghui and P. Dan Cook; Mesmaeker et al., 1994, Bioorganic & Medicinal Chem. Lett. 4:395; Jeffs et al., 1994, J. Biomolecular NMR 34:17); and non-ribose backbones, including those described in U.S. Patents Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in antisense Research," Ed. Y. S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition

of nucleic acids that may be used as agents as described herein. Several nucleic acid analogs are described in Rawls, C & E News Jun. 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc.

[00402] As described above generally for proteins, nucleic acid agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes may be used as outlined above for proteins.

[00403] The agents may be obtained from combinatorial chemical libraries, a wide variety of which are available in the literature. By "combinatorial chemical library" herein is meant a collection of diverse chemical compounds generated in a defined or random manner, generally by chemical synthesis. Millions of chemical compounds can be synthesized through combinatorial mixing.

[00404] The determination of the binding of the agent to one of the various proteins such as OST-PTP, PTP-1B, or gamma-carboxylase may be done in a number of ways. In a preferred embodiment, the agent is labeled, and binding determined directly. For example, this may be done by attaching all or a portion of one of the various proteins to a solid support, adding a labeled agent (for example an agent comprising a radioactive or fluorescent label), washing off excess reagent, and determining whether the label is present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

[00405] By "labeled" herein is meant that the agent is either directly or indirectly labeled with a label which provides a detectable signal, e.g. a radioisotope (such as ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, or ^{125}I), a fluorescent or chemiluminescent compound (such as fluorescein isothiocyanate, rhodamine, or luciferin), an enzyme (such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase), antibodies, particles such as magnetic particles, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin, etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal. Only one of the components may be labeled. Alternatively, more than one component may be labeled with different labels.

[00406] Transgenic mice, including knock in and knock out mice, and isolated cells from them (especially osteoblasts) that over or under express the nucleic acids disclosed herein (e.g., cDNA for Esp, PTP-1B, osteocalcin, gamma-carboxylase) can be made using routine methods

known in the art. In certain instances, nucleic acids are inserted into the genome of the host organism operably connected to and under the control of a promoter and regulatory elements (endogenous or heterogeneous) that will cause the organism to over express the nucleic acid gene or mRNA. One example of an exogenous/heterogeneous promoter included in the transfecting vector carrying the gene to be amplified is alpha 1(I) collagen. Many such promoters are known in the art.

[00407] Human osteoblasts can be transfected with vectors carrying the cDNA for human Esp, human PTP-1B, or human osteocalcin (or fragments or variants thereof) operably linked to known promoters and regulatory elements that cause the transfected human osteoblast to overexpress osteocalcin (or fragments or variants thereof).

[00408] Disclosed herein are transgenic mice and mouse cells, and transfected human cells overexpressing osteocalcin (or fragments or variants thereof), OST-PTP, PTP-1B, or gamma-carboxylase. Also disclosed herein are double mutant mice that have deletions of one or both alleles for osteocalcin, Esp, and gamma-carboxylase, and various combinations of double mutants.

[00409] Also disclosed herein are vectors carrying the cDNA or mRNA encoding the proteins for insertion into the genome of a target animal or cell. Such vectors can optionally include promoters and regulatory elements operably linked to the cDNA or mRNA. By "operably linked" is meant that promoters and regulatory elements are connected to the cDNA or mRNA in such a way as to permit expression of the cDNA or mRNA under the control of the promoters and regulatory elements.

[00410] Antisense and small interfering RNAs for use in reducing expression of OST-PTP, PTP-1B, and/or gamma-carboxylase, thereby treating or preventing a disorder related to reproduction in a male mammal can be made that specifically hybridize to the gene and/or mRNA encoding OST-PTP, PTP-1B, or gamma-carboxylase, respectively. The sequence for mouse (OST-PTP, Ptprv) cDNA is set forth in SEQ ID NO:10. The amino acid sequence for OST-PTP, Ptprv) protein is set forth in SEQ ID NO:11. This cDNA, or antisense and small interfering RNAs based on this cDNA, will hybridize with mRNA for OST-PTP and thereby interfere with its translation. Reducing OST-PTP expression will increase the level of undercarboxylated/uncarboxylated osteocalcin, thereby providing a therapeutic benefit with respect to disorders related to reproduction in male mammals. The sequence for human PTP-1B cDNA is set forth in SEQ ID NO:16. The amino acid sequence for human PTP-1B protein is set forth in SEQ ID NO:17. This cDNA, or antisense and small interfering RNAs based on this cDNA, will hybridize with mRNA for human PTP-1B and thereby interfere with its translation. Reducing human PTP-1B expression will increase the level of undercarboxylated/ uncarboxylated osteocalcin, thereby providing a therapeutic benefit with respect to disorders related to reproduction in male mammals. The cDNA for mouse gamma-carboxylase is identified by SEQ ID NO:8, and its amino acid sequence is SEQ ID NO:9. This cDNA, or antisense and small interfering RNAs based on this cDNA, will hybridize with mRNA for gamma-carboxylase and thereby interfere with its translation and is a preferred embodiment. The cDNA for human gamma-carboxylase is identified by SEQ ID NO:6, and the amino acid sequence is SEQ ID NO:7. Human gamma-carboxylase cDNA can be used therapeutically to

reduce gamma-carboxylase expression to treat or prevent a disorder related to reproduction in male humans.

[00411] The invention is illustrated herein by the following examples, which should not be construed as limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference. Those skilled in the art will understand that this invention may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will fully convey the invention to those skilled in the art. Many modifications and other embodiments of the invention will come to mind in one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing description. Although specific terms are employed, they are used as in the art unless otherwise indicated.

EXAMPLES

[00412] **Example 1 - Male *Osteocalcin*-deficient mice have decreased fertility**

[00413] Male mice in which both alleles of osteocalcin were disrupted and non-functional (*Osteocalcin*-/- mice) that were crossed with wild-type (WT) littermates show impaired fertility. Whether the *Osteocalcin*- mutation was on the C57Bl/6J or on the 129sv/ev genetic background, very few litters were obtained over the course of 3 months. Moreover, the litters were of significantly smaller size than those obtained when crossing WT male mice with WT female mice. When 8 *Osteocalcin*-/- male mice were placed with 2 WT female mice each from 6 to 12 weeks of age, only 17 pups were obtained and the litter size was 4.25 pups per litter. In contrast,

when 8 WT male mice were placed with 2 WT female mice each for the same period of time, 63 pups were obtained and the litter size was also significantly larger (7.93 pups per litter) (Figure 1). Furthermore, it was observed that after 6 months of age, and unlike what is the case for WT mice, male *Osteocalcin* -/- mice were totally infertile. This reproduction phenotype was not observed in O*steocalcin* +/- mice (mice having a single allele of osteocalcin disrupted).

[00414] **Example 2 -Male mice have abnormal spermatogenesis in the absence of osteocalcin**

[00415] Testis weight was measured at different ages in male *Osteocalcin*-deficient mice. As early as at 6 weeks of age, male *Osteocalcin* -/- mice had significantly smaller testes than their WT littermates and this phenotype progressively worsened over time (Figure 2A). Sperm count in the seminal fluid of *Osteocalcin*-/- and WT littermate male mice was also measured. It was found that sperm count was already decreased by 37% in 6 weeks old *Osteocalcin* -/- mice and that this decrease reached 60% of the sperm count in WT mice at 6 months of age (Figure 2B).

[00416] **Example 3 - *Esp*-deficient and *Osteocalcin*-deficient mice have opposite reproductive phenotypes**

[00417] *Esp* encodes a phosphatase which decreases osteocalcin bioactivity to such an extent that *Esp*-/- and *Osteocalcin*-/- mice display metabolic abnormalities that are the mirror image of one another (Hinoi et al., 2008, J. Cell Biol. 183:1235-1242; Ferron et al., 2008, Proc. Natl. Acad. Sci. USA 105:5266-5270; Lee et al., 2007, Cell 130:456-469). *Esp*-/- mice were tested for abnormalities of spermatogenesis. As shown in Figure 3A, *Esp*-/- mice had

significantly bigger testes than WT littermates at both 6 and 12 weeks of age. Moreover, their sperm count was significantly increased (Figure 3B). These data strongly suggest, although they do not prove, that, as is the case for energy metabolism, *Esp* and *Osteocalcin* are in the same genetic pathway and that it is the uncarboxylated form of osteocalcin that regulates spermatogenesis.

[00418] **Example 4 - Osteocalcin regulates germ cell apoptosis**

[00419] The proliferation and apoptosis of germ cells in *Osteocalcin* -/- or in *Esp* -/- mice were studied. When using BrdU labeling *in vivo*, no abnormalities in germ cell proliferation in *Osteocalcin* -/- or in the *Esp* -/- mice were detected at any time point (Figure 4A). In contrast, study of apoptosis by TUNEL assay consistently showed a significant increase in the apoptosis of germ cells in *Osteocalcin* -/- mice as young as 2 weeks of age (Figure 4B). In 12 week-old *Osteocalcin* -/- mutant mice, there was a 50% increase in germ cell apoptosis compared to WT mice (Figure 4B). Conversely, a decrease was observed in the number of apoptotic germ cells in *Esp* -/- mice compared to WT littermates at all time points analyzed (Figure 4C). Molecularly, it could be shown that Caspase 3, one of the main effectors of apoptosis, was significantly more abundant in *Osteocalcin* -/- than in WT testes (Figure 4D).

[00420] The morphology of Leydig cells was studied by immunostaining of 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (3β-HSD). The number of Leydig cells was not significantly affected by the absence of osteocalcin or Esp, nor was expression of genes affecting cell proliferation (Figure 14A and data not shown). Nevertheless, Leydig cells appeared hypotrophic in *Ocn-/-* testes as determined by the significant decrease of the ratio between the

Leydig cells and interstitial areas observed in *Ocn-/-* compared to WT testes (Figure 14B-C). Conversely, this ratio was increased in *Esp-/-* testes (Figure 14B-C).

[00421] The size of the epithelium in testis tubules was significantly decreased in *Ocn-/-* mice, a feature suggesting that osteocalcin regulates, presumably through its effect on testosterone biosynthesis, germ cell numbers (Figure 14D). In view of this result, and since testosterone inhibits germ cell apoptosis (Brinkworth et al., 1995, J. Reprod. Fertil. 105:25-33; Henriksen et al., 1995, Endocrinology 136:3285-3291; Sinha Hikim and Swerdloff, 1999, Rev. Reprod. 4:38-47), TUNEL assays were performed. Those assays showed a 50% increase in germ cell apoptosis in *Ocn-/-* compared to WT mice and a 50% decrease in *Esp-/-* testes (Figure 14F). *In vivo* BrdU labeling did not reveal any abnormalities in germ cell proliferation in either *Ocn-/-* or *Esp -/-* mice (Figure 20A).

[00422] Since osteocalcin favors testosterone synthesis by Leydig cells, whether osteocalcin affects the expression of enzymes necessary for testosterone biosynthesis such as S*tAR, Cyp11a, Cyp17,* and *3β-HSD* was tested. Uncarboxylated osteocalcin increased expression of these genes in Leydig cell cultures (Figure 14G and 14H). Accordingly, their expression was significantly decreased in *Ocn-/-* and increased in *Esp-/-* testes (Figure 14H), while it was unaffected in *Ocn-/-* and *Esp-/-* ovaries or adrenal glands (Figure 21B-E). Of note, there was no change in expression of *Cyp19*, the gene encoding the testosterone aromatase, or of *HSD-17,* in *Ocn-/-* and *Esp-/-* testes (Figure 14G and 14H).

[00423] Further support for the notion that osteocalcin influences germ cell apoptosis through testosterone was provided by an examination of the expression of *Gonadotropin*

Regulated Testicular Helicase (*Grth*). This gene has emerged as an essential regulator of spermatogenesis whose expression in germ cells is regulated by testosterone and inhibits germ cell apoptosis (Dufau and Tsai-Morris, 2007, Trends Endocrinol. Metab. 18:314-320; Sheng et al., 2006, J. Biol. Chem. 281:35048-35056; Tsai-Morris et al., 2007, Mol. Hum. Reprod. 13:887-892). *Grth* expression was decreased in *Ocn-/-* and increased in *Esp-/-* testes (Figure 14I). GRTH inhibits activation of *Caspase 3*, a determinant of apoptosis (Gutti et al., 2008, J. Biol. Chem. 283:17055-17064) and favors expression of tACE, a protein favoring germ cell maturation. Consistent with these notions, Western blot analyses showed an increase of cleaved caspase 3 protein accumulation and a decrease of tACE in *Ocn-/-* testes (Figure 14J).

[00424] **Example 5 - Osteocalcin regulates testosterone production**

[00425] The low sperm count without any abnormality in proliferation and the increase in apoptosis of germ cells suggested that the reproduction phenotype of the *Osteocalcin-/-* males could be due to a decrease in testosterone secretion or action. To determine if that was the case, testosterone levels were measured in *Osteocalcin-/-*, *Esp-/-*, and WT mice. As shown in Figure 5, there was a 70% decrease in the level of circulating testosterone in *Osteocalcin-/-* male mice while, in contrast, this level was increased in *Esp-/-* male mice.

[00426] To further prove that osteocalcin is a regulator of testosterone synthesis, a well-characterized mouse Leydig cell line, the TM3 cell line (Mather, 1980, Biol. Reprod. 23:243-252), was used. These cells have been extensively used as a model of Leydig cells for *in vitro* studies and have been shown to express SF-1, an important transcriptional regulator of most genes involved in testosterone biosynthesis (Mather, 1980, Biol. Reprod. 23:243-252; Cammas,

1997, Mol. Endocrinol. 11:867-876; Dakhova et al., 2009, Endocrinology 150:404-412). TM3 cells were treated with increasing amounts of osteocalcin and assayed for the expression of genes encoding enzymes of the steroidogenic cascade. As shown in Figure 6, osteocalcin specifically increased the expression of *StAR*, a cholesterol shuttle molecule, and of two cytochrome P450 steroid hydroxylases (*Cyp11a* and *Cyp17)*. Thus, whether looked at by *in vivo* or cell-based assays, osteocalcin promotes testosterone biosynthesis.

[00427] In a further series of experiments, supernatants of mesenchymal cell cultures were assayed for their ability to affect hormone production by testes and/or ovaries. In these cell-based assays (Figure 11A), of all those tested, the supernatants of osteoblast cultures increased testosterone secretion by testis explants to the largest extent (over 3 fold), while not affecting estradiol and progesterone secretion by testes or ovaries (Figure 11B-G). Since testosterone is produced by Leydig cells of the testes, whether osteoblast-derived molecule(s) act directly on Leydig cells was tested by culturing primary mouse Leydig cells in the presence or absence of supernatants of osteoblast cultures or cultures of other mesenchymal cell types. In the conditions of this assay, supernatants of osteoblast cultures were the only ones able to increase testosterone production by Leydig cells significantly (more than 4 fold) (Figure 11H). These experiments indicate that osteoblasts are the cells of mesenchymal origin that affect testosterone biosynthesis to the largest extent, and that they do so through secreted molecule(s) acting on Leydig cells of the testis. This novel endocrine function of osteoblasts was restricted to androgen production. Adipocytes also enhanced sex steroid hormone secretion, albeit to a lesser extent.

[00428] Several lines of evidence indicated that osteocalcin is the osteoblast-derived hormone enhancing testosterone secretion by Leydig cells. First, supernatants of wild type (WT) but not of *Osteocalcin* (*Ocn) -/-* osteoblast cultures increased testosterone production by testis explants and mouse Leydig cells (Figure 12A-B). Second, treating testis explants or Leydig cells with increasing amounts of uncarboxylated osteocalcin, the active form of the hormone, resulted in a dose-dependent increase in testosterone secretion (Figure 12C-D). Third, injection of osteocalcin in WT mice increased circulating levels of testosterone (Figure 12E). Fourth, to determine if osteocalcin regulates male fertility *in vivo*, loss- (*Ocn-/-* mice) and gain-of-function (*Esp-/-* mice) mouse models for osteocalcin (Lee et al., 2007, Cell 130:456-469) were used. When *Ocn-/-* males were crossed with WT female mice, the litter sizes were nearly two-fold smaller than when WT males were crossed with WT female mice (Figure 12F). Conversely, the number of pups per litter was consistently increased when *Esp-/-* males were bred with WT female mice (Figure 12F). The frequency of litters over a period of 8 weeks was also decreased in the case of the loss-of-function model and increased in the gain-of-function model (Figure 12G). Testis size and weight were significantly decreased in *Ocn-/-* and increased in *Esp-/-* mice at 3 months of age (Figure 12H and 12I). The weights of epididymides and seminal vesicles as well as sperm count were also significantly decreased in *Ocn-/-* and increased in *Esp-/-* mice (Figure 12J-L). These abnormalities worsened over time (Figure 12I and 12L).

[00429] Motility of sperm from both WT and *Ocn-/-* males was assessed by videomicroscopy immediately after dissemination from the caudal epididymis or after 2 hours of incubation under conditions known to prepare sperm for fertilization (Suarez and Osman, 1987, Biol. Reprod. 36:1191-1198). In both cases, the percentage of motile sperm did not differ

between *Ocn*-/- and WT mice (Figure 18A). Likewise, the percentage of abnormally shaped or dead sperm was similar in WT and *Ocn*-/- mice (Figure 18B-C).

[00430] Consistent with the fact that osteocalcin favors testosterone synthesis in Leydig cells *ex vivo*, circulating levels of testosterone were markedly decreased in *Ocn*-/- and increased in *Esp*-/- mice. Circulating progesterone levels were similar in *Ocn*-/- and WT mice and, although circulating levels of estradiol were higher in *Ocn*-/- than in WT mice, they remained within the normal range (Figure 12M). The most likely explanation for this mild increase in circulating estradiol levels in the *Ocn*-/- mice is that the increase in the number of adipocytes caused by *Osteocalcin* inactivation may result in an increase in the aromatization of testosterone into estrogen in fat (Nelson and Bulun, 2001, J. Am. Acad. Dermatol. 45:S116-124; Simpson et al., 2000, Trends Endocrinol. Metab. 11:184-188; Simpson, 2003, J. Steroid Biochem. Mol. Biol. 86:225-230). Estradiol levels were not affected in *Esp*-/- mice. As predicted by the co-culture assays, female fertility, ovary weight, morphology of the uterus, follicle numbers, and circulating levels of steroid sex hormones were normal in *Ocn*-/- female mice (Figure 18D-L). Taken together, these cell biology and genetic experiments identify osteocalcin as a secreted molecule favoring male fertility by increasing testosterone production by Leydig cells.

