Original Article

Odanacatib restores trabecular bone of skeletally mature female rabbits with osteopenia but induces brittleness of cortical bone: a comparative study of the investigational drug with PTH, Estrogen and Alendronate †

Mohd. Parvez Khan M.Sc. 1#, Atul Kumar Singh M.Tech. 2#, Abhishek Kumar Singh M.Sc. 3, Pragya Shrivastava M.Sc. 2, Mahesh Chandra Tiwari M.Sc. 1, Geet Kumar Nagar B.Sc. 1, Himangshu Kousik Bora M.V.Sc. 4, Venkitanarayanan Parameswaran PhD 5, Sabyasachi Sanyal PhD 3, Jayesh R. Bellare PhD 2,6 and Naibedya Chattopadhyay PhD 1*.

1 Division of Endocrinology and Centre for Research in Anabolic Skeletal Targets in Health and Illness (ASTHI), CSIR-Central Drug Research Institute, 10 Janakipuram Extn, Sitapur Road, Lucknow-226031, India; 2 Centre for Research in Nanotechnology & Science, Indian Institute of Technology-Bombay, Mumbai-400076, India; 3 Division of Biochemistry, CSIR-Central Drug Research Institute, Lucknow, India; 4 Department of Laboratory Animal, CSIR-Central Drug Research Institute, Lucknow, India; 5 Mechanical Engineering Department, Indian Institute of Technology-Kanpur 208016, India; 2,6 Department of Chemical Engineering, Indian Institute of Technology-Bombay, Mumbai-400076, India.

#: Contributed equally for this work.

Supporting grants: Council of Scientific and Industrial Research and Indian Council of Medical Research, Government of India.

*Correspondence: NaibedyaChattopadhyay; Tel: +91-522 2613894; Fax: +91-522 2623938; Email: n_chattopadhyay@cdri.res.in.

Disclosures: Although in no way related to this study, NC has received research support from GlaxoSmithKline Consumer Health Care, Gurgaon, India; and served as an Advisory Board Member of Alkem Laboratories Ltd, India. All other authors have no disclosure to declare.

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jbmr.2520]

Additional Supporting Information may be found in the online version of this article.
Abstract

Cathepsin K (CK), a lysosomal cysteine protease is highly expressed in mature osteoclasts and degrades type 1 collagen. Odanacatib (ODN) is a selective and reversible CK inhibitor that inhibits bone loss in preclinical and clinical studies. Although an anti-resorptive, ODN, does not suppress bone formation which led us to hypothesize that ODN may display restorative effect on the osteopenic bones. In a curative study, skeletally mature New Zealand rabbits were OVX and following induction of bone loss were given a steady-state exposure of ODN (9 μM/day) for 14 weeks. Sham operated and OVX rabbits treated with alendronate (ALD), 17β-estradiol (E2) or PTH served as various controls. Efficacy was evaluated by assessing BMD, bone microarchitecture (using microcomputed tomography), fluorescent labeling of bone and biomechanical strength. Skeletal Ca/P ratio was measured by scanning electron microscopy (SEM) with X-ray microanalysis, crystallinity by X-ray diffraction, and bone mineral density distribution (tissue mineralization) by backscattered SEM. Between the sham and ODN-treated osteopenic groups, lumbar and femur metaphyseal BMD, Ca/P ratio, trabecular microstructure and geometric indices, vertebral compressive strength, trabecular lining cells, cortical parameters (femoral BMD, area and thickness, and periosteal deposition) and serum P1NP were largely comparable. Skeletal improvements in ALD or E2-treated groups fell significantly short of the sham/ODN/PTH group. However, the ODN group displayed reduced ductility and enhanced brittleness of central femur, which might have been contributed by higher crystallinity and tissue mineralization. Rabbit bone marrow stromal cells expressed CK and when treated with ODN displayed increased formation of mineralized nodules and decreased apoptosis in serum-deficient medium compared with control. In vivo, ODN did not suppress remodeling but inhibited osteoclast activity more than ALD. Taken together, we show that ODN reverses BMD, skeletal architecture and compressive strength in osteopenic rabbits however, increases crystallinity and tissue mineralization thus leading to increased cortical bone brittleness. This article is protected by copyright. All rights reserved

Key words: Cathepsin K; Odanacatib; Tissue mineralization; Crystallinity; Stromal cells.
Introduction

Cathepsin K (CK), a lysosomal cysteine protease, has a high level of expression in osteoclasts and is critical for bone resorption\(^1,2\). Persuasive evidence for a role of CK in bone resorption has been obtained in a rare human genetic disease, pycnodysostosis that is linked to several loss-of-function mutations in the CK gene\(^3\). Bones in pycnodysostosis are abnormally dense (osteosclerosis) and are osteopetrotic thus making them more fragile. Ablation of CK gene in mice resulted in marked reduction in the resorptive activity of osteoclasts and caused osteopetrosis. These mice had higher bone mass in both cortical and trabecular bone and greater cortical and trabecular thickness than wild type mice\(^4\). Conversely, overexpressing CK gene in mice resulted in accelerated bone turnover and increased trabecular bone loss compared to wild type mice\(^5\). Together, the biochemical, morphological and genetic evidences provide a compelling case in favor of pharmacological inhibition of CK to be a novel therapeutic option for treating postmenopausal osteoporosis.

Odanacatib (ODN) is a selective and reversible inhibitor of CK and is currently in phase 3 trials for postmenopausal osteoporosis. ODN is unique compared with other anti-resorptives available so far because it inhibits bone resorption at the terminal stage of the resorption process (close to the reversal phase of the remodeling cycle) and leaves osteoclast number unaffected while bisphosphonates and anti-RANKL antibody act more upstream (at the resorption phase of the remodeling cycle) resulting in reduced osteoclast number\(^6\). Further, unlike other anti-resorptives, ODN does not alter bone formation in preclinical models of post-menopausal osteopenia\(^7\). In OVX non-human primates (NHP), ODN has been reported to increase periosteal bone formation and cortical thickness that are reminiscent of the skeletal action of intermittent PTH\(^8,9\). In a randomized, double-blind, 2-year trial of 214 postmenopausal women with low BMD, those that received ODN displayed increased femur
Five years of continued ODN therapy in a phase 2 study on postmenopausal women with low BMD showed a trend of elevation of procollagen type 1 N-terminal propeptide (P1NP; a serum marker of bone formation) over the baseline values\(^{(11)}\). Bone formation is a dominant event during skeletal growth and ODN increased distal femoral BMD in growing rabbits\(^{(12)}\). Adult mice in which CK gene has been deleted displayed higher mineral apposition and bone formation rates in addition to having higher trabecular and cortical bone mass than their wild type counterparts\(^{(13)}\). In human bone biopsy samples, CK immunoreactivity was demonstrated in osteoblasts, osteocytes and lining cells and CK activity was shown in cultured osteoblasts. Moreover, when induced to form mineralized matrix, osteoblasts displayed dramatic reduction of CK expression, suggesting that downregulation of CK could enhance osteoblast differentiation\(^{(14)}\). From these preclinical and clinical data it is tempting to speculate that ODN has bone restorative effect in the osteopenic skeleton.

In preclinical setting, the traditional rat models of OVX-induced osteopenia is not suitable for testing the efficacy of CK inhibitors because of low homology between the rat and human CK active sites\(^{(15)}\). Structures of CK and its active sites are almost identical in humans, NHP and rabbits and hence the latter two species have been used to demonstrate the ability of ODN to prevent bone loss caused by estrogen (E2) deficiency. In comparison to NHP, the rabbit as a preclinical model has advantages such as rapid achievement of skeletal maturation (6-9 months) and short remodeling period, allowing significant bone loss or gain to be achieved more rapidly than the primates\(^{(16)}\).

There is no systematic study evaluating a probable restorative effect of ODN (or for that matter any experimental human CK inhibitors) in the osteopenic skeleton. Accordingly, the present study was designed to assess the effects of ODN in rabbits with established osteopenia using static and dynamic histomorphometries, evaluation of biomechanical...
competence, assessment of biomaterial composition and biochemical parameters relevant to bone metabolism. E2 and alendronate (ALD) were included as anti-resorptive reference treatments and intermittent injections of human PTH as a bone anabolic reference treatment.

**Materials and Methods**

**Reagents and chemicals**

Cell culture media and supplements were from Invitrogen (Carlsbad, CA); all fine chemicals from Sigma Aldrich (St. Louis, MO, USA); human PTH (1-34) from Calbiochem (San Diego, CA, USA); rabbit carboxy terminal collagen crosslinks (CTX), procollagen I N-terminal propeptide (P1NP) and tartrate-resistant acid phosphatase 5b (TRAPc5b) ELISA kits were from MyBioSource (San Diego, CA, USA); and Odancatib (MK-0822) was purchased from Selleckchem (Houston, TX, USA; catalogue # S1115). Antibodies against runt-related transcription factor-2 (Runx-2) and CK were from Abcam (Cambridge, MA, USA), and β-actin and CD68 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP conjugated anti-rabbit and anti-mouse secondary antibodies were from Santa Cruz. Anti-rabbit TRITC-, anti-mouse FITC- and anti-mouse TRITC conjugated antibodies were from Invitrogen Inc. (USA). Dentine discs were purchased from Immunodiagnostic Systems (IDS GmbH, Frankfurt, Germany).

**Experimental design**

Studies described here were approved by Institutional Animal Ethical Committee guidelines (approval # IAEC/2012/65). New Zealand White (NZW) rabbits (2.9±0.50 kg) that were 7-8 months old underwent ovariectomy (OVX) or were sham-operated. Thirteen weeks after surgery, rabbits were weight randomized into six groups and the groups that received pharmacological agents were administered in dosages and regimens as reported before: sham operated (ovary intact)+vehicle, OVX+vehicle, OVX+E2 (17β-estradiol, 0.05 mg/kg s.c., 3x/week)\(^7\), OVX+ALD (300 µg/kg s.c., 2x/week)\(^7\), OVX+ODN (0.004% of the diet, 3x/week)\(^7\).
maintaining steady state of ODN AUC= 9µM/day)(7) and OVX+PTH (1–34) 40µg/kg s.c. 5×/week for next 14 weeks(7,9). Each group had 6 rabbits. Double-calcein fluorochrome injections (i.p.) were given on the twelfth and second days to rabbits before necropsy. At necropsy, femurs and lumbar vertebrae (LV3–5) were fixed and stored in 70% ethanol. Euthanasia and disposal of carcass were in compliance with the IAEC guidelines (for additional details see supplementary information on animal procedure, p.2).

Culture of rabbit long bone-derived osteoclasts and pit assay

Purified rabbit osteoclasts were prepared from all four limbs of 2- to 4-day-old rabbits as described before(17,18). Briefly, a mixed cell suspension was obtained by mincing bones in α-MEM. Mixed cell suspension was plated onto multi-well plates on dentine discs. After a 12-h incubation allowed for cells to adhere, non-osteoclast cells (comprising of fibroblasts and macrophages) were removed by vigorous whirling on a rocker for 2 h followed by flushing the plates multiple times with the medium using wide bore pipette. The remaining adhered cells were enriched with osteoclasts. Purity of multinucleated cells (>3 nuclei) in these culture preparations routinely exceeded 90% as determined from tartrate-resistant acid phosphatase (TRAP) staining. According to the prior reports of inhibition of pit resorption by rabbit osteoclasts by the drugs, 1nM ODN(19), 10µM ALD(20) and 1nM E2(21) were added in fresh αMEM containing 10% FBS and cells were incubated for 48 h(22). 1% DMSO in the medium served as control (vehicle). Pit number and area on dentine discs were first stained with toluidine and then calculated using with Image-Pro Plus 6.1 software.

BMSC mineralization

Femurs from adult rabbits were used for harvesting bone marrow cells. 2 × 10^6 cells/well in 6-well plates were first cultured in osteogenic medium (DMEM containing 10% FBS with 10−7M dexamethasone, 10mM β-glycerophosphate and 50µg/ml L-ascorbic acid)(23). After 7 days, cells were trypsinized and seeded in chamber slide (Lab-Tek 154534) to determine the
purity of osteoblastic cells through Runx-2 immunostaining which routinely yielded ~90% Runx2 positive cells (designated as bone marrow stromal cells, BMSC). In the osteogenic medium, BMSC (2×10^4 cells/well) were seeded in chamber slide and treated with ODN (1nM), bone morphogenetic protein-2 (BMP-2, 100ng/ml) or vehicle (1% DMSO) by changing the treatments every third day. After 21 d, cells were fixed with 4% paraformaldehyde and stained with alizarin red-S dye. Following the extraction of dye using an optimized protocol, O.D. was read at 595nm.

TUNEL Assay
This assay was performed as described before with some modifications. Briefly, BMSC in osteoblast differentiation medium (10^-7 M dexamethasone, 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid with 10% FBS in αMEM) were grown for 10 days, trypsinized and seeded (5 × 10^3 cells/well) in Lab-Tek Chamber Slide (Nunc, Thermo scientific, Rockford, IL). Cells were treated with vehicle or increasing concentrations of ODN in serum-starved medium (0.5% FBS in αMEM) for 48 h. Cells were fixed in paraformaldehyde and TUNEL assay was performed according to manufacturer’s instructions provided with TUNEL kit from Roche Applied science (Indianapolis, USA). Image-Pro plus 6.1 software (MediaCybernetics) was used for quantification of TUNEL positive cells from random fields by two independent researchers blinded to experimental design.

Sulforhodamine B (SRB) assay and trypan staining
BMSC were cultured in osteoblast differentiation medium for 10 days. Cells were then trypsinized and seeded in 48-well plates (5 × 10^3 cells/well) and allowed to attach overnight. Cells were then treated with vehicle or varying concentrations of ODN in growth medium (αMEM with 10% FBS) for 48 h. Following this incubation, the adherent cells were fixed,
washed and stained with SRB. SRB is an anionic dye that binds electrostatically to basic amino acid residues of cellular proteins and thus correlates with total protein synthesis rate and cell proliferation. The bound stain was solubilized and the absorbance was measured at 510 nm in a microplate reader\(^{(25)}\). For trypan staining, BMSC were incubated with trypan dye and the dye excluded (live) cells were counted using a hemocytometer.