[00431] **Example 6 - Daily osteocalcin injections affect sperm counts and germ cell apoptosis**

[00432] WT mice (n=6) were injected daily with either 3 ng/g or 30 ng/g of uncarboxylated osteocalcin for 12 weeks. Uncarboxylated osteocalcin, at both doses, significantly increased testis weight and sperm count and decreased sperm cell apoptosis (Figure

7A-C). Likewise, serum testosterone levels were increased in osteocalcin-injected mice compared to vehicle-injected mice (Figure 7D). Hence, whether looked at by genetic models of loss-of-function or gain-of-function of osteocalcin or by pharmacological means to increase osteocalcin serum levels, osteocalcin acts as a regulator of testosterone production and male germ cell production.

[00433] **Example 7 - *Osteocalcin* is not expressed in testis**

[00434] Given the nature and severity of the *Osteocalcin*-/- phenotype, a possible concern was that *Osteocalcin* could be in fact expressed at low, but nevertheless biologically important, levels in some cell types of the testis. This concern was even more legitimate since *Esp* is known to be expressed in Sertoli cells (Dacquin et al., 2004, Dev. Dyn. 229:826-834). To begin addressing this concern, several different experiments were performed. First, *Osteocalcin* expression in bone versus testes was compared by quantitative PCR. A 1,000 fold higher expression in bone than in testis was observed (Figure 8A). Second, *in situ* hybridizations were performed for *Osteocalcin* expression but failed to detect any signal in the testis (Figure 8B). Third, the *Cherry* gene, a fluorescent reporter gene, was knocked into the *Osteocalcin* locus to create *Ocn-Cherry* mice. Using this reporter, a strong signal could be detected in osteoblasts but staining could not be detected in testes (Figure 8C). Taken together, these results, albeit negative, strongly suggest that osteocalcin is not expressed in any cell type of the testes.

[00435] Gene expression and cell-specific gene deletion experiments were performed to further determine that osteocalcin regulates male fertility as an osteoblast-secreted molecule, not as a testis-secreted factor. When comparing *Osteocalcin* expression in bone, testes, and ovaries

by quantitative PCR (qPCR), it was observed that *Osteocalcin* expression was more than 750 fold higher in bone than in gonads; furthermore, *Osteocalcin* transcript or protein was not detected in testes by *in situ* hybridization or Western blot analyses (Figure 13A-C). To be able to trace *Osteocalcin*-expressing cells *in vivo,* the m*Cherry* fluorescent reporter gene was knocked into the *Ocn* locus (*Ocn-mCherry* mice) (Figure 19A-B). While the expected strong signal was observed in osteoblasts, there was no detectable m*Cherry* fluorescence in testes (Figure 13D). Thus, through multiple assays, *Osteocalcin* expression was not detected in testes.

[00436] **Example 8 - Generation of a floxed allele for *Osteocalcin***

[00437] Although the expression study described above did not identify *Osteocalcin* expression in Sertoli, germ cells or Leydig cells of the testes, there remained a concern that the sensitivity of the techniques used was not sufficient to detect a very low expression of *Osteocalcin* in these cells and that such expression could be the true cause of the reproduction phenotype observed in the *Osteocalcin* -/- mice. To more formally exclude this possibility, *Osteocalcin* cell-specific knockout lines may be generated and analyzed. The first step toward this goal is to generate a floxed allele of the *Osteocalcin* locus. Following the same deletion strategy previously used to create the complete knockout allele (Ducy et al., 1996, Nature 382:448-452), a targeting vector harboring LoxP sites flanking the *Osteocalcin* locus was generated (Figure 9A). Upon recombination by the Cre recombinase specifically expressed in a particular cell type, both *Osteocalcin* genes should be deleted in those cells. As shown in Figure 9B, mice harboring this floxed allele have already been obtained; such mice can be used to generate and analyze mice lacking osteocalcin in a cell-specific manner.

[00438] Cell-specific loss- and gain-of-function models of osteocalcin were genereated by crossing mice harboring floxed alleles of *Ocn* (Figure 19C-D) or *Esp* with either the *α1(I) Collagen-Cre* transgenic mice or the *Cyp17-iCre* transgenic mice to delete genes in osteoblasts or in Leydig cells only, respectively (Bridges et al., 2008, Dev Dyn. 224:245-251). Testis size and weight, epididymides and seminal vesicle weights, sperm count, and circulating testosterone levels were all reduced in 12 week-old Ocn_{Osb}-/- mice while none of these parameters were affected in mice lacking *Osteocalcin* in Leydig cells only (Figure 13E-I). There was a tight correlation between osteocalcin and testosterone circulating levels in Ocn_{Osb}-/- mice (Figure 13J). Conversely, Esp_{Osb}-/- mice displayed testis abnormalities identical to those of *Esp*-/- mice and that were the mirror image of *Ocn*-/- or Ocn_{Osb}-/- mice. Inactivation of *Esp* in Sertoli cells, where this gene is expressed (Dacquin et al., 2004, Dev. Dyn. 229:826-834; Jamin et al., 2003, Mol. Cell. Endocrinol. 211:15-19), had no detectable deleterious consequence on testis biology (Figure 13K-N). Hence, it is only through its expression in osteoblasts that osteocalcin promotes male fertility.

[00439] **Example 9 - Esp-deficient mice**

[00440] Esp-deficient mice are mice in which one (+/-) or both alleles (-/-) for OST-PTP have been inactivated in all of the cells in the animal. The mice were made by homologous recombination of a targeted OST-PTP allele with a transgene having a sequence encoding a nuclear-localized LacZ cassette, which is homologously recombined into exon 6 of the OST-PTP allele, such that the transgene is in frame with the OST-PTP gene, and expression of the transgene is operably linked to the native gene expression regulatory sequences of the OST-PTP

allele. The production of Esp-deficient mice is described in more detail in International Patent Publication No. WO 2008 033518; Ducy et al., 1996, Nature 382:448-452; and Lee et al., 2007, Cell 130:456-469.

[00441] **Example 10 - *Osteocalcin*-deficient and other mice**

[00442] "Osteocalcin-deficient mice" as used herein means a strain of mice in which both osteocalcin alleles were deleted. Generation of *Osteocalcin*-/- mice was previously reported (Ducy et al., 1996, Nature 382:448-452). Exon 4 of osteocalcin gene 1 (OG1), coding for the mature protein, and the entire osteocalcin gene 2 (OG2) sequence, were deleted, while osteocalcin-related gene (ORG) was left in place. Correct targeting resulted in the replacement of the entire mature osteocalcin protein-coding sequences by the pGKNeo selection cassette.

[00443] All experiments giving rise to the data shown in Figures 11-22 were performed on the 129-Sv (Taconic) genetic background. Control littermates were used in all these experiments. Mouse genotypes were determined by PCR. Strategies for generating transgenic mice are depicted in Figures 9, 19, and 22.

[00444] **Example 11 - Laboratory measurements**

[00445] Blood was collected by heart puncture of isoflurane anesthetized mice. Osteocalcin levels were quantified by IRMA (Immunotopics kit).

[00446] **Example 12 - Gene expression analyses**

[00447] Gene expression analyses were performed using real time PCR. DNAse I-treated total RNA was converted to cDNA with the SuperScript III kit (Invitrogen). Real-time PCR were performed using the Taq SYBR Green Supermix with ROX (Biorad) on an MX3000 instrument (Stratagene); beta-actin amplification was used as an internal reference for each sample. All primers were from SuperArray.

[00448] **Example 13 - Recombinant osteocalcin**

[00449] Recombinant osteocalcin was bacterially produced and purified on glutathione beads according to standard procedures. Osteocalcin was then cleaved from the GST subunit using thrombin digestion. Thrombin contamination was removed using an affinity column. The purity of the product was qualitatively assessed by SDS-PAGE. Bacteria do not have a gamma-carboxylase gene. Therefore, recombinant osteocalcin produced in bacteria is always completely undercarboxylated at all three sites.

[00450] **Example 14 – ELISA to measure undercarboxylated Osteocalcin**

[00451] Carboxylation levels of osteocalcin in the serum are usually assessed indirectly by hydroxyapatite (HA) pull down followed by measurement of the unbound fraction of osteocalcin using a commercially available radioimmunoassay (RIA). The HA assay is based on the principle that undercarboxylated osteocalcin has a decreased binding affinity for HA compared to carboxylated osteocalcin. HA-based measurements of undercarboxylated osteocalcin have some limitations, as this method requires a relatively large volume of serum. Moreover, the HA pull down assay of osteocalcin is a semi-quantitative method, as it does not precisely quantify the serum concentration of undercarboxylated osteocalcin, but only estimates

the percentage of osteocalcin having a low HA affinity. In order to measure more precisely undercarboxylated osteocalcin, an enzyme-linked immunosorbent assay (ELISA) system was developed for the quantification of mouse undercarboxylated osteocalcin.

[00452] Goats were immunized with full-length bacterially produced fully uncarboxylated osteocalcin. Using affinity columns, polyclonal antibodies were purified from these goat anti-sera that recognized either the osteocalcin C-terminal region between amino acids 25 and 46 (OC25-46) or the uncarboxylated central region between amino acids 11 and 26 (unOC11-26). Dot blot analysis verified that the OC25-46 antibodies recognized both uncarboxylated and carboxylated osteocalcin, while the unOC11-26 recognized specifically uncarboxylated osteocalcin. The OC25-46 antibodies were next conjugated to horseradish peroxidase (HRP), while the unOC11-26 antibodies were coated on ELISA plates using standard procedures. To test the specificity of the ELISA system, different concentrations of uncarboxylated or carboxylated osteocalcin were incubated on the plate for 18h at 4°C, then the wells were washed 5 times and incubated in the presence of the OC25-46 HRP conjugated antibodies for 1h at room temperature. Following 5 washes, the immunocomplex was incubated with an HRP substrate, TMB, which is converted to a blue compound by peroxidases. The reaction was stopped with 0.18 M H_2SO_4 and absorbance (O.D.) at 450 nm was measured using an ELISA plate reader. As shown in Figure 10, this assay allows the specific quantification of uncarboxylated osteocalcin in a physiological concentration range (1 to 100 ng/ml).

[00453] **Example 15 – Preparation of primary Leydig cells and testis explants**

[00454] Adult mouse Leydig cells were isolated by mechanical dissociation of the testes followed by purification on a 0-90% Percoll gradient (Hunter et al., 1982, Mol. Cell. Endocrinol. 25:35-47; Schumacher et al., 1978, FEBS Lett. 91:333-338). Primary Leydig cells were cultured in Minimal Essential Medium (MEM + GlutaMAX, Invitrogen) supplemented with 1x PenStrep, 25 mM HEPES, pH 7.4 and 0.07% BSA at 33°C in 5% CO_2. After 3 hours of attaching and starvation, cells were washed once with culture medium and then used for experiments. The preparation of testis explants was adapted from Powlin et al., 1998, Toxicol. Sci. 46:61-74. Explants were washed 3 times with PBS 1X and placed in serum free RPMI medium for 2 hours before being used for expriments.

[00455] **Example 16 – Osteocalcin stimulation of Leydig cells or testis explants**

[00456] Primary Leydig cells and testis explants were washed 3 times with PBS 1X and stimulated with different doses of recombinant osteocalcin prepared as previously described (Ferron et al., 2008, Proc. Natl. Acad. Sci. USA 105:5266-5270) or with human chorionic gonadotropin (hCG) as a positive control. After 1 hour, an aliquot of medium was collected for measurements of testosterone. Cells were then maintained for 3 additional hours and lysed in 1 ml TRIZOL® (Invitrogen) for RNA isolation.

[00457] **Example 17 – Sperm counts and hormone measurements**

[00458] Caudal epididymides were minced in 1 ml PBS and the number of cells released counted after 1 hour. The total sperm count was assessed in the final suspension by using a hemacytometer (Dakhova et al., 2009, Endocrinology 150:404-412). Circulating levels of testosterone, estradiol (E2), and progesterone were measured by radioimmunoassay (RIA) from

Diagnostic Systems Laboratories (Testosterone RIA DSL-4000, Estradiol RIA DSL-43100, and Progesterone RIA-3900).

[00459] **Example 18 – Histology**

[00460] One testis or ovary from each mouse was randomly selected for molecular analysis and the other one was used for histology. Specimens were collected, weighed, and fixed in Bouin's fixative for histological analyses before being dehydrated through graded ethanol, processed for paraffin embedding, and serially sectioned at 5 μm. For histological analysis, sections of testes and ovaries were stained with periodic acid-Schiff and counterstained with hematoxylin. TUNEL labeling was performed using the ApopTag Peroxydase In Situ Apoptosis detection kit (Millipore-S7100). Apoptotic indices were determined by counting the total number of TUNEL-positive cells or the number of TUNEL positive spermatocytes for all stage tubules. Approximately 500 tubules were counted on at least 4 cross-sections located at midtestis for each animal.

[00461] **Example 19 – Gene expression studies**

[00462] RNA was purified from tissues, primary Leydig cells, or cultured cells using TRIZOL® (Invitrogen). RNA isolation, cDNA preparation, and qPCR analysis was carried out following standard protocols. qPCR analyses were performed using specific quantitative PCR primers from SABiosciences.

[00463] **Example 20 – cAMP quantification**

[00464] For cAMP measurements, TM3 Leydig cells were plated in 6 cm dishes (10^7 cells per dish) a day before experiment. Cells were serum starved for 16 hours (in the presence of 0.1% BSA) then pre-incubated in the presence of 0.5 mM IBMX for 30 minutes and stimulated with the indicated concentration of osteocalcin (also in the presence of 0.5 mM IBMX) for 30 minutes. cAMP concentration was measured with the Parameter cAMP kit (R&D Systems, KGE002).

[00465] **Example 21 – Receptor binding assays**

[00466] For binding studies, testes from 8-week old mice were snap frozen in liquid nitrogen and 20 μm thick sections were prepared and dessicated overnight at +4°C under vacuum. On the following day, sections were rehydrated in ice-cold binding buffer (50 mM TrisHCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.1% BSA) for 15 minutes and incubated for 1 hour in the presence of biotinylated osteocalcin. For competition assays, a 100-fold molar excess of unlabeled osteocalcin, glycine, lysine, or hCG was added. After 3 washes in cold PBS, sections were incubated for 1 hour in the detection system containing 0.1%BSA (ABC Elite, Vector Laboratories), washed again, and incubated with DAB peroxidase substrate kit (Vector Laboratories) according to the manufacturer's protocol. After a final wash, sections were mounted in water-based mounting medium. As negative controls, sections incubated with the detection system only (ABC Elite and DAB) or *OstR*-/- testis sections (Basura et al., 2008, Hear. Res. 244:45-50) were used.

[00467] **Example 22 – Preparation, purification, and culture of primary mesenchymal cells**

[00468] Primary osteoblasts were isolated from 5 day-old *Ocn-/-* or WT littermate calvaria bones. Calvaria bones were dissected and placed in digestion medium (αMEM, 1 mg/ml collagenase P (BM), 2.5% trypsin/EDTA) for 60 minutes at 37°C under vigorous shaking. The cell suspension was transferred, free of bone pieces, into a culture plate and cultured for 1 week in αMEM/10% FBS (pH 6.9) and in mineralization medium (αMEM/10% FBS, 5mM β-glycerophosphate and 10 μg/ml ascorbic acid) thereafter. Upon complete differentiation, cells were cultured for 24 hours in a specific medium for primary Leydig cells or tissue explants (testis or ovary) in the presence of 0.1% BSA. Supernatants were collected, centrifuged to remove cell debris, and stored at –80°C.

[00469] Primary adipocytes were isolated from visceral fat pads (white adipocytes), dissected, and minced in PBS. Tissues were redigested for 1 hour at 37°C in 1 mg/ml collagenase (in KRP buffer; 20 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 2.5 mM d-glucose, 2% BSA, pH 7.4) as described (Ferron et al., 2008, Proc. Natl. Acad. Sci. USA 105:5266-5270; Lee et al., 2007, Cell 130:456-469). Isolated white fat adipocytes were then directly cultured for 2 hours in αMEM supplemented with 0.1% BSA. After 3 washes with PBS 1X, adipocytes were cultured for at least 8 hours in a specific medium for primary Leydig cells or tissue explants (testis or ovary) in the presence of 0.1% BSA. Supernatants were collected, centrifuged to remove cell debris, and stored at –80°C.

[00470] Primary fibroblasts were isolated from 5 day-old mice as described (Hakkinen et al., 2001, Methods Cell Sci. 23:189-196). Purified fibroblasts were cultured for at least 24 hours

in a specific medium for primary Leydig cells or tissue explants (testis or ovary) in the presence of 0.1% BSA. Supernatants were collected, centrifuged to remove cell debris, and stored at –80°C.

[00471] Primary myoblasts were isolated from 5 day-old mice as described (Springer and Blau, 1997, Somat. Cell Mol. Genet. 23:203-209). Purified myoblasts were cultured for at least 24 hours in a specific medium for primary Leydig cells or tissue explants (testis or ovary) in the presence of 0.1% BSA. Supernatants were collected, centrifuged to remove cell debris, and stored at –80°C.

[00472] **Example 23 – Assays with testis and ovary explants**

[00473] Preparation of testis explants was adapted from Powlin et al., 1998, Toxicol. Sci. 46:61-74). Testes from WT animals were decapsulated and 30 mg of testicular parenchyma was placed into a 10 ml glass scintillation vial containing 5 ml of culture medium (RPMI-1640, 5% FCS, 50 mg/ml soybean trypsin inhibitor) and used for each different condition. The vial was capped, briefly vortexed, and incubated vertically for 2 hours at 34°C under vigorous shaking (175 rpm). Testes explants were then washed 3 times with PBS 1X, placed in fresh serum free RPMI medium for 2 hours, washed again 3 times with PBS 1X, and incubated for 1 hour with supernatants collected from various mesenchymal cell cultures. At the end of the incubation period, 1.4 ml of cultured medium were collected and centrifuged at 14,000 g for 5 minutes (4°C) to pellet all remaining testicular parenchyma. The resulting supernatant was frozen at -80°C until radioimmuno assays (RIA) were performed to measure the circulating levels of testosterone, estradiol, or progesterone. As a positive control, testis explants were cultured with

medium containing hCG (1 IU/ml). hCG binds to the LH receptor on Leydig cells to stimulate testosterone production. This stimulation confirmed the viability of the explants.