**QPCR and Western blotting**

QPCR studies were performed using SYBR green chemistry as previously described\(^{(26)}\). Rabbit CK (accession number: NM_001082641.1) primer pairs were 5′gAATCCAgCCAAC3′ (forward), 5′TCATTCCACTgCATCATTgTAgA3′ (reverse); and rabbit β-actin (accession number: AF404278.1) were 5′gTgggCATgggTCAgAAG3′ (forward), 5′TAgTgATTCgTGCTCgATgg3′ (reverse).

Western blotting was performed following our optimized protocol\(^{(26)}\). Briefly, whole cell lysates (50μg protein) were resolved in 12% SDS-PAGE and blotted onto PVDF membrane (Millipore Inc.). Dilutions of primary antibodies were; anti-Runx-2 (1:2000) and anti-CK(1:2000). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse (both 1:8000 dilution). Bands were detected by incubating the membranes with ECL substrate (Millipore Inc.) following the secondary antibody treatments using ImageQuant LAS 4000 (GE Healthcare, Sweden).

**Immunostaining**

These experiments were performed following an optimized protocol\(^{(24)}\). Briefly, BMSC were grown in osteoblast differentiation medium for 10 days and 1×10⁵ cells/well were seeded in Lab-Tek Chamber Slide. RAW 264.7 cell line were also grown to same cell density in Lab-Tek Chamber Slide. Paraffin-embedded, 5μm lung tissue sections were deparaffinized and processed for staining. Fixed cells and hydrated tissue sections were washed and was
incubated with the specific antibodies against Runx2 (1:1500 dilution), CK (1:1500 dilution) and CD68 (1:1000 dilution) overnight at 4°C in humidified chamber. After washing, slides were incubated with secondary antibodies (anti-mouse FITC conjugated for Runx2 and anti-rabbit TRITC conjugated for CK; both 1:2000 dilution). Anti-mouse TRITC conjugated secondary antibody (dilution, 1:1000) was used for CD68. Immunostained cells and lung sections were mounted with Vectashield and viewed under fluorescence microscope (Nikon 80i, Japan) at 40x and images were captured digitally with NIS Elements F 3.0 camera (Nikon, Japan). H&E staining on tissue sections was performed as described before(27). Images were taken using a fluorescent microscope (Nikon Eclipse Ni-U, Japan).

Microcomputed tomography (μCT)

μCT analysis of excised bones was carried out using the SkyScan 1076 CT scanner (Aartselaar, Belgium). Femurs and vertebrae were scanned (both 2-D and 3-D) at nominal resolution (pixels) of 18μm and the x-ray source was set at 86 kV and 110μA. Reconstruction was carried out on a modified Feldkamp algorithm using the SkyScan NRecon software. One hundred projections were obtained over an angular range of 180°. Image slices were reconstructed using the cone-beam reconstruction software version 2.6 based on the Feldkamp algorithm (SkyScan). At femur metaphysis, 200 slices were selected leaving 50 slices from the start of the growth plate as a reference point. Trabecular bone volume (BV/TV; percentage), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp; mm) and trabecular thickness (Tb.Th; mm) were calculated according previously published protocol with necessary modification for rabbit bone analysis(28). Three-dimensional parameters with connectivity density (Conn. D; 1/mm³), trabecular pattern factor (Tb.Pf; 1/mm) and structure model index (SMI) were recorded and analyzed with Marching cubes-type model with a rendered surface as described earlier(28). Cortical area (Ct.Ar; mm²) and cortical thickness (Ct.Th; mm) were calculated by 2-D analysis of femur mid-diaphysis.
Calculation of diaphyseal areas and perimeters was based on the Pratt algorithm. To maintain consistency, 100 slices were selected in the cortical region leaving 400 slices as offset from the start of growth plate as a reference point.

Volumetric BMD (vBMD) was determined from the VOI using µCT scans. Hydroxyapatite (HA) phantom rods of 2mm diameter with known BMD (0.25g/cm³ and 0.75 g/cm³) were employed for calibration as described earlier^{29,30}.

Assessment of the bone lining cells

Sections through distal femur (5µm) were made after decalcification and stained with hematoxylin to determine the number of surface lining cells as described before^{31}.

Determination of osteoclast number and surface area

Longitudinal sections through distal femur (5µm, decalcified) were stained for TRAP as described previously^{32}. Number of osteoclasts (OC)/bone perimeter (N.Oc/B.Pm) and OC surface/ bone surface (%) were analysed using BIOQUANT OSTEO MPR software version 12.5.6 (Nashville, TN).

Biomechanical testing

Femur three-point bending and LV-5 compression tests were performed using a Universal Testing Machine (SSTM-25KN, Huntington Beach, CA). In both tests, displacement rate was 0.5mm/min. The load-displacement curves were used to calculate ultimate load (N), energy to fracture (N-mm), stiffness (N/mm), post yield energy (N-mm) and post yield deflection (mm) according to the methods described before^{33,34}.

Dynamic histomorphometry

Mineralizing surface per bone surface (MS/BS), mineral apposition rate (MAR) and periosteal bone formation rate/bone surface (pBFR/BS) of femur mid-diaphysis were performed according to our previously published protocol on cross sections (50μM) of undecalcified bone that was in accordance to ASBMR protocol^{23,35}. To avoid label escape...
error, level of bone surface actively mineralizing was calculated as sum of the double label surface plus half of the single label surface as previously described\(^{(36)}\).

Serum biochemistry

Serum levels of collagen type 1 cross-linked C-telopeptide (CTX), tartrate-resistant acid phosphatase (TRAPc5) and N-terminal type 1 procollagen (P1NP) were determined by ELISA following protocols provided by the manufacturer (MyBioSource).

Energy Dispersive X-Ray (EDX) analysis

Excised bones were embedded in an acrylic material and cross sections through proximal femur region (50µm) were made and coated with gold sputter coater (SC7640, Quorum Technologies Ltd, UK). EDX analysis of bone matrix was performed with an X-ray detector system attached to field emission scanning electron microscope (JEOL JSM-7600F FE-SEM). Operating voltage and illuminating current were 15kV and 914pA. Ca and P weight (%) and Ca/P molar ratio were determined with reference standard (hydroxyapatite, HA) and synthetic pure samples having similar Ca/P ratio (tricalcium phosphate). The standard EDX graph produced was used to correct all observed ratio for all groups in the study. As established before, Ca/P ratio for the standard HA was 1.67\(^{(37)}\). Data obtained was corrected by the ZAF (Z=atomic number, A=absorption and F=fluorescent excitation) correction method\(^{(38)}\).

X-Ray Diffraction (XRD) analysis

Crystallinity index of bone powder from femur samples were recorded using an X-ray diffractometer (PAN analytical X’PertPro). CuKα monochromatic radiation (\(\lambda=1.541\) Å) was used with operating parameters of 40kV voltage and 30mA current. XRD patterns were recorded between 10° and 70° (2θ) in step of 0.0167 intervals with 1 second counting interval following our previously published protocol with appropriate modification\(^{(39)}\). Two different crystallographic planes were analyzed:002 and 310 reflections. The 002 reflection was related
to the length axis (c-axis) and the 310 reflection to the dimension perpendicularly to the c-axis (crystal width). Full width at half maximum (FWHM) of 002 and 310 reflections were observed after curve-fitting with OriginPro8 software.