[00474] Preparation of ovary explants was adapted from (Powlin et al., 1998, Toxicol. Sci. 46:61-74). Ovaries from WT animals were removed, cleared of fat, weighed, thoroughly minced with scissors into approximately < $1mm^3$, and placed into a 10 ml glass scintillation vial containing 1 ml of culture medium (RPMI-1640, 5% FCS, 50 mg/ml soybean trypsin inhibitor). Two ovaries were used for each different condition. The vial was capped, briefly vortexed, and incubated vertically for 2 hours at 34°C under vigorous shaking (175 rpm). Ovary explants were then washed 3 times with PBS 1X, placed in fresh serum free RPMI medium for 2 hours, washed again 3 times with PBS 1X, and incubated for 1 hour with supernatants collected from various mesenchymal cell cultures. At the end of the incubation period, the culture medium was centrifuged at 14,000 g for 5 min (4°C) to pellet all remaining tissues. The resulting supernatant was frozen at -80°C until analyzed for testosterone, estradiol, or progesterone levels.

[00475] **Example 24 – Assessment of estrus cycle**

[00476] Weanling WT or *Ocn-/-* female mice were inspected daily for vaginal opening and estrus cycling was determined in sexually mature WT or *Ocn-/-* female mice by light microscope analysis of vaginal epithelial cell smears (Walters et al., 2007, Endocrinology. 148:3674-3684). To define estrus cycle length, daily vaginal samples were collected for 14 consecutive days.

[00477] **Example 25 – *In situ* mRNA hybridization**

[00478] Ten μm coronal sections of mouse testis or bone were cut in a cryostat and mounted on positively charged microscope slides. For hybridization, cryosections were incubated with DIG-labelled riboprobe at 69°C, followed by incubation with alkaline phosphatase-conjugated anti-DIG antibody. Color development was performed by incubation with NBT/BCIP.

[00479] **Example 26 – Immunohistochemistry and immunofluorescence**

[00480] Immunohistochemistry was performed according to Qin et al., 2008, PLoS One 3:e3285. Mouse testes were fixed overnight in Bouin's fixative, dehydrated through graded ethanol, processed for paraffin embedding, and sectioned at 5 μm. Goat polyclonal anti-3β-HSD (Santa Cruz Biotechnology) was used as primary antibody and a biotinylated rabbit anti-goat antibody (Jackson ImmunoResearch) was used as secondary antibody. The VECTASTAIN ABC KIT® (Vector laboratories) and 3,3'–diaminobenzidine (DAB) substrate kit (Vector laboratories) were used for reaction development.

[00481] For immunofluorescence studies, animals were anesthetized and perfused transcardially with ice-cold saline, followed by PFA 4%/PBS. Mouse testes and ovaries were dissected, postfixed overnight in PFA 4%/PBS, and then cryoprotected by overnight immersion in a 20% sucrose solution. Frozen testes or ovaries were sliced in 20 μm coronal sections using a cryotome, dried at room temperature for 20 minutes, washed with PBS, and blocked with appropriate serum for 1 hour. Sections were then incubated with rabbit anti-OstR for 24 hours at 4°C, rinsed, and incubated with a donkey anti-rabbit antibody (Cy3; Jackson immunoresearch).

[00482] **Example 27 – Germ cell proliferation**

[00483] Proliferation analysis was performed by BrdU staining in 2 week-old WT and *Ocn-/-* mice (Wang et al., 2003, Endocrinology 144:5058-5064). Two hours before sacrifice, mice received an intraperitoneal injection of BrdU (40 μg/g body weight). BrdU staining was performed on sections prepared as above using a BrdU staining kit (Invitrogen, 93-3943) and hematoxylin counterstaing.

[00484] **Example 28 – Leydig cell analysis**

[00485] Testes of WT, *Ocn-/-,* and *Esp-/-* mice were dissected and fixed in Bouin's fixative for histological analyses. Tissues were dehydrated through graded ethanol, processed for paraffin embedding, serially sectioned at 5 μm, and stained by immunohistochemistry with anti-3β-HSD as described above. The ratio between Leydig cell area (reflected by immunostaining) and interstitially area was measured by computer analysis using a 40x objective lens and an 8x ocular lens with test grid by counting 100 test points in 35–40 sites, as described (Dakhova et al., 2009, Endocrinology 150:404-412). The ratio percentage was obtained by counting points over Leydig cells area and dividing by the total number of points counted over interstitial area. The total number of Leydig cells per testis was calculated by dividing the total area of Leydig cells by the total number of Leydig cells in this area (number of Leydig cells/mm^2).

[00486] **Example 29 – Sperm preparation and hyperactivation**

[00487] All routine chemicals and compounds were purchased from Sigma-Aldrich with exceptions noted below. A mouse sperm capacitating medium (Suarez and Osman, 1987, Biol.

Reprod. 36:1191-1198; Suarez., 2008, Hum. Reprod. Update 14:647-657) was used for incubating and washing sperm. The medium consisted of 110 mM NaCl, 2.68 mM KCl, 0.36 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (EMD), 5.56 mM glucose, 1.0 mM pyruvic acid, 0.006% penicillin G (Na), 2.4 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 20 mg/ml BSA (EMD). The medium was adjusted to pH 7.6 and 290-310 mOsm/kg).

[00488] Sperm were obtained from freshly dissected epididymides as follows. A 100 microliter droplet of medium was covered by mineral oil in a 35 X 10 mm Petri dish (Falcon), which was equilibrated in a 37°C, 5% CO_2 incubator prior to use. Caudal epididymides were cleaned of fat and then blood was gently pushed out of surface vessels. The epididymides were placed under the mineral oil in the Petri dish. Several cuts were made in the coiled tubules near the vas deferens and the thick fluid containing sperm was gently pulled out of each cut using forceps and transferred under the oil to the medium droplet. Sperm were allowed to disperse for 10 minutes in the incubator and then were diluted with medium to 5 x 106/ml. To promote capacitation and hyperactivation, sperm were incubated at 37°C and 5% CO_2 for 2 hours.

[00489] **Example 30 – Analysis of sperm motility**

[00490] Samples of sperm were placed on slides on a 37°C stage of a Zeiss Axiovert 35 microscope and videotaped at 30 Hz using 200X bright field microscopy and stroboscopic illumination provided by a 75 W xenon flash tube (Chadwick-Helmuth Co., El Monte, CA). Videotaping was conducted using a black-and-white Dage CCD 72 video camera (Dage-MTI, Inc., Michigan City).

[00491] **Example 31 – Gpcr expression analysis in testis and ovary**

[00492] Expression of the following 103 orphan GPCRs: Gpr1, Gpr101, Gpr107, Gpr108, Gpr110, Gpr111, Gpr112, Gpr113, Gpr114, Gpr115, Gpr116, Gpr119, Gpr12, Gpr120, Gpr123, Gpr124, Gpr125, Gpr126, Gpr128, Gpr132, Gpr133, Gpr135, Gpr137, Gpr137b, Gpr137c, Gpr139, Gpr141, Gpr142, Gpr143, Gpr144, Gpr146, Gpr149, Gpr15, Gpr150, Gpr151, Gpr152, Gpr153, Gpr155, Gpr156, Gpr158, Gpr160, Gpr161, Gpr162, Gpr165, Gpr17, Gpr171, Gpr172b, Gpr173, Gpr174, Gpr175, Gpr176, Gpr177, Gpr179, Gpr18, Gpr180, Gpr182, Gpr183, Gpr19, Gpr20, Gpr21, Gpr22, Gpr25, Gpr26, Gpr27, Gpr3, Gpr31, Gpr31c, Gpr33, Gpr34, Gpr35, Gpr37, Gpr37l1, Gpr39, Gpr4, Gpr44, Gpr45, Gpr50, Gpr52, Gpr55, Gpr56, Gpr6, Gpr61, Gpr62, Gpr63, Gpr64, Gpr65, Gpr68, Gpr75, Gpr77, Gpr81, Gpr82, Gpr83, Gpr84, Gpr85, Gpr87, Gpr88, Gpr89, Gpr97, Gpr98, Gprc5a, Gprc5b, Gprc5c, Gprc5d, Gprc6a, Tmem181, Lgr5, Lgr4, Lancl1, Mrgprh, Mrgprg, pgr15l were tested by qPCR in testes and ovaries isolated from 8 week-old WT mice. The 22 most highly expressed genes in testes were tested for expression in primary Leydig cells and their expression in Leydig cells was compared with their expression in whole testes. The expression of four genes were specifically enriched in Leydig cells.

[00493] **Example 32 – Western blotting**

[00494] Western blotting was performed according to standard procedures. Frozen testes were homogenized and lysed with 1X RIPA buffer. Membranes were blocked and then incubated overnight with primary antibody in TBST-5%BSA, followed by incubation with appropriate HRP-conjugated secondary antibody. Signals were visualized with ECL. The

following primary antibodies were used: anti-phospho-tyrosin (9416), anti-pERK1/2 (4370), anti-ERK1/2 (9102), anti-pCREB (9198), anti-CREB (4820), anti-Cleaved Caspase-3 (Asp175) (9661) (all from Cell Signaling), and anti-tACE (H-300): sc-13973 and anti-CHD5 (H-185): sc-68390 from Santa Cruz; the anti-osteocalcin was described in Ferron et al., 2010, Biochem. Biophys. Res. Comm. 397:691-696.

[00495] **Example 33 – Measurement of intracellular calcium**

[00496] Isolated primary Leydig cells were resuspended in buffer containing 95 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, 25 mM $NaHCO_3$, 1.7 mM $CaCl_2$, 0.25 mM sodium purivate, 1x PenStrep, 20 mM HEPES, 0.3% BSA, pH 7.4 at 37°C (reagents from Sigma Aldrich, except for sodium pyruvate, PenStrep and HEPES from Invitrogen), plated at the density of 17-25000 cells/well on 96-well plate and placed in an incubator at 33°C, 5% CO_2 for 2 hours. Afterward, the FLIPR dye (Calcium Plus Assay Kit Dye, Molecular Devices) diluted in the same buffer supplemented with 2.5 mM probenecid, pH 7.4 was exchanged for the previous medium and incubated with the cells for an additional 30-45 minutes. Following the incubation with the dye, cells were placed in a BD Pathway 855 High-Content Bioimager, recorded for 10-20 seconds to get baseline fluorescence, and then stimulated with doses of osteocalcin ranging from 0.3 to 500 ng/ml as well as control substances: medium, calcium ionophore A23187, 10% FBS, 400 μM $Arginine^{2+}$, 100 μM ATP/UTP mix and recorded for an additional 2-5 minutes. The probing time was varied between 1 and 5 seconds. Obtained images were analyzed for the change in fluorescence using ImageJ 1.41.

[00497] **Example 34 – Chromatin immunoprecipitation**

[00498] Chromatin immunoprecipitation was performed on lysates from TM3 Leydig cells using the ChIP AssayKit (Millipore, #17-295) and an anti-CREB antibody (Cell Signaling, #4820). CREB binding sites in the promoters of indicated genes were obtained from previous bioinformatics analyzes (Zhang et al., 2005, Proc. Natl. Acad. Sci. U S A. 102:4459-4464). Binding of CREB to the indicated regions of DNA was detected by PCR using the following primers: Cyp11a hCRE-1364 forward 5'-CTCAGGTCTTCATGA TTGTGG -3' (SEQ ID NO:18), reverse 5'-CGAAAGAGAGTGTATCCACC-3' (SEQ ID NO:19); Cyp11a hCRE-4176 forward 5'-CCTTTACGTGGAATAACATTCA-3' (SEQ ID NO:20), reverse 5'-ATAGGGAATCACGG TGTAGC-3' (SEQ ID NO:21); 3β-HSD hCRE-993 forward 5'-GCAGCTTCAAGGATTACGTAA-3' (SEQ ID NO:22), reverse 5'-CATCTTGTGAACTGGTGGCT-3' (SEQ ID NO:23); HSD3beta hCRE-3109 forward 5'-TCCATAGA ACAGACTACCTAC-3' (SEQ ID NO:24), reverse 5'-GATCACAGCTGAGGAAGGC-3' (SEQ ID NO:25); StAR hCRE-40 forward 5'-TGATGCACCTCAGTTACTGG-3' (SEQ ID NO:26), reverse 5'-GCTGTGCATCATCA CTTGAG-3' (SEQ ID NO:27) and the region which did not contain CREB binding sequence as a negative control forward 5'-CATACGTGCACTGTCTTAGC-3' (SEQ ID NO:28), reverse 5'-ACTCCTCCAGTA ACTCCTTC-3' (SEQ ID NO:29).

[00499] **Example 35 – OstR, a G-protein coupled receptor transducing osteocalcin signals in Leydig cells**

[00500] To better understand osteocalcin's molecular mode of action, a search for a receptor expressed in Leydig cells that could transduce osteocalcin's signal (OstR) was carried out. To that end, a two-steps experimental strategy was used.

[00501] First, the signal transduction pathway used by osteocalcin in Leydig cells was defined. For that purpose, Leydig cells were treated with uncarboxylated osteocalcin and assayed for tyrosine phosphorylation, ERK activation, intracellular calcium accumulation, and cAMP production using in each case an appropriate positive control. Osteocalcin consistently induced cAMP production in Leydig cells to a level comparable to that induced by human chorionic gonadotropin (hCG), the positive control, but did not induce tyrosine phosphorylation, ERK activation, or intracellular calcium accumulation in these cells (Figure 15A-D). Since these data implied that the osteocalcin receptor may be a G-protein coupled receptor (GPCR), the second step of this experimental strategy took advantage of the dichotomy of function of osteocalcin between males and females. Specifically, a search was done for orphan GPCRs that were expressed in testis at a level at least 5-fold higher than in ovary. Twenty-two out of 103 orphan GPCRs tested were predominantly expressed in testes; out of these 22, only 4 were enriched in Leydig cells (Figure 15E-F). Among them, GPRC6a stood out because its deletion in all cells results in a metabolic and fertility phenotype reminiscent of the one observed in *Ocn-/-* mice (Pi et al., 2008, PLoS One 3:e3858).

[00502] Immunohistochemistry verified that *Gprc6a* is expressed only in Leydig cells in testes and not in follicular cells of the ovary (Figure 15G and Figure 21A). Post-natally, *Gprc6a* expression peaked within the first week of life, when testosterone circulating levels are elevated.

Gprc6a expression then decreased but increase again at 6 weeks of age, when circulating levels of testosterone also rebound (Figure 15I). Binding assays were performed on mouse testes using biotinylated osteocalcin as a ligand. Under the conditions of this assay, osteocalcin bound to Leydig cells and the specificity of this binding was confirmed by several criteria (Figure 15J). First, there was no signal when using avidin-biotin alone; second, there was no signal in other cellular compartments of the testicular tubules; third, no binding was detected when using *GPRC6a*-deficient testes; fourth, osteocalcin binding could be competed away by an excess (100 fold) of unlabeled osteocalcin but not by the same excess of hCG or of other molecules proposed as ligands of GPRC6a (Wellendorph and Brauner-Osborne, 2004, Gene 335:37-46) (Figure 15J). These data identify GPRC6a as a receptor of osteocalcin in Leydig cells. Therefore, GPRC6a is also referred to herein as OstR.

[00503] To define OstR function in Leydig cells *in vivo,* $OstR_{Leydig}$-/- mice were generated. Prior to analyzing these $OstR_{Leydig}$-/- mice, it was verified that *OstR* had been deleted, although partially, in testis but not in other organs (Figure 22A-C). In $OstR_{Leydig}$-/- male mice, testes size and weight, epididymides and seminal vesicle weight, sperm count, circulating testosterone levels, and Leydig cell area were all reduced, as was the expression of *Grth* and the 3 genes controlling testosterone biosynthesis that are regulated by osteocalcin (Figure 16A-I). Accordingly, the number of apoptotic germ cells increased compared to WT testes (Figure 16J). To establish genetically that OstR may be the signaling receptor for osteocalcin in Leydig cells, compound mutant mice lacking one allele of *Ocn* and one allele of *OstR* in Leydig cells only (*Ocn*+/- ; $OstR_{Leydig}$+/- mice) were analyzed. Whether looking at testes, epididymides and

seminal vesicle weight, or sperm count, *Ocn*+/- ; $OstR_{Leydig}$+/- mice had a phenotype identical to the one observed in $OstR_{Leydig}$-/- and Ocn_{osb}-/- mice (Figure 16A-I).

[00504] **Example 36 – CREB is a transcriptional effector of osteocalcin signaling in Leydig cells**

[00505] The observations that cAMP production increases in Leydig cells treated with osteocalcin and that OstR is a GPCR implied that CREB could be a transcriptional mediator of osteocalcin functions in Leydig cells. The fact that osteocalcin treatment of Leydig cells favors CREB phosphorylation supported this hypothesis (Figure 17A). This contention was tested further through the generation of mice lacking CREB in Leydig cells only ($Creb_{Leydig}$-/- mice).

[00506] Twelve week-old $Creb_{Leydig}$-/- male mice displayed a reduction in testis size and weight, in epididymides and seminal vesicle weight, in sperm count, and in circulating testosterone levels similar to the one seen in *Ocn*-/- and $OstR_{Leydig}$ -/- mice (Figure 17B-G). $Creb_{Leydig}$-/- mice also demonstrated a strong decrease in the expression of *Grth* and of the 4 genes involved in testosterone biosynthesis whose expression is regulated by osteocalcin (Figure 17H-I). In agreement with these data, binding sites for CREB were identified in the promoters of *Cyp11a, 3β-HSD* and *StAR* (Zhang et al., 2005, Proc. Natl. Acad. Sci. U S A. 102:4459-4464) and CREB could bind to those promoter sites (Figure 17J). To establish that CREB acts downstream of OstR in Leydig cells to regulate male fertility, compound heterozygous mice lacking one copy of *Creb* and one copy of *OstR* in Leydig cells were generated. The fertility phenotype of these $Creb_{Leydig}$+/-; $OstR_{Leydig}$+/- male mice was similar to that observed in

$Creb_{Leydig}$-/- or $OstR_{Leydig\ cell}$ -/- male mice (Figure 17B-G). This decrease in male fertility was not observed in single heterozygous mutant mice. Thus, CREB is a transcriptional mediator of osteocalcin regulation of testosterone biosynthesis in Leydig cells.

WHAT IS CLAIMED IS:

1. A method of treating disorders related to reproduction in male mammals comprising administering to a male mammal in need of treatment for a disorder related to reproduction a pharmaceutical composition comprising a therapeutically effective amount of undercarboxylated/uncarboxylated osteocalcin and a pharmaceutically acceptable carrier or excipient.

2. The method of claim 1 where the male mammal is a human and the osteocalcin is human osteocalcin.

3. The method of claim 1 or 2 where the disorder is male infertility, low sperm count, impaired sperm motility, impaired sperm viability, low testosterone levels, reduced libido, erectile dysfunction, underdevelopment of testes, or excess apoptosis in testes.

4. The method of any one of claims 1-3 where at least one of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin is not carboxylated.

5. The method of claim 4 where all three of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin are not carboxylated.

6. The method of claim 3 where the undercarboxylated/uncarboxylated osteocalcin is a preparation of undercarboxylated/uncarboxylated osteocalcin in which more than about 20% of the total Glu residues at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin in the preparation are not carboxylated.

7. The method of claim 1 where the undercarboxylated/uncarboxylated osteocalcin shares at least 80% amino acid sequence identity with mature human osteocalcin when the undercarboxylated/uncarboxylated osteocalcin and mature human osteocalcin are aligned for maximum sequence homology.

8. The method of claim 1 where the undercarboxylated/uncarboxylated osteocalcin shares about 90% amino acid sequence identity with mature human osteocalcin when the undercarboxylated/uncarboxylated osteocalcin and mature human osteocalcin are aligned for maximum sequence homology.

9. The method of claim 1 where the undercarboxylated/uncarboxylated osteocalcin differs at 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues from mature human osteocalcin.

10. The method of claim 1 where the undercarboxylated/uncarboxylated osteocalcin is a polypeptide selected from the group consisting of:

(a) a fragment comprising mature human osteocalcin missing the last 10 amino acids from the C-terminal end;

(b) a fragment comprising mature human osteocalcin missing the first 10 amino acids from the N-terminal end;

(c) a fragment comprising amino acids 62-90 of SEQ ID NO:2;

(d) a fragment comprising amino acids 1-36 of mature human osteocalcin; and

(e) variants of the above.

11. The method of claim 1 where the undercarboxylated/uncarboxylated osteocalcin polypeptide comprises an amino acid sequence

YLYQWLGAPVPYPDPLX$_1$PRRX$_2$VCX$_3$LNPDCDELADHIGFQEAYRRFYGPV (SEQ ID NO:13)

wherein

X_1, X_2 and X_3 are each independently selected from an amino acid or amino acid analog, with the proviso that if X_1, X_2 and X_3 are each glutamic acid, then X_1 is not carboxylated, or less than 50 percent of X_2 is carboxylated, and/or less than 50 percent of X_3 is carboxylated,

or said osteocalcin polypeptide comprises an amino acid sequence that is different from SEQ. ID. NO:13 at 1 to 7 positions other than X_1, X_2 and X_3; and/or

wherein said amino acid sequence of SEQ. ID. NO:13 includes one or more amide backbone substitutions.

12. A method of treating disorders related to reproduction in male mammals comprising administering to a male mammal in need of treatment for a disorder related to reproduction a pharmaceutical composition comprising an agent that modulates the OST-PTP signaling pathway or the PTP-1B signaling pathway, where the agent reduces OST-PTP phosphorylase expression or activity or reduces PTP-1B phosphorylase expression or activity, reduces gamma-carboxylase expression or activity, or increases the level of undercarboxylated/uncarboxylated osteocalcin, wherein the pharmaceutical composition comprises the agent in an amount that produces an effect in a male mammal selected from the group consisting of increasing fertility, raising sperm count, increasing sperm motility, increasing sperm viability, increasing serum testosterone levels, increasing libido, ameliorating erectile dysfunction, reducing underdevelopment of testes, and reducing excess apoptosis in testes.

13. The method of claim 12 where the male mammal is a human.

14. The method of claim 12 or 15 where the agent is selected from the group consisting of a small molecule, an antibody, or a nucleic acid.

15. The method of claim 14 where the agent is a small molecule selected from the group consisting of warfarin, vitamin K inhibitors, and biologically active fragments or variants thereof.

16. The method of claim 15 where the agent is warfarin.

17. The method of claim 14 where the agent is an antibody or antibody fragment that binds to and inhibits the activity of OST-PTP, PTP-1B, or gamma-carboxylase.

18. The method of claim 17 where the antibody or antibody fragment is a monoclonal antibody.

19. The method of claim 17 or 18 where the antibody or antibody fragment binds to the extracellular domain of OST-PTP or PTP-1B.

20. The method of any one of claims 17-19 where the OST-PTP is human OST-PTP or the PTP-1B is human PTP-1B.

21. The method of any one of claims 17-19 where the agent reduces OST-PTP phosphorylase expression or activity and the OST-PTP is the mouse OST-PTP of SEQ ID NO:11 or an OST-

PTP having an amino acid sequence that is substantially homologous or identical to SEQ ID NO:11.

22. The method of claim 21 where the OST-PTP is an OST-PTP having an amino acid sequence that is at least 70% homologous or identical to SEQ ID NO:11.

23. The method of claim 17 or 18 where the agent reduces PTP-1B phosphorylase expression or activity and the PTP-1B is human PTP-1B of SEQ ID NO:17 or a PTP-1B having an amino acid sequence that is substantially homologous or identical to SEQ ID NO:17.

24. The method of claim 23 where the PTP-1B is a PTP-1B having an amino acid sequence that is at least 90% homologous or identical to SEQ ID NO:17.

25. The method of claim 14 where the agent is a nucleic acid that inhibits the expression or activity of OST-PTP, PTP-1B, or gamma-carboxylase.

26. The method of claim 25 where the nucleic acid is an antisense oligonucleotide or a small interfering RNA (siRNA).

27. The method of claim 25 where the nucleic acid is an isolated nucleic acid that is selected from the group consisting of an antisense DNA, antisense RNA, and siRNA, which nucleic acid is sufficiently complementary to SEQ ID NO:10 or a sequence that is substantially homologous

or identical to SEQ ID NO:10 to permit specific hybridization to SEQ ID NO:10 or a sequence that is substantially homologous or identical to SEQ ID NO:10, and wherein the hybridization prevents or reduces expression of OST-PTP in osteoblasts.

28. The method of claim 25 where the nucleic acid is an isolated nucleic acid that is selected from the group consisting of an antisense DNA, antisense RNA, and siRNA, which nucleic acid is sufficiently complementary to SEQ ID NO:16 or a sequence that is substantially homologous or identical to SEQ ID NO:16 to permit specific hybridization to SEQ ID NO:16 or a sequence that is substantially homologous or identical to SEQ ID NO:16, and wherein the hybridization prevents or reduces expression of PTP-1B in osteoblasts.

29. A method of diagnosing a patient as having or being at risk of developing a disorder related to reproduction in male mammals comprising:

(i) determining the ratio of undercarboxylated/uncarboxylated osteocalcin to total osteocalcin in a biological sample from the patient; and

(ii) comparing the ratio to a standard ratio;

wherein, if the patient ratio is lower than the standard ratio, the patient is diagnosed as having or being at risk of developing a disorder related to reproduction in male mammals.

30. The method of claim 29 comprising the further step of administering to the patient diagnosed as having or being at risk of developing a disorder related to reproduction in male

mammals a therapeutic agent to treat or prevent a disorder related to reproduction in male mammals.

31. The method of claim 29 or 30 where the biological sample is blood.

32. The method of any one of claims 29-31 where the standard ratio is 5%-10%.

33. The use of an agent that reduces OST-PTP phosphorylase activity, reduces PTP-1B phosphorylase activity, reduces gamma-carboxylase activity, and/or increases undercarboxylated/uncarboxylated osteocalcin for treating or preventing a disorder related to reproduction in male mammals.

34. The use of claim 33 where the agent inhibits phosphorylation of gamma-carboxylase.

35. The use of claim 33 or 34 where the agent increases the level of undercarboxylated/ uncarboxylated osteocalcin.

36. The use of any one of claims 33-35 where the agent increases the ratio of undercarboxylated/uncarboxylated osteocalcin compared to carboxylated osteocalcin.

37. The use of claim 36 where the agent inhibits carboxylation of osteocalcin.

38. The use of claim 36 where the agent decarboxylates osteocalcin.

39. The use of claim 33 where the agent is undercarboxylated/uncarboxylated osteocalcin.

40. The use of claim 39 where the undercarboxylated/uncarboxylated osteocalcin increases fertility, raises sperm count, increases sperm motility, increases sperm viability, increases serum testosterone levels, increases libido, ameliorates erectile dysfunction, reduces underdevelopment of testes, or reduces excess apoptosis in testes.

41. The use of claim 39 or 40 where at least one of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin is not carboxylated.

42. The use of claim 41 where all three of the glutamic acids in the undercarboxylated/uncarboxylated osteocalcin at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin are not carboxylated.

43. The use of claim 39 or 40 where the undercarboxylated/uncarboxylated osteocalcin is a preparation of undercarboxylated/uncarboxylated osteocalcin in which more than about 20% of the total Glu residues at the positions corresponding to positions 17, 21 and 24 of mature human osteocalcin in the preparation are not carboxylated.

44. The use of any one of claims 39-43 where the undercarboxylated/uncarboxylated osteocalcin shares about 90% amino acid sequence identity with mature human osteocalcin when the undercarboxylated/uncarboxylated osteocalcin and mature human osteocalcin are aligned for maximum sequence homology.

45. The use of any one of claims 39-43 where the undercarboxylated/uncarboxylated osteocalcin differs at 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues from mature human osteocalcin.

46. The use of claim 33 where the agent is selected from the group consisting of a small molecule, an antibody, or a nucleic acid.

47. The use of claim 46 where the agent is a small molecule selected from the group consisting of warfarin, vitamin K inhibitors, and biologically active fragments or variants thereof.

48. The use of claim 47 where the small molecule is warfarin.

49. A method of contraception for use in male mammals comprising administering to a male mammal in need of contraception a pharmaceutical composition comprising a therapeutically effective amount of an antagonist of undercarboxylated/uncarboxylated osteocalcin and a pharmaceutically acceptable carrier or excipient.

50. The method of claim 49 where the antagonist is an antagonist of human undercarboxylated/uncarboxylated osteocalcin.

FIGURE 1

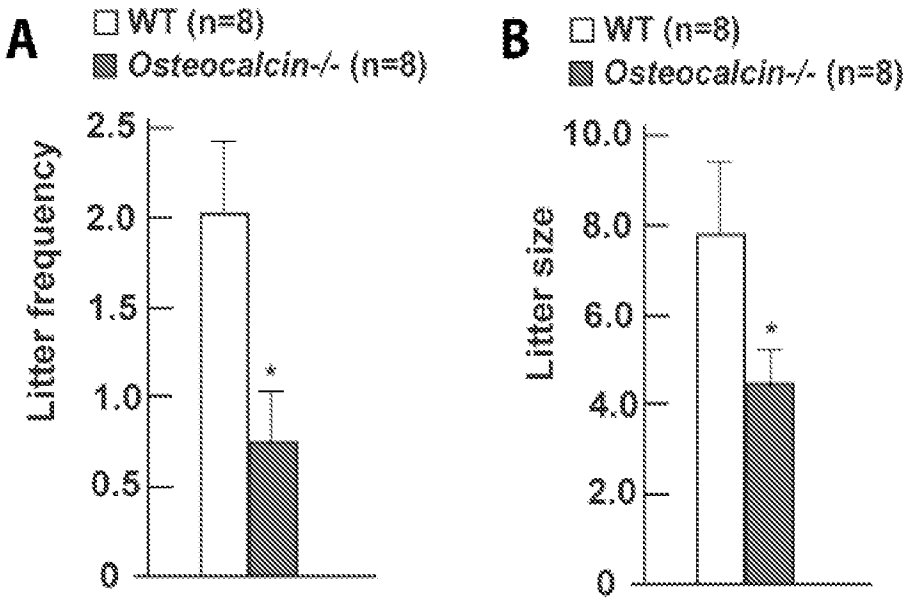

A
WT (n=8)
Osteocalcin-/- (n=8)
Litter frequency
2.5
2.0
1.5
1.0
0.5
0
*
B
WT (n=8)
Osteocalcin-/- (n=8)
Litter size
10.0
8.0
6.0
4.0
2.0
0
*

FIGURE 2

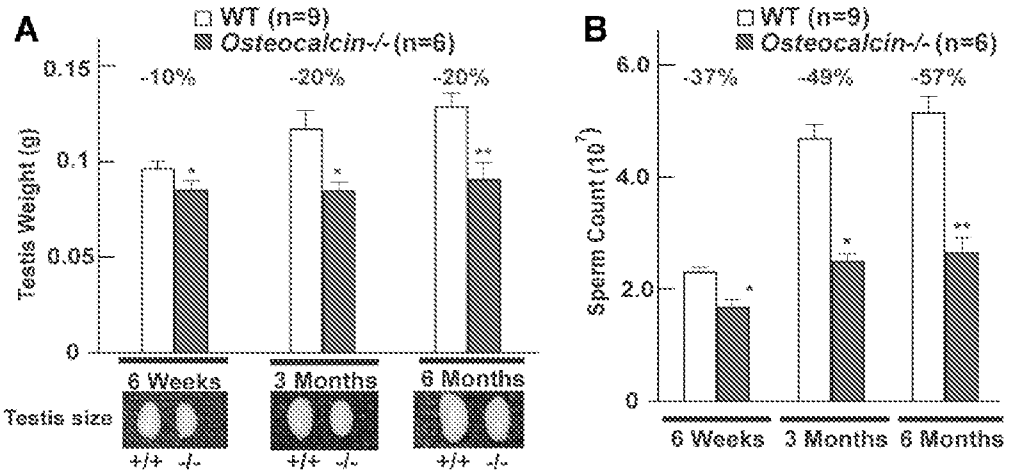

A
WT (n=9)
Osteocalcin-/- (n=6)
Testis Weight (g)
0.15
0.1
0.05
0
-10%
-20%
-20%
*
*
**
6 Weeks
3 Months
6 Months
Testis size
+/+
-/-
B
WT (n=9)
Osteocalcin-/- (n=6)
Sperm Count (10^7)
6.0
4.0
2.0
0
-37%
-48%
-57%
*
*
**
6 Weeks
3 Months
6 Months

FIGURE 3

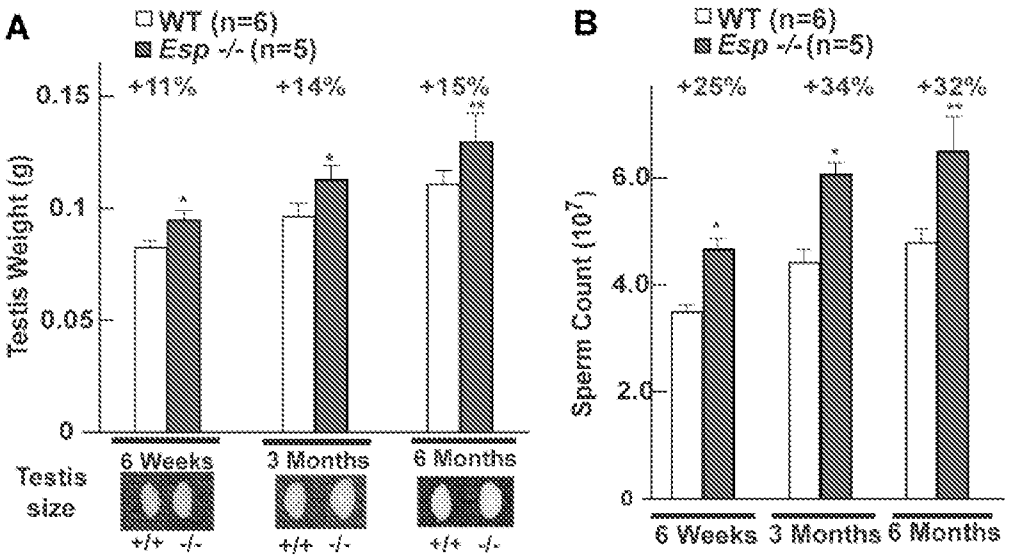

A
WT (n=6)
Esp -/- (n=5)
+11%
+14%
+15%
Testis Weight (g)
0.15
0.1
0.05
0
6 Weeks
3 Months
6 Months
Testis size
+/+ -/-
B
WT (n=6)
Esp -/- (n=5)
+25%
+34%
+32%
Sperm Count (10^7)
6.0
4.0
2.0
0
6 Weeks
3 Months
6 Months