**Backscattered Electron Imaging (BEI) analysis**

BEI was performed on sections of femur diaphysis (50µm) with FE-SEM (JEOL JSM-7600F) equipped with a four quadrant semiconductor LABE detector (JEOL). The electron beam accelerating voltage used was 15 kV. BE images were captured at a nominal magnification of 800×. The working distance in the SEM was adjusted to 15mm. Digital BE images had a gray-level resolution of 256 gray-level steps. During each imaging session, bone section from sham group was imaged every time before and after collecting images from other groups in order to ensure beam stability.

**Bone Mineral Density Distribution (BMDD) analysis**

Digital images from BEI were processed and analyzed by using Image J software (NIH, Bethesda) and EXCEL (Microsoft) with custom made routines. Gray-level histograms were derived from the digital BE images by counting the number of pixels with gray levels falling into a given interval or bin using Image J software. Total number of pixels was reported to demonstrate the amount of bone tissue scanned for each specimen. X-axis of histograms indicated gray-level values and Y-axis represented percent of pixels (corresponded to the mineralized bone, characterized as BMDD). The curves were drawn as best fit Gaussian line curves connecting the values of each bin. Two variables were extracted from the BMDD curves: a) $C_{\text{peak}}$ which was the modal value of calcium concentration (the peak position of the histogram) and represented the most frequently occurring calcium concentration of the scanned bone area and b) $C_{\text{width}}$, which was the full width at half maximum (FWHM) of the calcium distribution, representing the variation in mineralization density or heterogeneity\(^{40}\).

**Statistics**

This article is protected by copyright. All rights reserved
Data are expressed as mean ± SEM unless otherwise indicated. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by Tukey’s multiple comparison test of significance using GraphPad Prism 3.02 software. Qualitative observations have been represented following assessments made by two individuals blinded to the design of the experiments.

Results

Establishment of osteopenia in rabbits
Thirteen-week post-OVX, body weight was increased by ~4% (Fig. 1A) and uterine weight was decreased by ~46% compared with the ovary intact sham operated rabbits (sham) (Fig. 1B). At this stage, which was considered as baseline (BL), a significant reduction in vBMD of lumbar vertebra (LV3-5) (Fig. 1C) and femur metaphysis was observed in OVX rabbits compared with the sham group (Fig. 1D). Serum CTX (a bone resorption marker) levels were higher in the OVX group than the sham (Fig. 1E). Having confirmed osteopenia in the OVX group, at this time various treatments were given for another 14 weeks (total post-OVX span was 27 weeks). Post-OVX, CTX was increased with time, significantly higher at 27 weeks (end point, EP) compared with 13 weeks (BL) (Fig. 1E). Results in the subsequent sections describe effects of various treatments on these OVX rabbits at EP.

Effect on osteoclast function
Consistent with earlier reports showing that ODN failed to suppress increased osteoclast population in E2-deficient animals(6), we found that OVX + vehicle and OVX + ODN groups had comparable osteoclast number (Oc.N/B.Pm) (Fig. 2A) and osteoclast surface (Oc.S/B.Pm) in femur metaphysis (Fig. 2B), and serum TRAPc5 (a surrogate of osteoclast number) (Fig. 2C). Both ALD and E2 treatments strongly suppressed all three parameters (Fig. 2A-C). Serum CTX was elevated in the OVX group compared with the sham and ODN
suppressed OVX-induced CTX to the level that was significantly lower than the sham (Fig. 2D). CTX levels in the E2 or ALD were comparable to the sham (Fig. 2D). None of the osteoclast and resorption parameters were different between the OVX and PTH groups (Fig. 2A-D).

Because, serum CTX was greater suppressed in ODN compared with ALD or E2, we compared the effect of these three drugs in vitro in a pit resorption assay. Reduction of pit number and area were both greater with ODN treatment than E2 or ALD (Fig. 2E and F).

Effects on trabecular bone

vBMD of LV3-5 covering trabecular part (designated as Tb.LV3-5) was reduced in OVX group compared with sham (Fig. 3A). Post-OVX, Tb.LV3-5 showed a trend of decrease from BL (13 wk) to EP (27 wk) however, did not attain statistical significance (data not shown). Tb.LV3-LV5 BMD was not different between the sham and ODN groups but it was significantly higher in the PTH group. E2 and ALD groups had higher BMD over OVX but lesser than sham. When Tb.LV4 and Tb.LV5 were individually measured, ODN was found to restore BMD at both sites to sham level however, PTH had higher BMD at Tb.LV5. E2 or ALD failed to restore BMD at these two lumbar vertebra sites to the sham level (Fig.3A).

LV5 trabecular data (3D μCT) showed that trabecular bone volume (BV/TV; %) was reduced in the OVX group which appeared to be contributed by reduced Tb.N and Tb.Th with consequent increase in Tb.sp (Fig.3B). Both ODN and PTH restored all the parameters to sham levels. Between sham, E2 and ALD groups, all parameters were comparable except Tb.N, which was less in the latter two groups than the sham (Fig.3B). The geometric
parameters at LV5 including Conn.D, Tb.Pf and SMI did not vary between the groups (data not shown).

vBMD of femur metaphysis was reduced in the OVX + vehicle, OVX + E2 and OVX + ALD groups when compared with the sham group (Fig. 2A). BMD of the sham, OVX + ODN and OVX + PTH was not different (Fig. 4A). Higher Ca/P ratio has been shown to be a reliable index of bone quality and the ratio is lower in humans with postmenopausal osteoporosis and rabbits with osteopenia when compared with E2-replete controls\(^{(38,41)}\). EDX-SEM microscopy of proximal femur showed that Ca/P ratio was decreased in the OVX group compared with the sham group (Fig. 4B) which was contributed by reduced total Ca in the OVX group relative to sham (Supplementary table 1). The Ca/P ratio was not different between sham and any of the drug treated groups (Fig. 4B).

The femoral BV/TV, Tb.N and Tb.Th were decreased and Tb.Sp increased in the OVX group compared with sham (Fig. 4C-F). ODN and PTH treatments in OVX rabbits reversed these parameters to the level of sham. E2 and ALD increased BV/TV and Tb.N in OVX rabbits but failed to restore these parameters to the level of the sham group (Fig. 4C and D). However, Tb.Th and Tb.Sp were comparable between the sham, E2 and ALD groups (Fig. 4E and F).

Conn.D is a geometric parameter which measures the degree of connectivity of trabeculae normalized by TV\(^{(29)}\). Conn.D was strikingly reduced in the OVX group compared with sham (Fig. 4G). Tb.Pf and SMI are also geometric parameters and their higher values indicate disconnected trabecular structure and more “rod-like” trabecular struts respectively. As expected, both Tb.Pf and SMI in OVX were higher than the sham (Fig. 4H, I). Remarkably, all three geometric indices were comparable between the sham, ODN and PTH groups. These
three geometric parameters showed only a partial improvement in the E2 and ALD groups as it failed to attain values of the sham group (Fig. 4G-I).