FIGURE 4

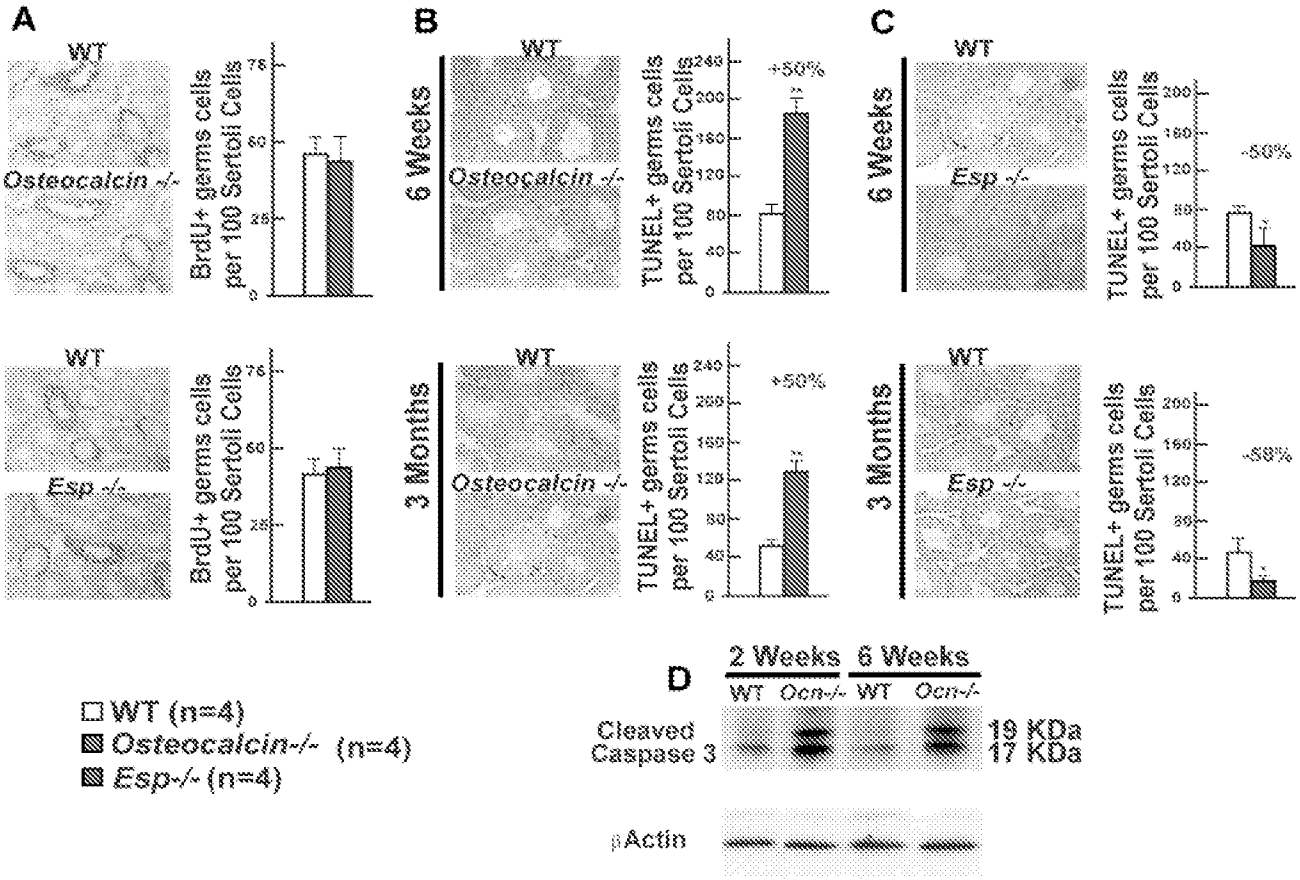

A
WT
Osteocalcin -/-
BrdU+ germs cells per 100 Sertoli Cells
B
6 Weeks
WT
Osteocalcin -/-
TUNEL+ germs cells per 100 Sertoli Cells
+50%
C
6 Weeks
WT
Esp -/-
TUNEL+ germs cells per 100 Sertoli Cells
-50%
3 Months
WT
Esp -/-
BrdU+ germs cells per 100 Sertoli Cells
3 Months
WT
Osteocalcin -/-
TUNEL+ germs cells per 100 Sertoli Cells
+50%
3 Months
WT
Esp -/-
TUNEL+ germs cells per 100 Sertoli Cells
-50%
WT (n=4)
Osteocalcin-/- (n=4)
Esp-/- (n=4)
D
2 Weeks
6 Weeks
WT
Ocn-/-
WT
Ocn-/-
Cleaved Caspase 3
19 KDa
17 KDa
βActin

FIGURE 5

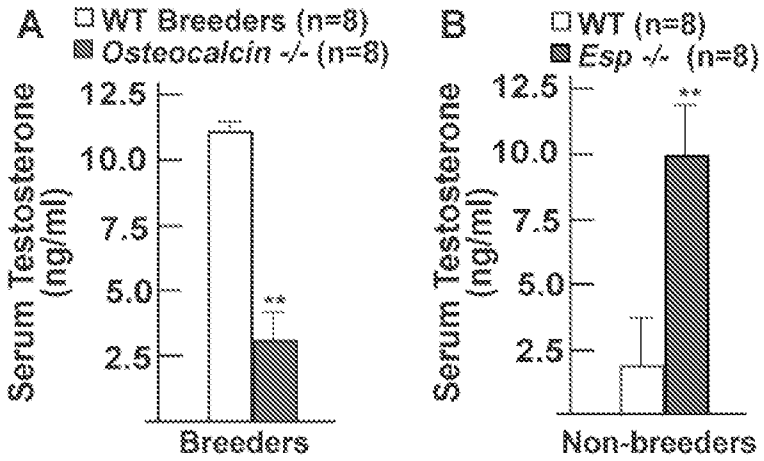

A
WT Breeders (n=8)
Osteocalcin -/- (n=8)
Serum Testosterone (ng/ml)
12.5
10.0
7.5
5.0
2.5
**
Breeders
B
WT (n=8)
Esp -/- (n=8)
Serum Testosterone (ng/ml)
12.5
10.0
7.5
5.0
2.5
**
Non-breeders

FIGURE 6

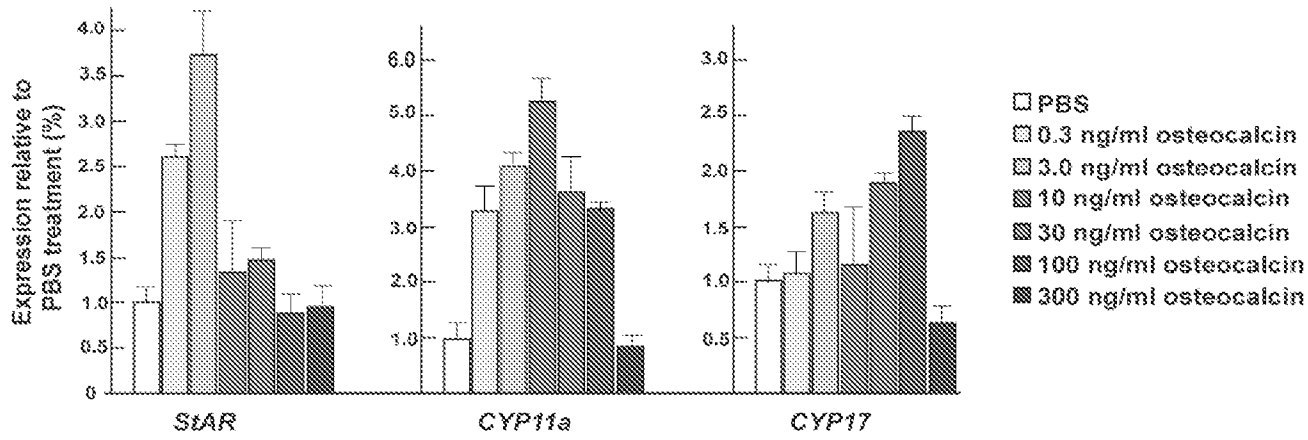

Expression relative to PBS treatment (%)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
StAR
6.0
5.0
4.0
3.0
2.0
1.0
CYP11a
3.0
2.5
2.0
1.5
1.0
0.5
CYP17
PBS
0.3 ng/ml osteocalcin
3.0 ng/ml osteocalcin
10 ng/ml osteocalcin
30 ng/ml osteocalcin
100 ng/ml osteocalcin
300 ng/ml osteocalcin

FIGURE 7

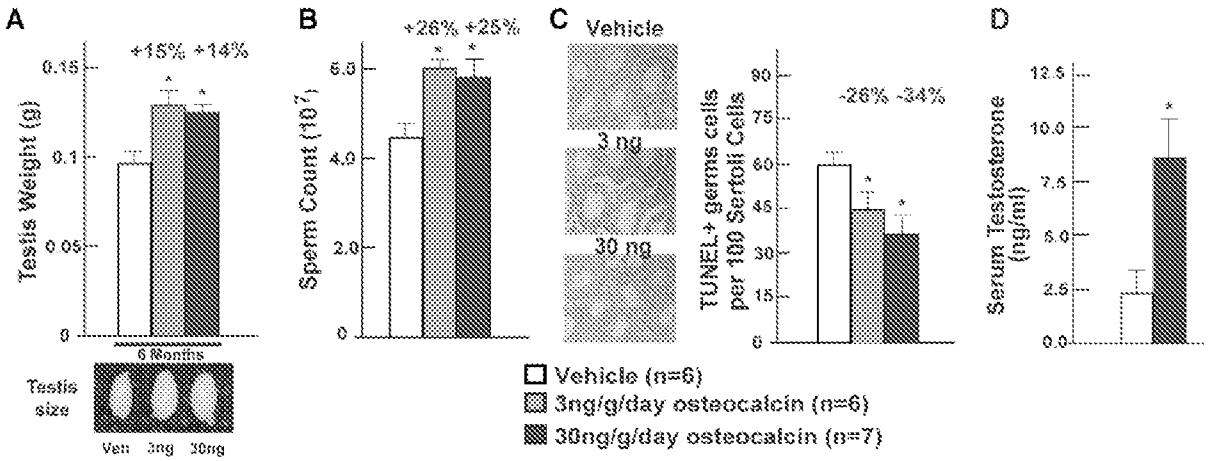

A
+15% +14%
Testis Weight (g)
0.15
0.1
0.05
0
6 Months
Testis size
Ven 3ng 30ng
B
+26% +25%
Sperm Count (10^7)
6.0
4.0
2.0
0
C
Vehicle
3 ng
30 ng
TUNEL+ germs cells per 100 Sertoli Cells
-26% -34%
90
75
60
45
30
15
0
D
Serum Testosterone (ng/ml)
12.5
10.0
7.5
5.0
2.5
0.0
Vehicle (n=6)
3ng/g/day osteocalcin (n=6)
30ng/g/day osteocalcin (n=7)

FIGURE 8

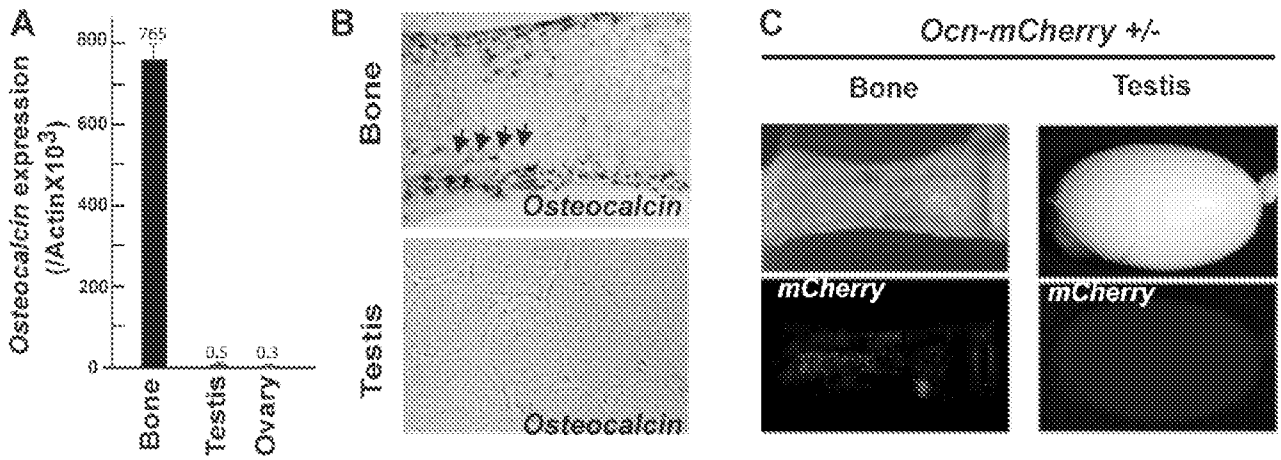

A
Osteocalcin expression (/ActinX10^3)
800
600
400
200
0
765
0.5
0.3
Bone
Testis
Ovary
B
Bone
Osteocalcin
Testis
Osteocalcin
C
Ocn-mCherry +/-
Bone
Testis
mCherry
mCherry

FIGURE 9

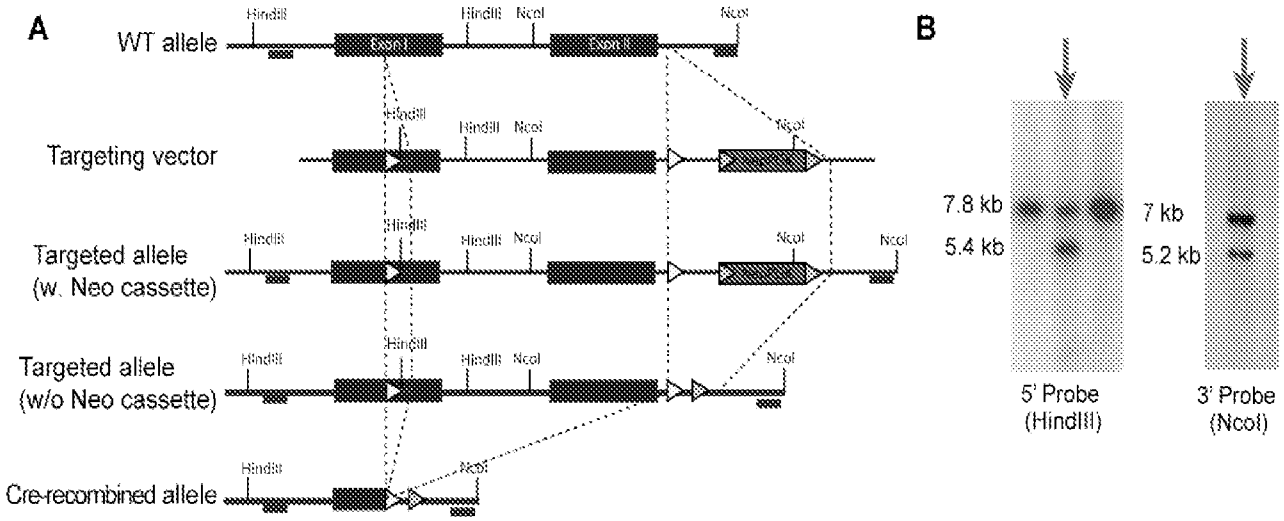

A
WT allele
Targeting vector
Targeted allele
(w. Neo cassette)
Targeted allele
(w/o Neo cassette)
Cre-recombined allele
HindIII
NcoI
B
7.8 kb
5.4 kb
7 kb
5.2 kb
5' Probe
(HindIII)
3' Probe
(NcoI)

FIGURE 10

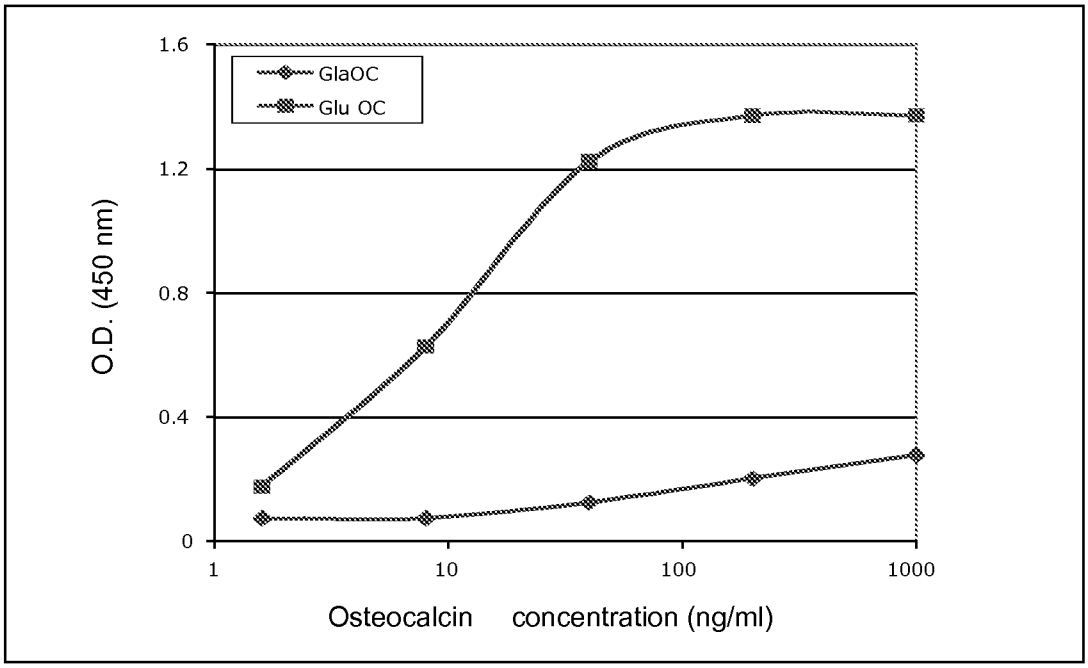

1.6
1.2
0.8
0.4
0
O.D. (450 nm)
GlaOC
Glu OC
1
10
100
1000
Osteocalcin concentration (ng/ml)

FIGURE 11

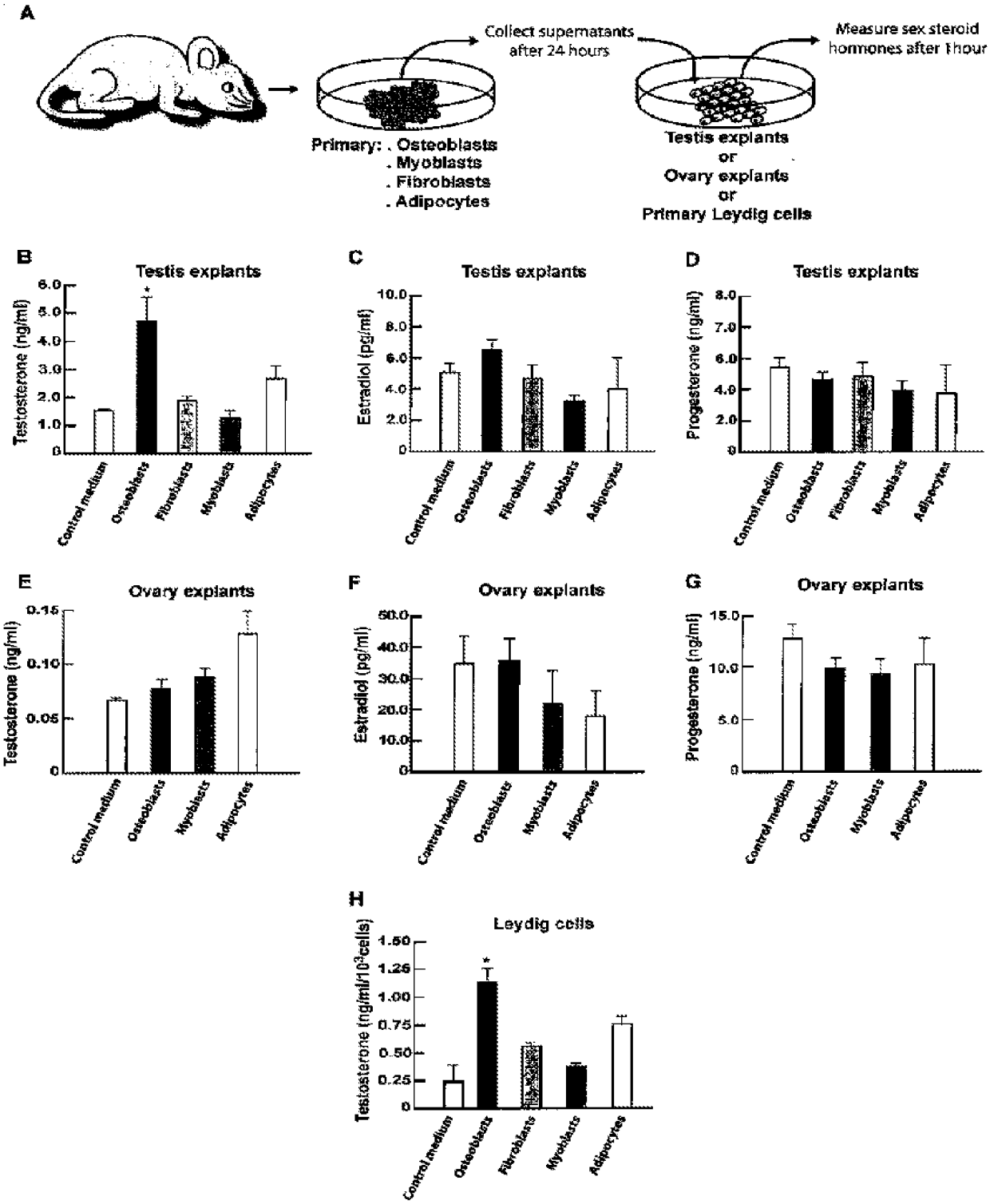
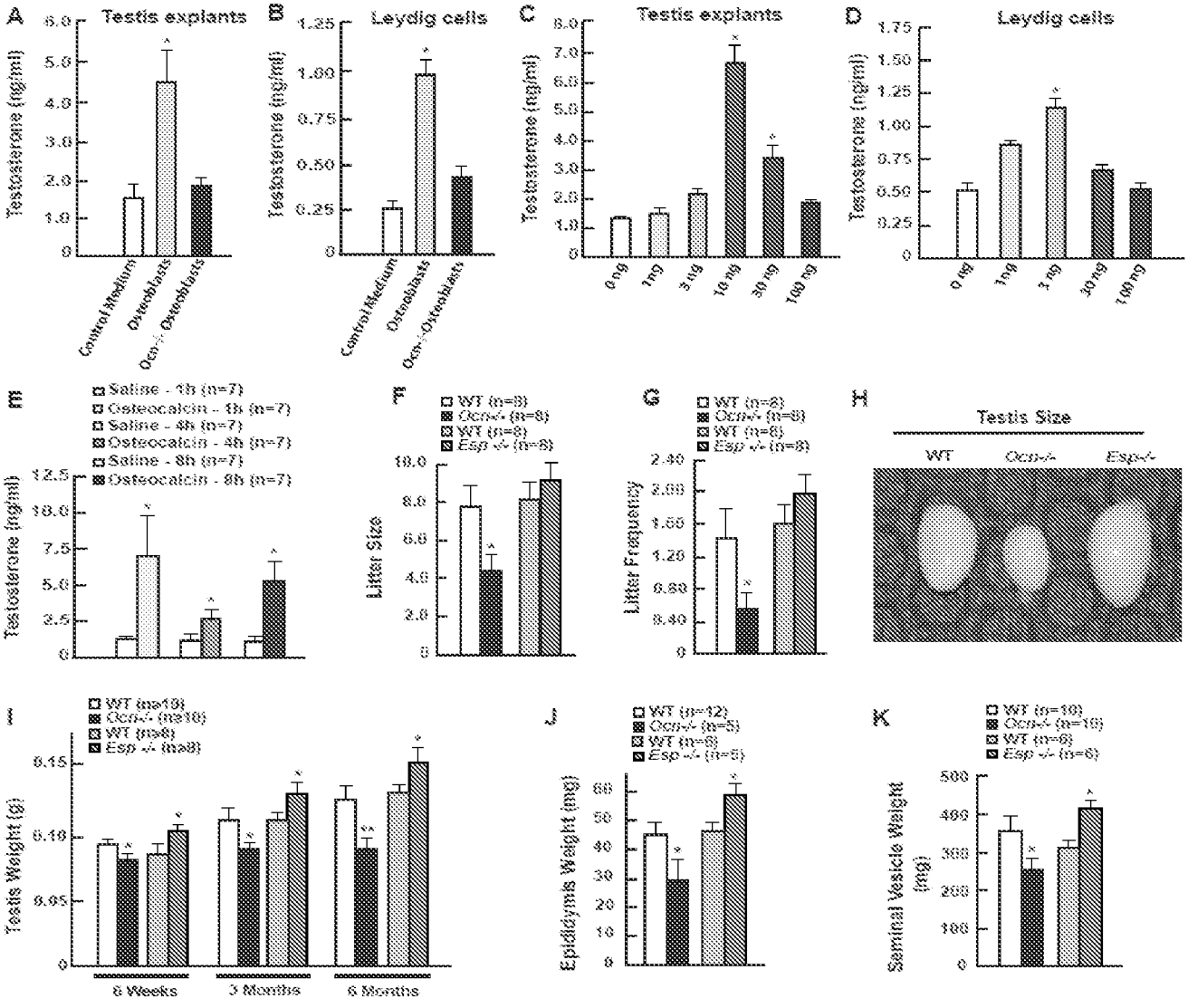

A
Collect supernatants after 24 hours
Measure sex steroid hormones after 1hour
Primary: . Osteoblasts
. Myoblasts
. Fibroblasts
. Adipocytes
Testis explants
or
Ovary explants
or
Primary Leydig cells
B
Testis explants
Testosterone (ng/ml)
C
Testis explants
Estradiol (pg/ml)
D
Testis explants
Progesterone (ng/ml)
E
Ovary explants
Testosterone (ng/ml)
F
Ovary explants
Estradiol (pg/ml)
G
Ovary explants
Progesterone (ng/ml)
H
Leydig cells
Testosterone (ng/ml/10^3cells)
Control medium
Osteoblasts
Fibroblasts
Myoblasts
Adipocytes

FIGURE 12



M

		Testosterone (ng/mL)	Estradiol (pg/mL)	Progesterone (ng/mL)
WT (Breeders)	(n=10)	13.60 ± 0.72	12.24 ± 0.82	13.14 ± 1.27
Ocn-/- (Breeders)	(n=9)	2.70 ± 0.53*	38.89 ± 2.89*	12.28 ± 1.59
WT (Non-Breeders)	(n=10)	2.07 ± 0.60	28.9 ± 3.27	
Ocn-/- (Non-Breeders)	(n=7)	0.49 ± 0.12*	61.60 ± 6.57*	
WT (Breeders)	(n=10)	13.34 ± 1.12	21.24 ± 2.27	8.78 ± 1.25
Esp-/- (Breeders)	(n=9)	23.50 ± 1.77*	18.54 ± 1.81	10.32 ± 0.70
WT (Non-Breeders)	(n=6)	2.03 ± 1.35	16.02 ± 1.64	
Esp-/- (Non-Breeders)	(n=5)	13.46 ± 1.19*	19.80 ± 1.82	

FIGURE 13

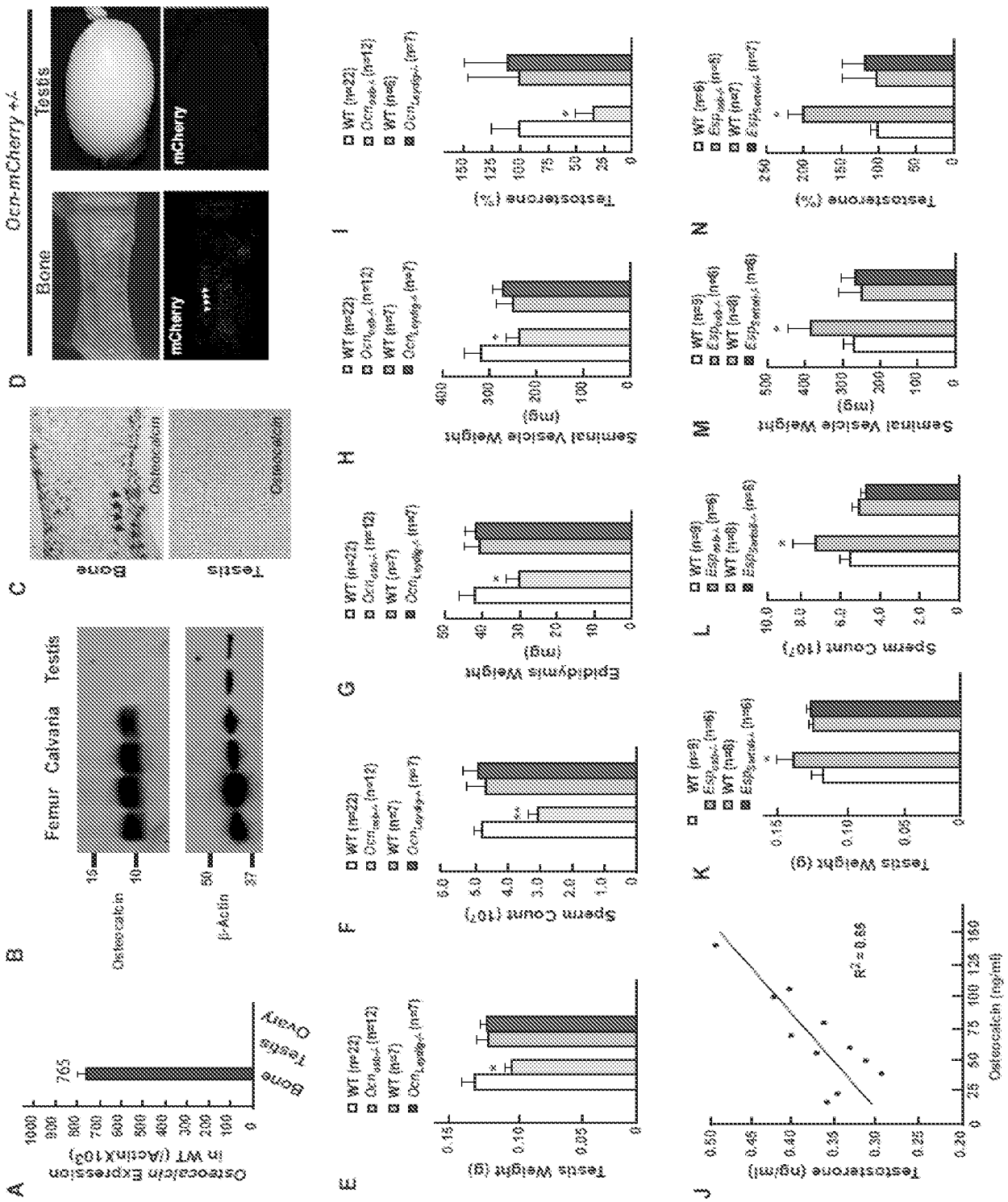

A
Osteocalcin Expression in WT (ΔActinX10³)
765
Bone
Testis
Ovary
B
Femur Calvaria Testis
Osteocalcin
β-Actin
C
Bone
Testis
Osteocalcin
D
Ocn-mCherry +/-
Bone
Testis
mCherry
E
Testis Weight (g)
F
Sperm Count (10⁷)
G
Epididymis Weight (mg)
H
Seminal Vesicle Weight (mg)
I
Testosterone (%)
J
Testosterone (ng/ml)
R² = 0.66
Osteocalcin (ng/ml)
K
Testis Weight (g)
L
Sperm Count (10⁷)
M
Seminal Vesicle Weight (mg)
N
Testosterone (%)

FIGURE 14

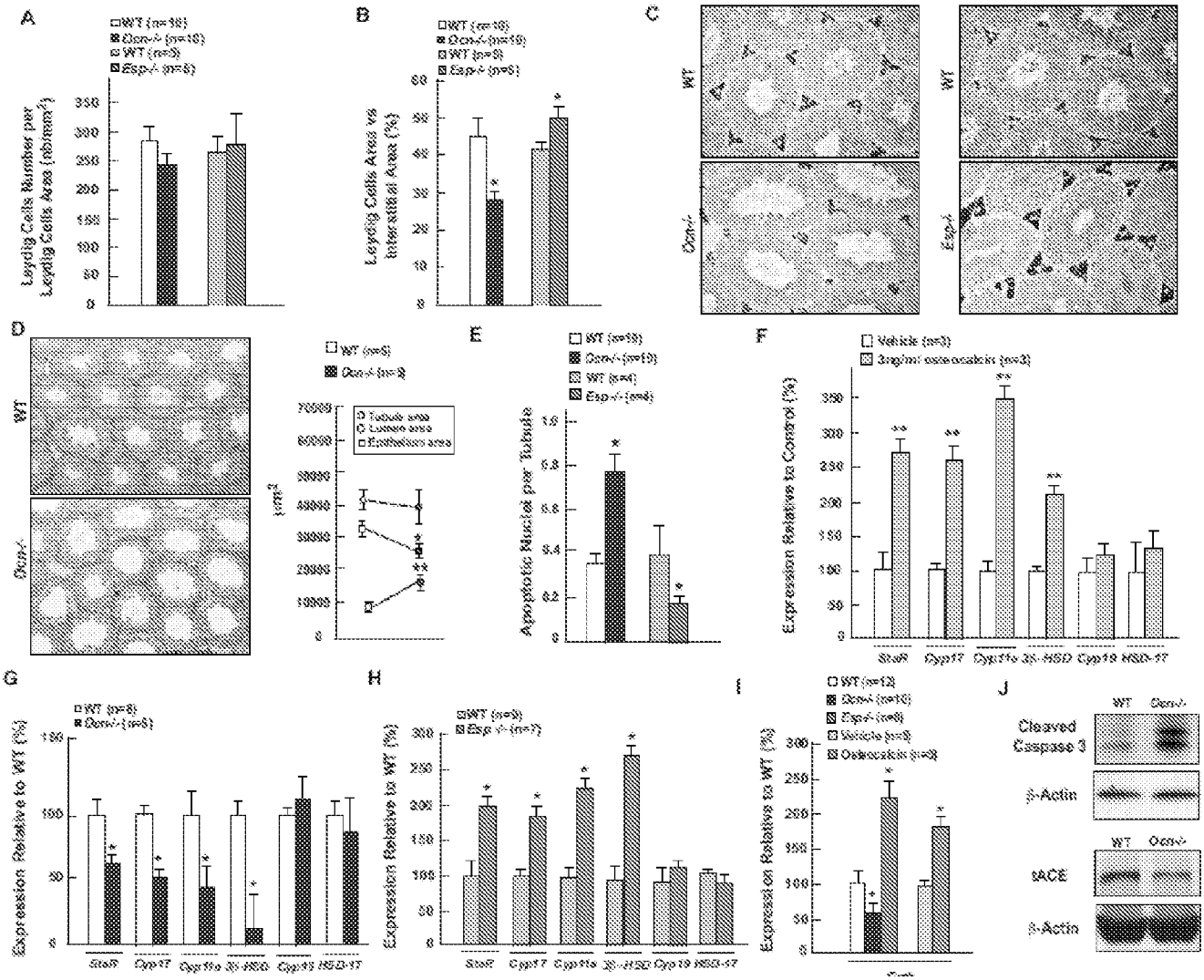

A
B
C
D
E
F
G
H
I
J
WT
Ocn-/-
Esp-/-
Leydig Cells Number per Leydig Cells Area (nb/mm²)
Leydig Cells Area vs Interstitial Area (%)
Apoptotic Nuclei per Tubule
Expression Relative to Control (%)
Expression Relative to WT (%)
Vehicle (n=3)
3ng/ml osteocalcin (n=3)
Cleaved Caspase 3
β-Actin
tACE

FIGURE 15

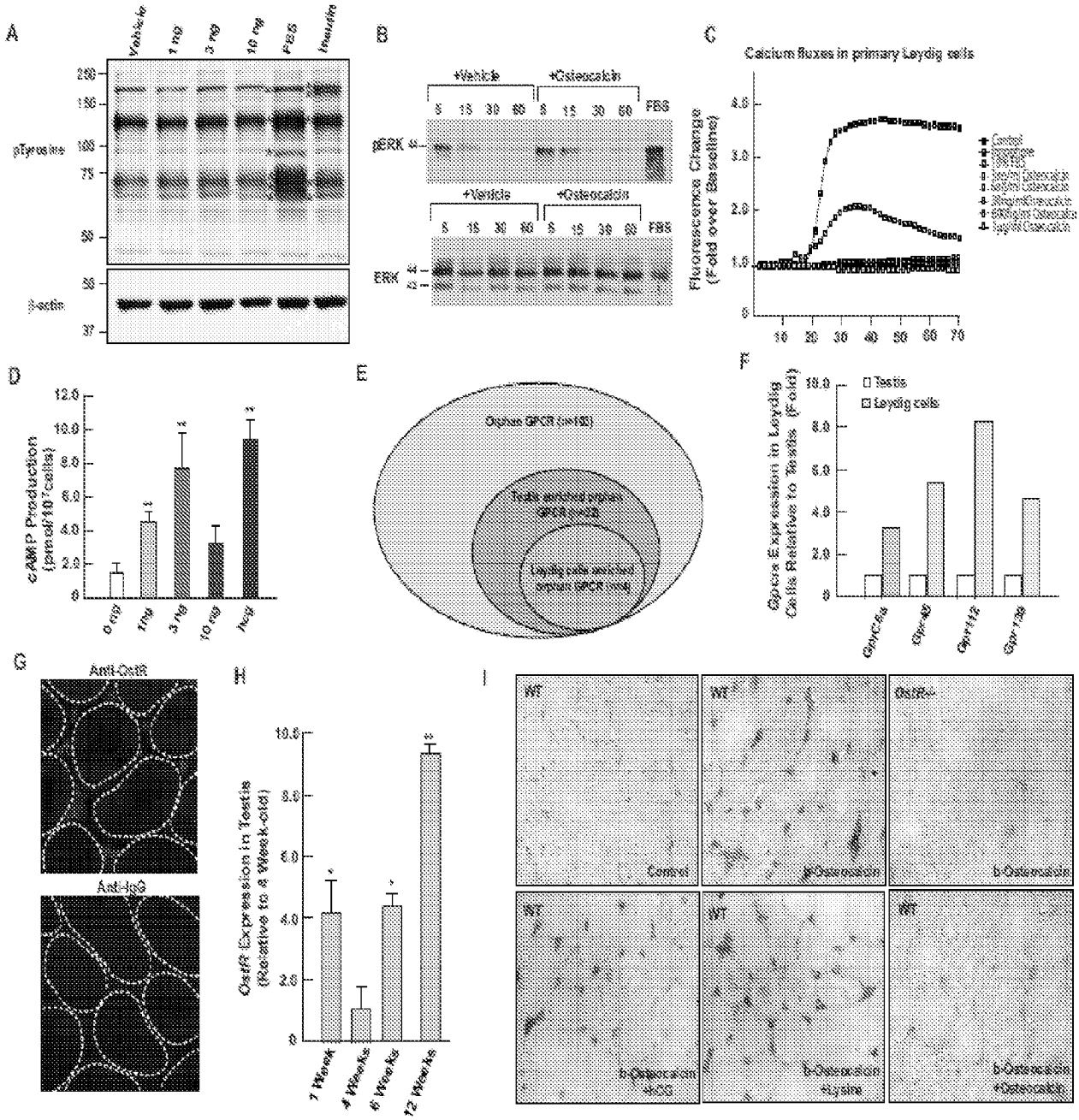

A
Vehicle
1 ng
3 ng
10 ng
FBS
Insulin
pTyrosine
β-actin
B
+Vehicle
+Osteocalcin
pERK
ERK
C
Calcium fluxes in primary Leydig cells
Fluorescence Change (Fold over Baseline)
D
cAMP Production (pmol/10^5 cells)
E
Orphan GPCR (n=58)
F
Gpcrs Expression in Leydig Cells Relative to Testis (Fold)
Testis
Leydig cells
G
Anti-GstR
Anti-IgG
H
GstR Expression in Testis (Relative to 4 Week-old)
1 Week
4 Weeks
6 Weeks
12 Weeks
I
WT
GstR-/-
Control
b-Osteocalcin
b-Osteocalcin +CS
b-Osteocalcin +Lysine
b-Osteocalcin +Osteocalcin