Trabecular lining cells respond to PTH treatment by reacquisition of bone forming ability and constitute a major osteogenic response\(^{(42)}\). The lining cell number was markedly reduced in the OVX group when compared with sham (Fig. 4J; for photomicrograph, see Supplementary Fig. S1). ODN treatment increased lining cell number in OVX animals to the level of sham (Fig. 4J). E2 and PTH treatments were both effective in increasing the lining cell number to the sham level however, ALD had no effect (Fig. 4J).

**Effects on cortical bone**

As shown in Fig. 5A, BMD of femur mid-diaphysis was reduced in the OVX group compared with sham. Diaphyseal BMD was not different between the sham, ODN and PTH groups. E2 or ALD treatment although increased BMD compared with the OVX but the values were less than the sham (Fig. 5A).

Static histomorphometry at femur mid-diaphysis showed that cortical bone area \([\text{Ct.Ar} = \text{cortical volume}/(\text{number of slices} \times \text{slice thickness})]\) and average cortical thickness (Ct.Th) were reduced in the OVX group compared with sham (Fig. 5B). These two parameters were not different between the sham, ODN and PTH groups. Ct.Ar was modestly but significantly increased in E2 and ALD groups over the OVX group. Ct.Th was not different between the sham and other drug treated groups (Fig. 5B).

Dynamic histomorphometry analysis of cortical bone at femur mid-diaphysis showed that OVX group had higher MS/BS, MAR and pBFR/BS than the sham group suggesting higher remodelling due to OVX (Fig. 5C). As expected, E2 and ALD suppressed OVX-induced...
bone remodelling as evidenced by decrease in all three parameters compared with OVX group. ODN and PTH however did not suppress OVX-induced increase in the formation parameters and both treatments rather increased pBFR/BS compared with the OVX group (Fig. 5C).

**Effects on biomechanical strength and crystallinity**

We studied whether the restoration of BMD and microarchitectural parameters of LV5 of OVX rabbits by ODN treatment translated to improved resistance against compressive load. As reported before\(^{(43)}\), OVX group had reduced mechanical strength compared with sham. Table 1 showed that peak load, area under the curve (AUC) and stiffness in the OVX group were less than the sham. All three parameters were comparable between the sham and ODN groups. In comparison to the sham, peak load and stiffness were decreased in the E2 and ALD groups but AUC was comparable between these three groups. None of the three parameters was different between the sham and PTH. However, when comparisons were made between the ODN and PTH groups, peak load was not different but AUC was higher and stiffness was lower in the PTH group. Although E2 or ALD treatment failed to achieve compression strength parameters equivalent to the sham but both were higher than the OVX group.

Because cortical BMD, thickness and area of femur mid-diaphysis, and periosteal deposition were increased by ODN treatment to OVX rabbits, we surmised that ODN will impart greater resistance against bending strain. Expectedly, compared to sham, OVX group had reduced strength parameters characterized by lower peak load and energy to fracture as determined by femoral three-point bending test (Fig. 6A). In ODN group, peak load was comparable to sham but energy to fracture was significantly lower. These two indices were not different.
between the sham, E2 and PTH groups. Peak load in ALD was decreased compared with the sham group but energy to fracture was comparable between the two groups (Fig. 6A).

Increase in brittleness leads to decreased deformation to failure\(^{(44)}\). As shown in Fig. 6B, load vs. displacement curve in the sham group consists of three segments; elastic behavior (a-b), post-yield region from start of yielding to reaching the ultimate load (b-d) and post-yield region from ultimate load to failure (d-e). Post-yield energy (PE) is area under the curve from point b to e and post-yield displacement (PYD) is the displacement from point b to e. In all groups shown in Fig 6B, there was a clear deviation of the response from linearity at point b and this corresponded to yielding. PYD provides direct insight into the ductility of a bone. The PE and PYD obtained from tests for the different groups are presented in table 2. PE and PYD in OVX were significantly lower than sham in addition to decreased strength (peak load). Even though strength in the ODN group was comparable to the sham however, PE and PYD in the ODN group were lower than sham and most remarkably PYD was even lower than OVX, thus suggesting low ductility in the ODN group. ALD and E2 groups had lower strength than sham but displayed PYD comparable to sham suggesting similar ductility. For PTH, the PYD was comparable to sham, whereas, both strength and PE were higher than sham. Thus PTH group displayed ductility similar to sham but strength was higher.

Full width at half maximum (FWHM) of the 310 reflection obtained from XRD is inversely related to crystallinity\(^{(45)}\). In this plane, FWHM was higher in the OVX group compared with sham (Fig. 6C), suggesting lower crystallinity in the former group. ODN group showed marked decrease in FWHM compared with the sham, suggesting higher crystallinity. FWHM in the PTH group was modestly but significantly decreased compared with sham. FWHM was increased in the E2 group when compared with sham and it was comparable between the
sham and ALD groups (Fig. 6C). There was no significant difference in 002 line broadening between the groups (data not shown).

Effects on bone mineral density distribution and osteoblast function

Due to the observed increase in crystallinity in the ODN group, we next assessed mineralization of the bone matrix by qBEI. Back-scattering electron (BE) gray-level index positively correlates with mineral content (Ca molwt%). The BE images show bright gray levels (white dotted area) and dark gray levels (solid white area) respectively representing high and low mineral content (Supplementary Fig. S2). The heterogeneity of mineral content is described by the well-established BMDD which can be measure by $C_a^{\text{peak}}$ (Ca maximum frequency) and $C_a^{\text{width}}$. BMDD histogram represented percentage mineral content with respect to gray level index such that deviation to the left indicated a lower mineralization profile and vice-versa with respect to sham (Fig. 7A). Amongst all the groups, the sham group had the highest $C_a^{\text{peak}}$ (pCa). ODN and PTH groups showed decreases in $C_a^{\text{peak}}$ compared with sham. We next evaluated $C_a^{\text{width}}$ (wCa) data, which represented shift in gray level index from the sham group that was based on FWHM of the respective groups. Right and left shift with respect to sham indicated hypo- and hyper mineralization, respectively and Fig. 7A showed that the PTH group had a left shift, indicating lower matrix mineralization and ODN group had a right shift, indicating higher mineralization.

Increased tissue mineralization could be an outcome of reduced osteoclast activity and/or increased osteoblast activity. Inhibition of osteoclast function by ODN appeared to be the likely cause of increased tissue mineralization however, stimulation of osteoblast function by the compound cannot be ruled out as CK expression has been reported in human osteoblastic cells. BMSC cultures having ~90% Runx-2 positive cells (see Materials and Methods)
were co-stained with CK and Runx-2 antibodies and we observed dual immunoreactivity in majority of the cells suggesting expression of CK in osteoblastic cells (Fig. 7B). In breast cancer cells, secreted pro-cathepsin D was shown to be endocytosed by neighboring cells and in lung adenocarcinoma exogenous addition of cathepsin G was located in the endosomes of cancer cells\textsuperscript{47,48} thus raising the possibility that through a similar mechanism CK secreted by macrophages/osteoclasts present as contaminating cells in stromal cell cultures could be endocytosed by stromal cells and detected by anti-CK antibody. To address this issue, we stained stromal cell cultures that showed ~90% Runx2 positive cells with anti-CD68 antibody (a macrophage/osteoclast marker) and found absence of CD68 immunoreactivity in these cultures in contrast to strong CD68 staining in RAW 264.7 (mouse monocyte/macrophage) cell line and in rabbit lung sections (Supplementary Fig S3). In these stromal cell cultures, we demonstrated expression of CK by Western blotting. Similar to mature osteoclasts obtained from rabbit long bones, BMSC expressed a pro-enzyme band (43 kDa) and a low molecular weight band (29 kDa) that represented the processed and secreted form of CK (Fig. 7C). Treating BMSC cultures with ODN for 48 h showed comparable levels of CK mRNA between the control and ODN treated cells, suggesting that ODN had no effect on the synthesis of CK (Fig 7D).