FIGURE 16

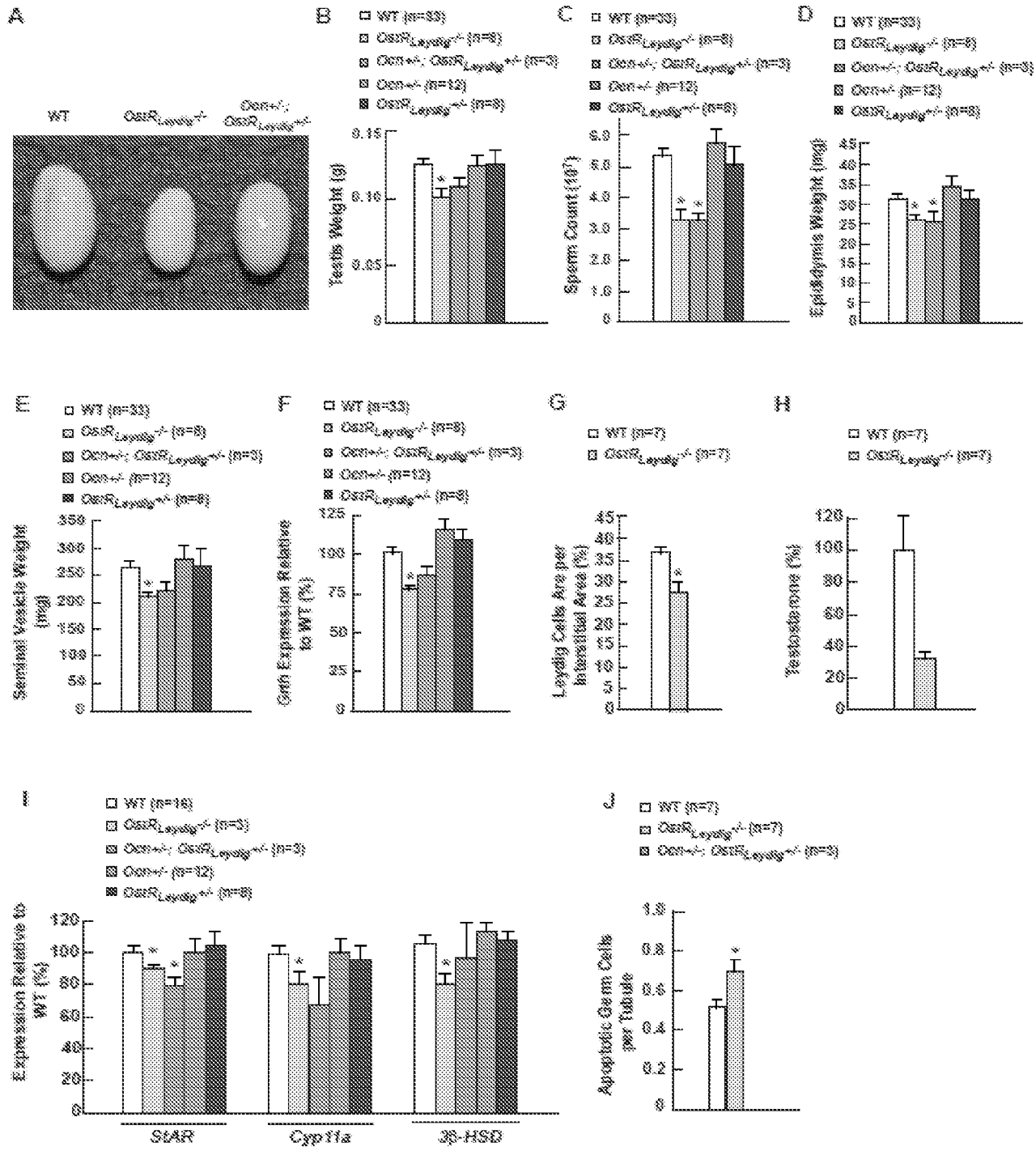

A
WT
Ocn+/-; OstR Leydig+/-
B
Testis Weight (g)
C
Sperm Count (10⁷)
D
Epididymis Weight (mg)
E
Seminal Vesicle Weight (mg)
F
Ocn Expression Relative to WT (%)
G
Leydig Cells Are per Interstitial Area (%)
H
Testosterone (%)
I
Expression Relative to WT (%)
StAR
Cyp11a
3β-HSD
J
Apoptotic Germ Cells per Tubule
WT (n=33)
OstR Leydig+/- (n=8)
Ocn+/-; OstR Leydig+/- (n=3)
Ocn+/- (n=12)
OstR Leydig+/- (n=8)
WT (n=7)
OstR Leydig+/- (n=7)
Ocn+/-; OstR Leydig+/- (n=3)
WT (n=16)
OstR Leydig+/- (n=5)

FIGURE 17

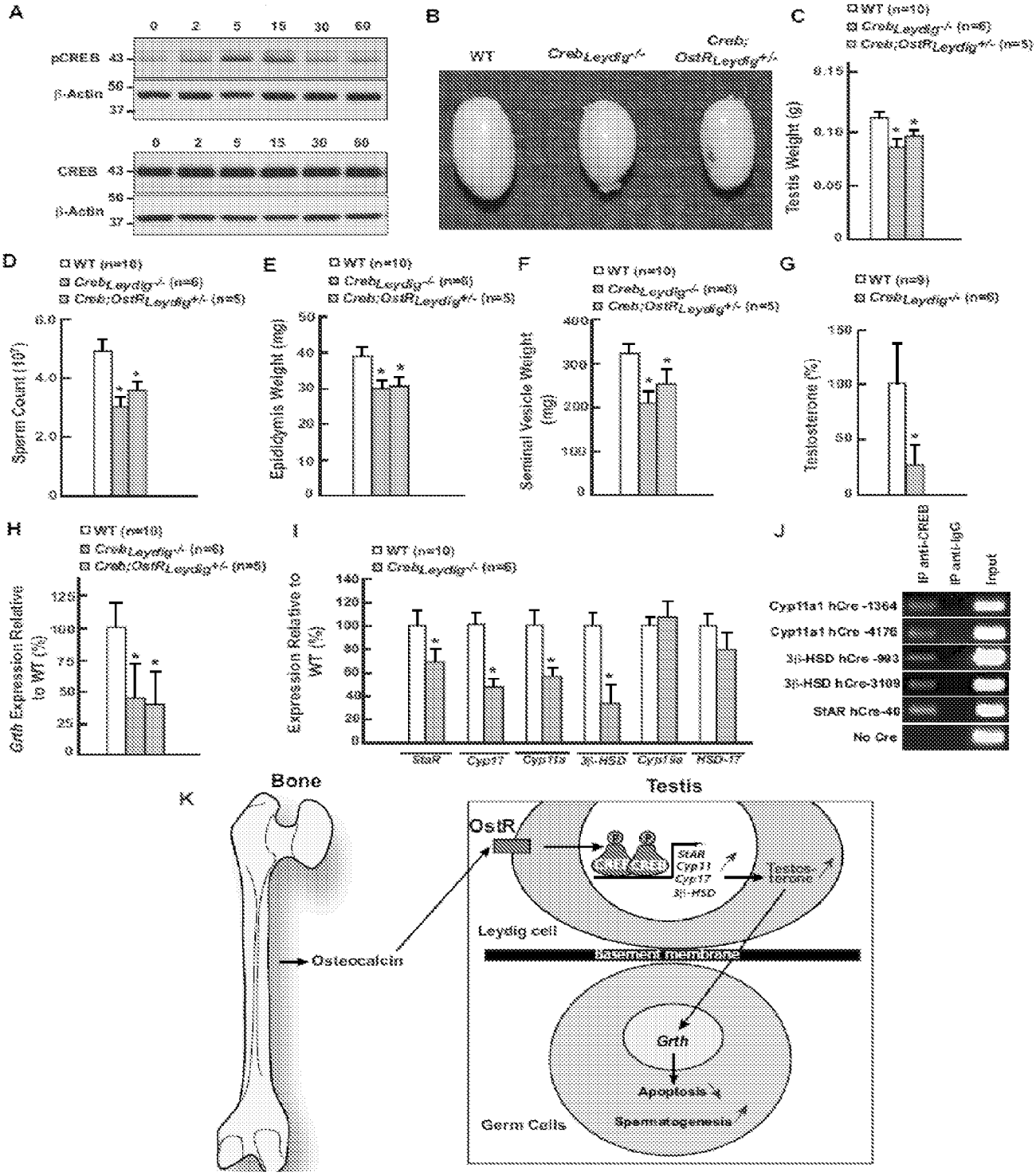
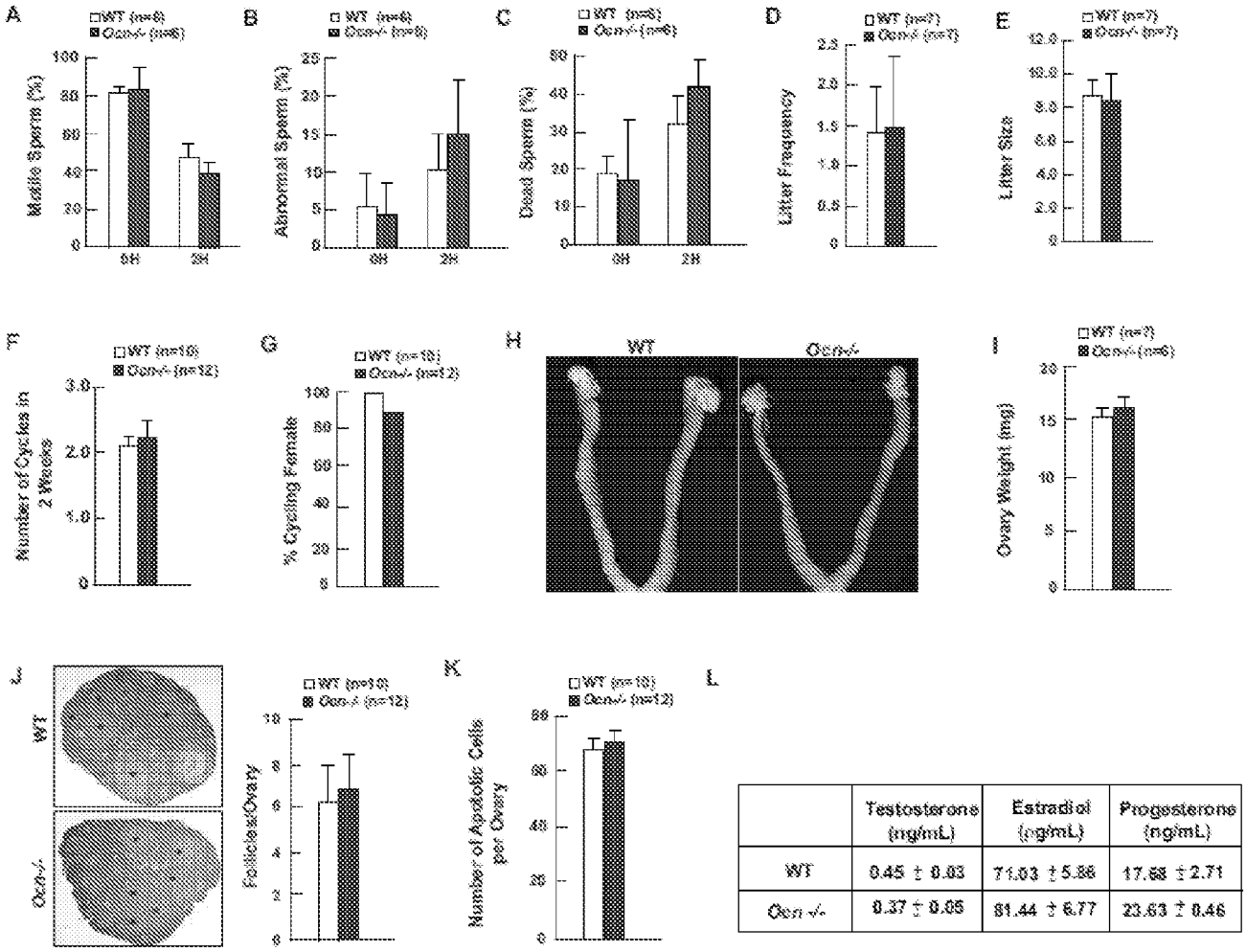

A
0 2 5 15 30 60
pCREB 43
β-Actin 56 37
CREB 43
β-Actin 56 37
B
WT
Creb Leydig-/-
Creb; OstR Leydig+/-
C
WT (n=10)
Creb Leydig-/- (n=6)
Creb;OstR Leydig+/- (n=5)
Testis Weight (g)
D
Sperm Count (10^7)
E
Epididymis Weight (mg)
F
Seminal Vesicle Weight (mg)
G
WT (n=9)
Creb Leydig-/- (n=6)
Testosterone (%)
H
Grth Expression Relative to WT (%)
I
Expression Relative to WT (%)
StAR
Cyp11
Cyp11b
3β-HSD
Cyp19a
HSD-17
J
IP anti-CREB
IP anti-IgG
Input
Cyp11a1 hCre -1364
Cyp11a1 hCre -4176
3β-HSD hCre -993
3β-HSD hCre-3108
StAR hCre-46
No Cre
K
Bone
Testis
OstR
Osteocalcin
Leydig cell
Basement membrane
Grth
Apoptosis
Spermatogenesis
Germ Cells

FIGURE 18



	Testosterone (ng/mL)	Estradiol (pg/mL)	Progesterone (ng/mL)
WT	0.45 ± 0.03	71.03 ± 5.88	17.68 ± 2.71
Ocn-/-	0.37 ± 0.05	81.44 ± 6.77	23.63 ± 0.46

FIGURE 19

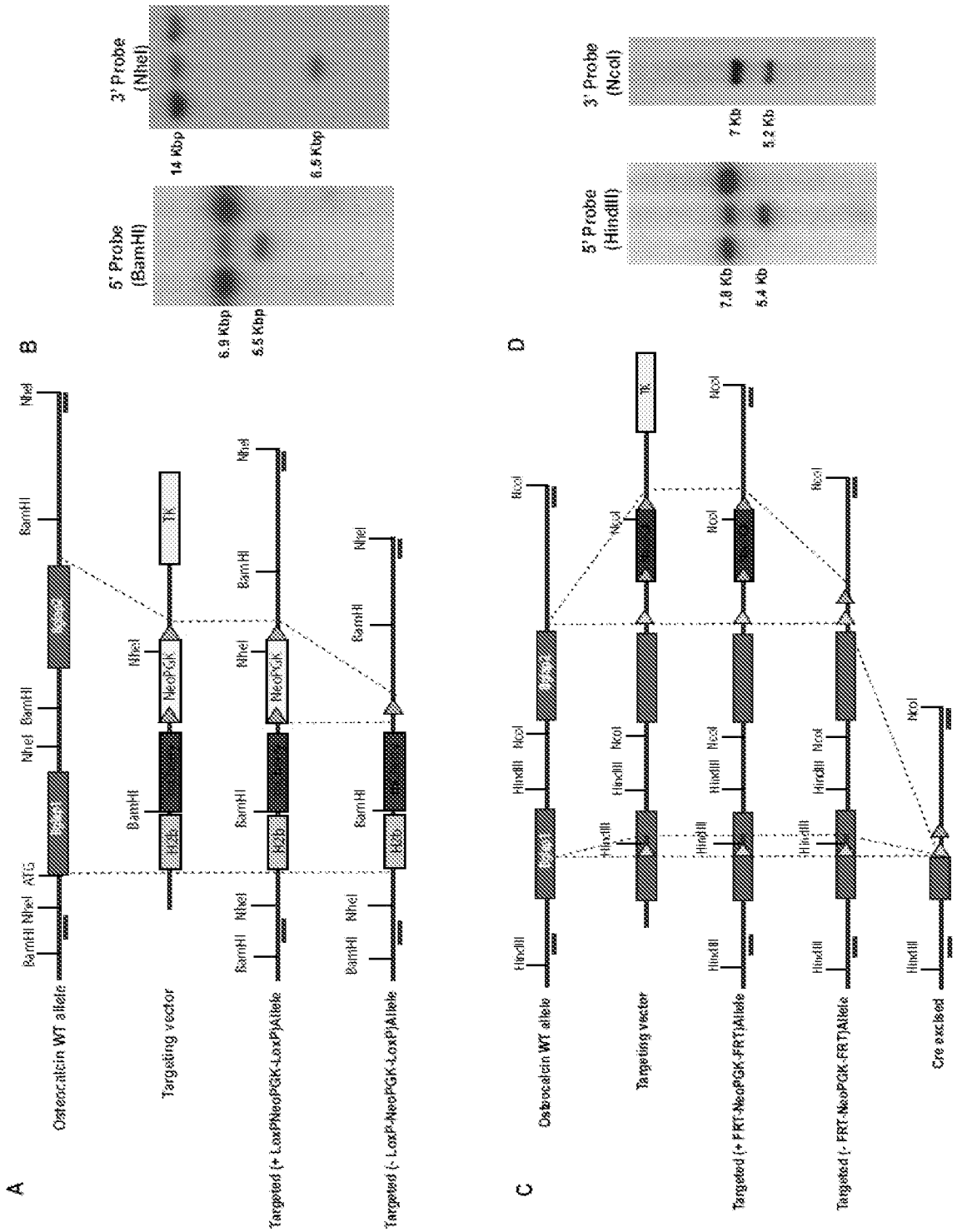

A
Osteocalcin WT allele
Targeting vector
Targeted (+ LoxPNeoPGK-LoxP)Allele
Targeted (- LoxP-NeoPGK-LoxP)Allele
B
5' Probe (BamHI)
3' Probe (NheI)
6.9 Kbp
6.5 Kbp
14 Kbp
6.5 Kbp
C
Osteocalcin WT allele
Targeting vector
Targeted (+ FRT-NeoPGK-FRT)Allele
Targeted (- FRT-NeoPGK-FRT)Allele
Cre excised
D
5' Probe (HindIII)
3' Probe (NcoI)
7.8 Kb
5.4 Kb
7 Kb
5.2 Kb

FIGURE 20

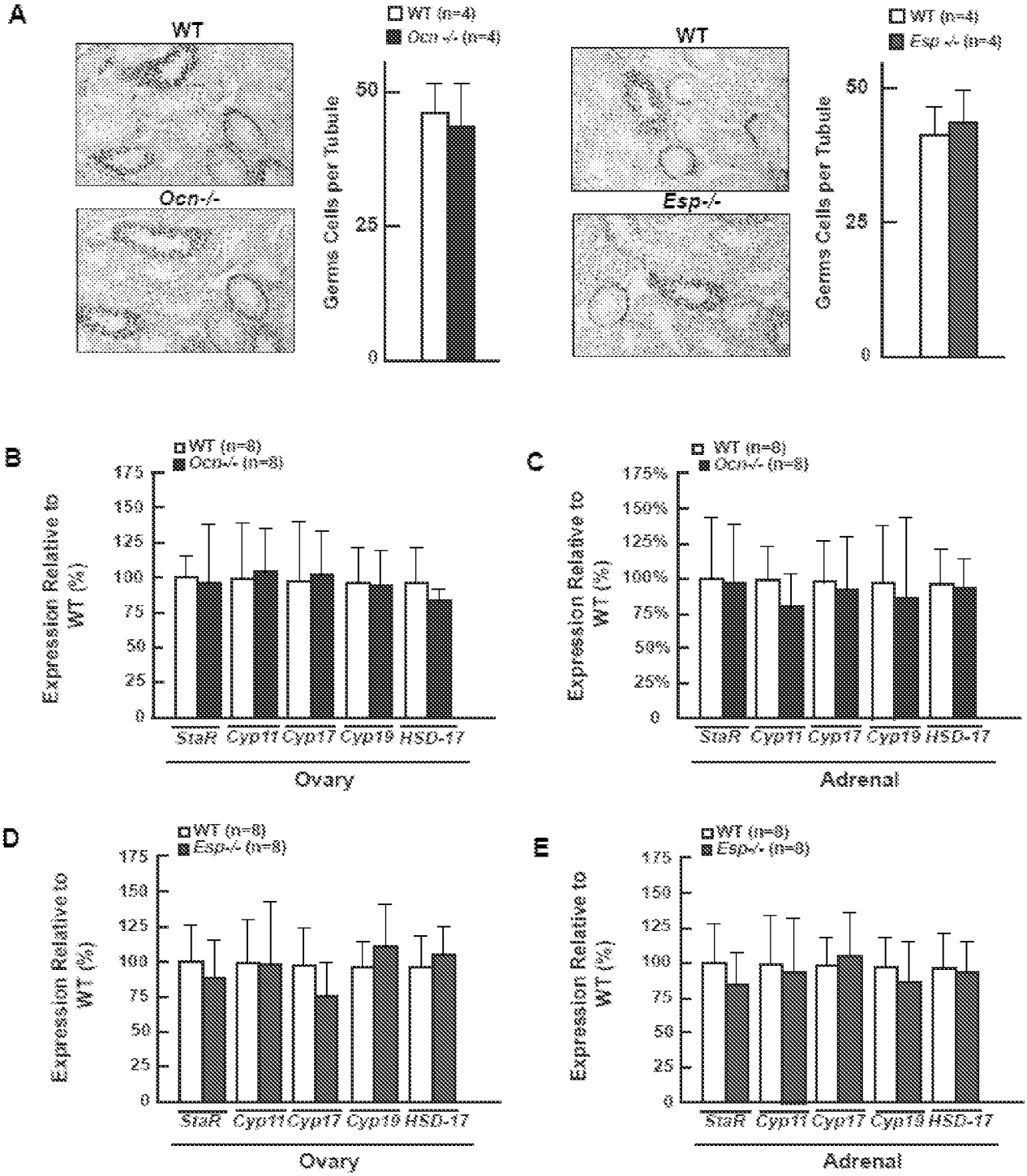

A
WT
Ocn-/-
WT (n=4)
Ocn -/- (n=4)
Germs Cells per Tubule
50
25
0
WT
Esp-/-
WT (n=4)
Esp -/- (n=4)
Germs Cells per Tubule
50
25
0
B
WT (n=8)
Ocn-/- (n=8)
Expression Relative to WT (%)
175
150
125
100
75
50
25
0
StaR Cyp11 Cyp17 Cyp19 HSD-17
Ovary
C
WT (n=8)
Ocn-/- (n=8)
Expression Relative to WT (%)
175%
150%
125%
100%
75%
50%
25%
0
StaR Cyp11 Cyp17 Cyp19 HSD-17
Adrenal
D
WT (n=8)
Esp-/- (n=8)
Expression Relative to WT (%)
175
150
125
100
75
50
25
0
StaR Cyp11 Cyp17 Cyp19 HSD-17
Ovary
E
WT (n=8)
Esp-/- (n=8)
Expression Relative to WT (%)
175
150
125
100
75
50
25
0
StaR Cyp11 Cyp17 Cyp19 HSD-17
Adrenal

FIGURE 21

A

Orphan Gpcr (n=103)

Testis enriched Orphan Gpcr (n=22)

Gpr65	Gpr110	Gpr176	Gpr141	Gpr175	Gpr37l1	Gpr22		Gpr113	Gpr50
Gpr162	Gpr35	Gpr108	Gpr55	Gpr25	Gpr173	Gpr126		Gpr15	Gpr6
Gpr180	Gpr151	Gpr111	Gpr114	Gpr146	Gpr97	Gpr77		GprC5d	Gpr37
Gpr171	Gpr52	Gpr33	Gpr125	Lgr4	Gpr12	Gprc5c		Gpr160	Gpr3
Gpr4	Gprc5b	Gpr85	Gpr128	Gpr161	Gpr116	Lgr5	Gpr179	Lancl1	Gpr87
Gpr172b	Gpr44	Gpr115	Gpr137b	Gpr1	Gpr39	Gpr124	Gpr27	Gpr158	Gpr137c
Mrgprg	Gpr150	Gpr34	Gpr56	Gpr182	Gpr143	Gpr135	Gpr153	Gpr137	Grp120
Gpr132	Gpr18	Gpr98	Gpr17	Gpr31c	Gpr174	Gpr119	Gprc152	Gpr61	Gprc6a
Gpr89	Gpr183	Gpr21	Gpr62	Gpr143	Gpr177	Gpr123		Gpr19	Gpr139
Gpr144	Gpr88	Gpr20	Pgr151	Gpr81	Gpr149	Gpr155		Gpr156	Grp112
Gpr107	Gpr31	Gprc5a	Mrgprh	Gpr64	Gpr165	Gpr101		Gpr26	Gpr45

B

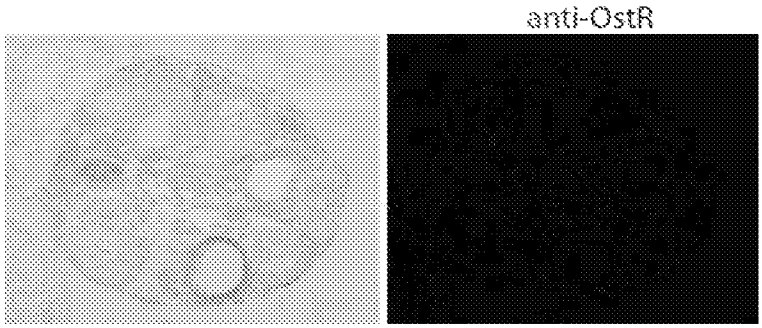


FIGURE 22

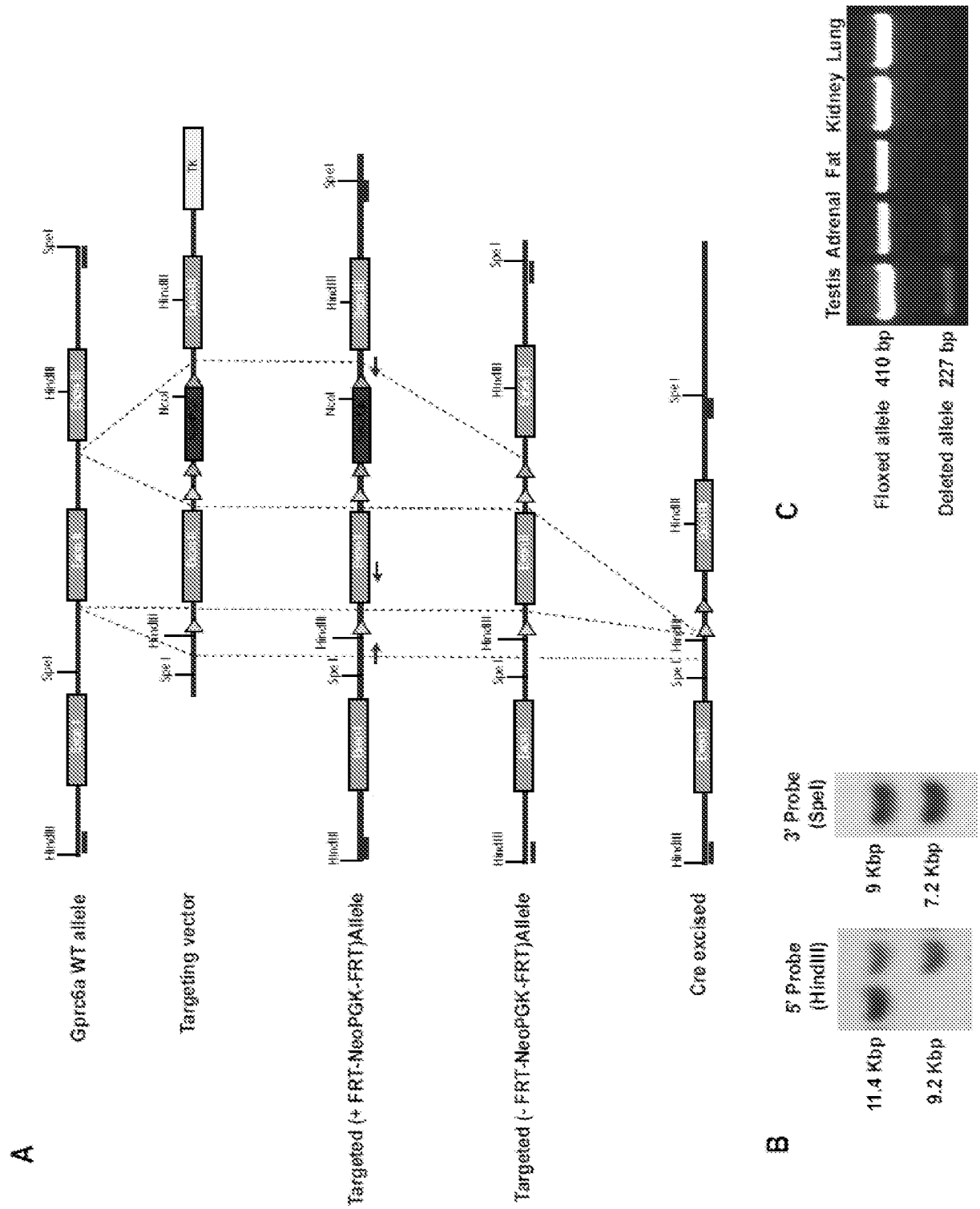
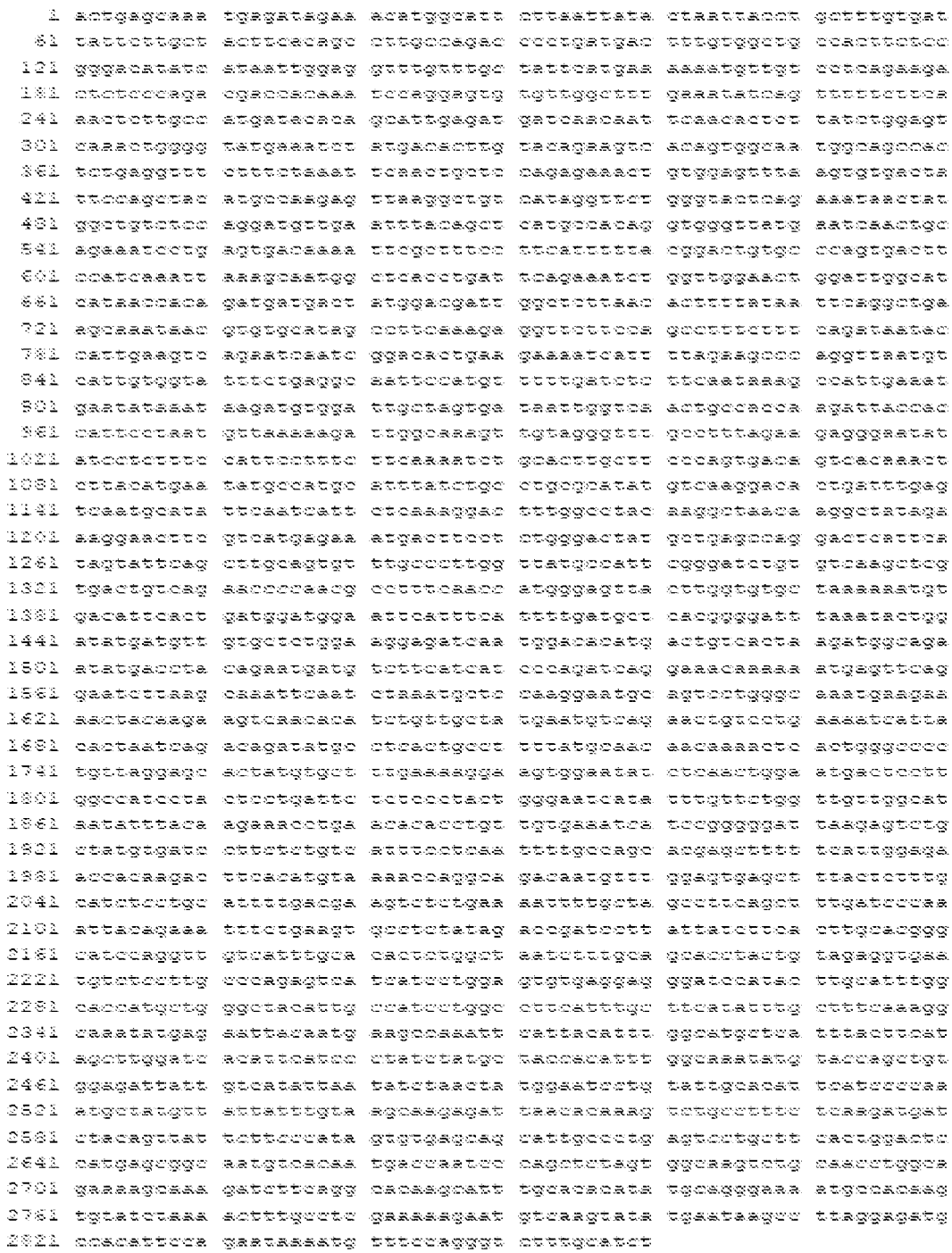

A
Gprc6a WT allele
Targeting vector
Targeted (+ FRT-NeoPGK-FRT)Allele
Targeted (- FRT-NeoPGK-FRT)Allele
Cre excised
SpeI
HindIII
NcoI
TK
B
5' Probe (HindIII)
3' Probe (SpeI)
11.4 Kbp
9.2 Kbp
9 Kbp
7.2 Kbp
C
Testis Adrenal Fat Kidney Lung
Floxed allele 410 bp
Deleted allele 227 bp

FIGURE 23



SEQ ID NO: 30

FIGURE 24

/translation="MAFLIILITCFVIILATSQPQQTPDDFVAATSPGHIIIGGLFAI
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LQLMPQVGYESTAEILSDKIRFPSFLRTVPSDFHQIKAMAHLIQKSGWNWIGIITTDD
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FLRQFHVFDLFNKAIEMNINKMWIASDNWSTATKITTIPNVKKIGNVVGFAFRRGNIS
SFHSFLQNLHLLPSDSHKLLHEYAMHLSACAYVNDTDLSQCIFNHSQRTLAVKANKAI
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KNVTFIDGNNSFHFDAHGDLNTGYDVVLWKEINGHMTVTKMAEYDLQNDVFIIPDQET
KNEFRNLKQIQSKCSKECSPGQNKKTIRSQHICCYECQNCPENHYTNQTDMPHCLLCN
NNTHWAPVRSTMCFEKEVEYLNWNDSLAILLLILSLLGIIFVLVVGIIFTRNLNIPVV
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KILLAFSFDPKLQKFLKCLYRPILIIFICTGIQVVICTLWLIFAAPTVEVNVSLPRVI
ILECEEGSILAFGTMLGYIAILAFICFIFAFNGKYENYNEAKFITFGMLIYFIAWITF
IPIYATTFGKYVPAVEIIVILISNYGILYCTFIPKCYVIICKQEINTKSAFLKNIYSY
SSHSVSSIALSPASLDSMSGNVTNTNPSSSGKSATWQKSKDLQAQAFAHICRENATSV
SNTLPRNRMSSI"

SEQ ID NO: 31