Reduced osteoblast survivability and function are major reasons of negative remodeling balance under E2 deficiency\textsuperscript{49}. BMSC cultured in complete growth medium when treated with ODN (ranging from 1nM to 10\textmu M) showed no effect on cell growth assessed by SRB and trypan blue cell counting assays (Supplementary Fig S4). However, when BMSC were cultured in serum-starved medium (0.5% FBS) to induce apoptosis, ODN treated cultures at all drug concentrations showed significantly reduced TUNEL-positive cells compared to control, suggesting an ati-apoptotic effect of ODN on osteoblastic cells (Fig 7E). Furthermore,
ODN at 1nM (the concentration that significantly suppressed pit resorption by osteoclasts) increased the formation of mineralized nodules by BMSC compared to vehicle treated BMSC cultures (Fig. 7F). The stimulatory effect of ODN on nodule formation was comparable to BMP-2. In addition, the serum level of bone formation marker P1NP was comparable between the ODN and sham groups. As expected, PTH restored P1NP to the sham level but E2 and ALD did not (Fig. 7G).

**Discussion**

The aim of the study was to determine whether a curative ODN treatment given to skeletally mature OVX rabbits with established osteopenia could reverse the changes in vBMD, microarchitecture, strength and biomaterial properties to the levels of E2-replete state as in sham group. Similar to Pennypecker et al.\(^7\), we observed osteoporosis like changes at lumbar spine at 13wk post-OVX (baseline). However, around the similar time after OVX, Castañeda et al. found a decreasing trend in spine BMD that did not achieve significance compared with the vary intact group\(^{50}\). Areal BMD measured by DXA in case of the report by Castañeda et al. could be lesser sensitive to detection of bone mass loss compared with vBMD measured by us using \(\mu\)CT. Since PTH restores lost bone by an anabolic mode of action, we compared the ODN effect with PTH on the osteopenic skeleton. To the best of our knowledge, no study has so far compared the skeletal effects of ODN or any CK inhibitor with PTH. Furthermore, we included ALD and E2 to assess whether skeletal restoration by ODN was beyond that of the suppressive effect on bone loss by these two agents. In addition, because assessment of therapeutic efficacy of anti-osteoporotic drugs is scarcely available in OVX rabbit skeleton with established osteopenia, this study be useful for comparison of skeletal effects of these drugs in the curative mode.
Our findings that a) no change in osteoclast number and its serum marker, TRAPC5 and b) greater suppression of serum CTX in ODN group than E2 and ALD groups underscored an effective anti-resorptive action of ODN in the therapeutic mode and appeared to be not different from its reported mode of action when given in the preventive regimen in preclinical setting. Moreover, ODN completely reversed spinal osteopenia when vBMD, microarchitecture and compressive strength were considered whilst E2 and ALD had partial effect. At femur metaphysis, ODN restored connectivity parameters (Tb.N, Tb.Th and Tb.Sp) to the sham level as did by other pharmacological agents. However, unlike E2 and ALD and similar to PTH, ODN restored the geometric parameters (Conn.D, Tb.Pf and SMI) to the sham levels. From these trabecular data, it appears that in contrast to other anti-resorptive agents, ODN could completely reverse osteopenia.

Jensen et al.\(^{6}\) reported that at the eroded surface, ODN but not ALD had higher osteoblast recruitment indicated by higher densities of mature osteoblasts and increased subpopulation of cuboidal osteoblasts that are favorable for bone formation. Complementing this favorable condition for bone formation, increase in the number of trabecular lining cells and a stimulatory effect of ODN on osteoblast differentiation in vitro and survival as observed by us might have contributed to the observed trabecular restoration by this investigational drug. Furthermore, osteoclast-specific deletion of CK in mice has been shown to increase trabecular number and bone mass due to increased levels of sphingosine-1-phosphate (S1P), a putative osteogenic molecule that is produced by osteoclasts and is negatively regulated by CK\(^{51}\). It is possible that pharmacological suppression of CK by ODN in rabbits could also enhance S1P production from osteoclasts thereby leading to bone formation.

Restoration of vBMD and Tb.N appeared to have translated to gain in LV5 compressive strength of ODN group comparable to the level of sham group. Ca/P ratio correlates well with HA content, which decreases with increasing bone turnover and may contribute to bone
strength in addition to microarchitecture\textsuperscript{(52)}. OVX rabbits had reduced Ca/P ratio in the trabecular bones compared with the sham. Ca/P ratio in none of the treatment groups was different from the sham and yet peak load in LV5 was restored to the sham level in ODN and PTH but not in ALD and E2 groups thus suggesting that Ca/P ratio did not contribute to restoration of vertebral compressive strength. Furthermore, LV5 data showed that the stiffness in ODN group was significantly higher than the anti-resorptive treatment groups which could be contributed by the increase in vBMD by the drug. Whether increased stiffness by ODN could predispose trabecular bones to the increased formation and lengthening of microcracks is required to be studied. Nonetheless, based on our data, it is reasonable to assume that ODN treatment to postmenopausal osteoporotic individuals could curtail the risk of vertebral fracture.

Sustained E2 deficiency leads to cortical thinning due to increased remodeling activity\textsuperscript{(53)} that proceeds with a negative balance at the BMU (basic multicellular unit). If the balance is to change to the positive by the deposition of more bone in a smaller pit so that reconstruction of bone may follow, it is preferred that bone remodeling activity continues to be high, as each remodeling event will result in deposition of osteoid. ODN treatment causes formation of shallower resorption pits and as shown by us, when this process is accompanied by increased remodeling activity and osteoblast function it is likely to produce a positive balance by each BMU. Active Haversian remodeling as present in rabbits allows testing bone anabolic agents such as PTH. ODN has been reported to cause greater Haversian remodeling over the gonad-intact animals which could lead to uncoupling of bone formation from bone resorption thereby favoring the former event\textsuperscript{(8)}. In OVX NHP, periosteal apposition occurs with ODN when given in preventive mode\textsuperscript{(54)} and we made a similar observation by therapeutic administration of this drug. Deletion of CK in cells of osteoclast lineage in mouse resulted in increased cortical bone volume by periosteal modeling-based bone formation thus reiterating
that loss of CK protein or suppression of its activity could favor cortical thickness\(^{(51)}\). Periosteal BFR was increased by ODN and PTH more than the OVX group suggesting greater induction of surface extent of remodeling of the periosteum by these two treatments. MAR is an index of increased osteoblast function although it may be influenced by the number of osteoblasts present within the BMU\(^{(55)}\). MAR was comparable between the ODN and PTH groups and in addition, our in vitro data showed that ODN increased osteoblast survival. Together, these results suggest an osteogenic effect of ODN. In addition, cortical thickness, cortical area and tissue mineralization density (by qBEI) were increased, and these favorable changes in bone morphology produced by ODN were expected to increase greater resistance to bending.

ODN group indeed displayed increased femoral stiffness and peak load which respectively indicated resistance to deformation and maximum stress to failure that were required to carry large loads, however, PE and PYD were significantly lower than the sham. A lower PYD and PE are indicative of high brittleness or decreased ductility. PYD in the ODN group was even lower than OVX. Increased tissue mineralization in the ODN group compared with the other groups as demonstrated by qBEI might have produced a matrix that was less ductile and hence less able to deform when loaded. In addition, ODN group showed increased mineral crystallinity (assessed by FWHM). Crystallinity has been reported to increase with age causing a decrease in torsional ductility in mouse femoral cortical bone\(^{(56)}\). CK null mice display osteopetrotic phenotype in rapidly remodeled bones and resemble human genetic disorder pycnodysostosis in several respects\(^{(3,4)}\). Osteopetrosis is the primary cause of enhanced bone fragility observed in individuals with pycnodysostosis\(^{(57)}\). Based on our biomechanical evaluation of central femur, it appeared that the ODN group displayed more brittleness (feature of osteopetrosis) compared with other groups likely caused by increased tissue mineralization and crystallinity. These data may help to explain the reported cases of a
typical femur shaft fracture in postmenopausal women treated in the ODN group but none in the placebo group in a Phase III Long-term Odanacatib Fracture Trial (LOFT)\(^{58}\).

As human osteoblasts have been reported to express CK and its expression was negatively associated with osteoblast differentiation\(^{(15)}\), we speculated that its pharmacological inhibition by ODN could help to understand functional significance of CK expression in osteoblasts. Rabbit BMSC cultures (>90% Runx2 positive cells and absence of anti-CD68 positive cells) displayed CK expression assessed by immuno-colocalization (with Runx2), western blotting and qPCR. As ODN treatment of these BMSC cultures augments cell survival and nodule formation compared with control cultures, it is possible that pharmacological inhibition of CK has osteogenic effect. Furthermore, because serum P1NP (the bone formation marker) level in ODN group was comparable to the sham and higher than E2/ALD groups, a bone anabolic effect of ODN as a consequence of observed increase in osteoblast survival and differentiation in vitro could be postulated. However, we cannot rule out the possibility that the observed trabecular restorative effect was due to the formation-sparing effect of ODN rather than a direct osteogenic effect in vivo.

We have a few caveats in the study. We have not measured mineral-to-matrix ratio, carbonate-to-phosphate ratio and collagen quality as compositional contributors of bone quality to mechanical competence. Second, the effects of ODN on BMDD and crystallinity were not studied in trabecular bones, in particular LV5. Third, we have not studied the effect of ODN on bone formation at the trabecular site, particularly wall thickness which represents an important parameter of new bone made in the remodeling cycle and an important indicator of bone anabolic response. Fourth, the use of 10mM β-glycerol phosphate, a stimulant for matrix calcification in the nodule formation assay with osteoblastic cells instead of physiological phosphate levels could lead to dystrophic calcification due to necrotic nodules.
Although, the use of β-glycerol phosphate for the induction of mineralization is widely reported and in our case it was present in all treatment groups, which made increased nodule formation only in the ODN treated osteoblastic cells due to dystrophic calcification unlikely yet it did not altogether eliminate such possibility. Lastly, we have not included L-235 (a lesser selective inhibitor of CK)\(^7\) in the study to assess whether the reversal of osteopenia by ODN was due to its greater selectivity to the enzyme.

In summary, our study for the first time demonstrates that ODN treatment results in a substantial restoration of trabecular BMD, microstructure and biomechanical properties in osteopenic rabbits. Although ODN increased bone formation and cortical thickness at the central femur, it however caused loss of heterogeneity likely due to increased mineralization and crystallinity which appeared to contribute to cortical brittleness. In vitro and in vivo evidences of increased osteoblast function by ODN might explain the observed skeletal restorative effects, however additional studies are required to firmly establish an osteogenic outcome by pharmacological inhibition of CK. Further studies focusing on the effect of CK inhibition on bone quality are required to determine whether it is a safe molecular target for postmenopausal osteoporosis therapy.

**Acknowledgements**

The authors are thankful for the technical assistance provided by Ms. Kavita Singh at the Confocal Microscopy Facility, SAIF division, CSIR-CDRI; Mr. Manoj Gautam at HSEML Lab, Department of Mechanical Engineering, IIT Kanpur for mechanical testing; Department of Physics, Indian Institute of Technology, Bombay for XRD facility.

**Authors’ roles:** Study design: NC, MPK, JB and AKS. Study conduct: MPK, AKS. Data analysis: MPK, JB and AKS. Data interpretation: SS, JS and NC. Drafting manuscript: NC, SS, MPK and AKS. Revising manuscript content: MPK, AKS, SS and NC. Approving final...
References


This article is protected by copyright. All rights reserved


**Figure legends**

This article is protected by copyright. All rights reserved.
**Figure 1.** Demonstration of osteoporosis like changes in rabbits. 13 weeks after OVX (designated as BL – baseline), various parameters were compared with ovary intact (sham operated) rabbits to confirm the appearance of osteoporosis like changes in rabbits. (A) body weight, (B) uterine weight, (C) vBMD (volumetric BMD) of entire segment of lumbar vertebrae encompassing trabecular (Tb) bone (LV3-5), (D) femur metaphyseal vBMD and (E) serum CTX showing continuous increase post-OVX (EP, end point at 27 wk). Values are expressed as mean ± SEM (n=6 rabbits/group); **P<0.01, *** P<0.001 vs. sham as determined by one way one way ANOVA followed by Tukey’s multiple comparison test. *P<0.05 as indicated.

**Figure 2.** Effect of ODN on various osteoclast parameters. At the end point, sections (5µM) were obtained from proximal femur and (A) number of osteoclasts/bone perimeter (N.Oc/B.Pm) and (B) osteoclast surface/bone surface (Oc.S/BS %) were determined. Resorption markers, (C) serum TRAPc5 (surrogate of osteoclast number) and (D) CTX (indicating resorption activity in vivo) were determined using ELISA. Rabbit long bone osteoclasts were cultured on dentine slice with indicated treatments; CNT – vehicle (1% DMSO), E2 – 1nM, ALD – 10µM and ODN – 1nM. (E) Resorption area and (F) number of pits were measured using Image-Pro Plus 6.1 software. Data pooled from 3 independent experiments. Values are expressed as mean ± SEM (n=6 rabbits/group); *P<0.05, **P<0.01, *** P<0.001 vs. sham as determined by one way one way ANOVA followed by Tukey’s multiple comparison test.

**Figure 3.** ODN restored vBMD and microarchitecture of trabecular region (Tb) of lumbar vertebra in osteopenic rabbits. (A) vBMD of entire and individual segment of lumbar vertebra from LV3-5. (B) µCT parameters of LV5. All values are expressed as mean ± SEM (n =6
rabbids/group); *P<0.05, **P<0.01, *** P<0.001 vs. sham as determined by one way ANOVA followed by Tukey’s multiple comparison test.

**Figure 4.** ODN improved various skeletal parameters at femur metaphysis in osteopenic rabbits. (A) vBMD, (B) Ca/P ratio (also see supplementary table 1 for individual Ca and P values), (C-I) various µCT parameters as indicated and (J) lining cells. Values are expressed as mean ± SEM (n=6 rabbits/group); *P<0.05, **P<0.01, ***P<0.001 vs. sham as determined by one way ANOVA followed by Tukey’s multiple comparison test.

**Figure 5.** ODN increased vBMD and cortical deposition at femur mid-diaphysis. (A) vBMD and (B) static morphometry by 2D µCT showing cortical bone area (Ct.Ar) and cortical thickness (Ct.Th). (C) Dynamic histomorphometric parameters at the periosteal region of the femur mid-diaphysis. Values are expressed as mean ± SEM (n=6 rabbits/group); *P<0.05, **P<0.01, ***P<0.001 vs. sham, ##P<0.01 and ###P<0.001 vs. OVX as determined by one way ANOVA followed by Tukey’s multiple comparison test.

**Figure 6.** ODN increased bone brittleness and crystallinity. (A) Determination of bone strength by three-point bending of whole femur. (B) Load vs. deformation curve generated by three-point bending of femur; elastic behavior (a-b), post-yield region from start of yielding to reaching the ultimate load (b-d) and post-yield region from ultimate load to failure (d-e). For post-yield resilience data derived from these curves, refer to table 2. (C) Assessment of crystallinity index on the basis of full width at half maximum (FWHM) of the 310 reflection. Values are expressed as mean ± SEM (n=6 rabbits/group); *P<0.05, **P<0.01, ***P<0.001 vs. sham as determined by one way ANOVA followed by Tukey’s multiple comparison test.

**Figure 7.** ODN stimulated bone mineralization and osteoblast function. (A) qBEI-based determination of BMDD showing Ca\textsubscript{peak} (pCa) and Ca\textsubscript{width} (wCa) and respective values for

This article is protected by copyright. All rights reserved
each group are as indicated. Data are average of 3 independent experiments (n=3 rabbits/group). (B) Immunofluorescence images of CK in Runx2-positive cells (20×). CK staining (red, left panel) with anti-rabbit antibody (1:1500 dilution) and Runx2 staining (green, middle panel) with anti-mouse antibody (1:1500 dilution). A composite image (merged, right panel) resulting from the superimposition of red and green fluorescence revealed CK immunoreactivity in Runx2-positive cells. White arrow – cells shown in inset at a higher magnification (40×). Bar scale-100µm. (C) Western blotting showing CK proteins in the whole cells lysates of BMSC. β-actin was used as loading control. Representative blot from three independent experiments with similar results is shown. (D) QPCR-based quantification of CK mRNA. ODN (1nM) treatment was given for 48 h to BMSCs cultured in osteogenic medium. The relative CK expression values are presented following β-actin normalization. Pooled data from 3 independent experiments is shown. (E) BMSC cultured in osteogenic medium for 10 days were treated with 0.1% DMSO (vehicle, CNT) or different concentrations of ODN in serum-starved medium (0.5% FBS in αMEM) for 48 h. Representative photographs of TUNEL-positive cells (scale bar, 100µm) are shown and the bar diagram showing quantified data from 3 independent experiments; *P<0.05. (F) BMSC cultured in osteogenic medium with vehicle, ODN (1nM) or BMP-2 (100ng/ml) with the change in medium every third day for 21 days. At the end of the treatments, alizarin red-S staining was performed and then the dye was extracted to quantify mineralization at O.D. 595 nm. Pooled data from 3 independent experiments is shown; **P<0.01 and ***P<0.001 vs. CNT. (G) Serum P1NP, a marker of bone formation was determined by ELISA. Values are expressed as mean ± SEM (n=6 rabbits/group); *P<0.05, **P<0.01, ***P<0.001 vs. sham as determined by one way ANOVA followed by Tukey’s multiple comparison test.
Table 1. LV5 compression test

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+ODN</th>
<th>OVX+E2</th>
<th>OVX+ALD</th>
<th>OVX+PTH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peak load</strong></td>
<td>3572.5±131.5</td>
<td>2277.2±146.6***</td>
<td>3822.4±190.2###</td>
<td>2779.1±34.4**,<strong>,</strong>,**$$$$</td>
<td>2745.1±40.4**,#,$$,$$</td>
<td>3332.3±160.8###</td>
</tr>
<tr>
<td><strong>Stiffness</strong></td>
<td>29771.1±1481</td>
<td>19516.9±1130***</td>
<td>32337.5±1757###</td>
<td>24558.2±1358**,#,$$,$$</td>
<td>2438.4±1162**,#,$$,$$</td>
<td>28229.2±965###</td>
</tr>
<tr>
<td><strong>AUC</strong></td>
<td>428.7±15.7</td>
<td>279.3±19.1***</td>
<td>374.5±18.6##</td>
<td>375.6±4.6##,$$</td>
<td>374.5±5.5##,$$</td>
<td>470.1±22.6###</td>
</tr>
</tbody>
</table>

Values represent mean±SEM; 6 bones/group. **P<0.01 and ***P<0.001 vs. sham; #P<0.05, ##P<0.01 and ###P<0.001 vs. OVX; $\$\$ P<0.01 and $$$P<0.001 vs. OVX+ODN; $P<0.05 and $$P<0.01 vs. OVX+PTH.
Table 2. Post-yield resilience of femur bone

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+ODN</th>
<th>OVX+E2</th>
<th>OVX+ALD</th>
<th>OVX+PTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-yield deflection</td>
<td>0.50±0.01</td>
<td>0.44±0.01**</td>
<td>0.38±0.01***#</td>
<td>0.47±0.01$$</td>
<td>0.46±0.02$$</td>
<td>0.48±0.01$$</td>
</tr>
<tr>
<td>(mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-yield energy</td>
<td>352.43±5.6</td>
<td>221.69±6.2***</td>
<td>259.94±14.8####</td>
<td>316.86±6.1###''</td>
<td>313.97±8.3###''</td>
<td>366.10±5.26###''</td>
</tr>
<tr>
<td>(N-mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean±SEM; n = 6 bones/group. **P<0.01 and ***P<0.001 vs. sham; #P<0.05 and ###P<0.001 vs. OVX; $P<0.01 and $$$P<0.001 vs. OVX+ODN and "P<0.01 vs. OVX+PTH.
Fig 3

(A) Tb.LV3-5

(B) µCT Parameters
Fig 4

(A) vBMD (g/cm³)

(B) CaP (mass ratio)

(C) BV/TV (%)

(D) Tb.Th (mm)

(E) Tb.Sp (mm)

(F) Tb.N (mm)

(G) Conn.D (1/mm)

(H) Tb.Pf (1/mm)

(I) SMI

(J) Lining cells (1/mm²)

μCT parameters (C-I)
Fig 6

(A) AUC (N-mm) and Peak Load (N) for SHAM, O VX, O VX+ODN, O VX+E2, O VX+ALD, O VX+PTH.

(B) Displacement (mm) for SHAM, O VX, O VX+E2, O VX+ALD, O VX+ODN, O VX+PTH.

FWHM (310) for SHAM, O VX, O VX+ODN, O VX+E2, O VX+ALD, O VX+PTH.