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의학박사학위논문

# DRG2 유전자의 골대사에 대한 영향

## The effect of developmentally regulated GTP-binding protein 2 on bone metabolism

(A gene related study with a review of current osteoporosis medications and a comparison between two kinds of BMP-2)

2019 년 8 월

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Yuan-Zhe Jin

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이 논문을 의학박사 학위논문으로 제출함
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## Abstract

# The effect of developmentally regulated GTP-binding protein 2 on bone metabolism

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Osteoporosis is caused by an imbalance between bone formation and bone resorption that results in low bone mass and deteriorated bone microstructure and finally elevates the risk of low-trauma fracture. For developing new therapies for managing osteoporosis, this study compromised 3 stages as follows: 1, the bone formation efficacy of recombinant human bone morphogenetic protein 2 (rhBMP-2) was investigated since bone substitute is necessary for osteoporosis patients; 2, the efficacy of currently available medications was analyzed via meta-analysis; 3, the impact brought by the mutation in *Drg2* in bone homeostasis was researched. Their methods and

results were as follow. In the first part, we compared the osteoinductivity of *Escherichia coli* rhBMP-2 (ErhBMP-2) with Chinese hamster ovary cell-derived rhBMP-2 (CrhBMP-2) with human mesenchymal stem cells and rat calvarial defect. In the second part, we systematically reviewed the effect of current osteoporosis medications on preventing secondary osteoporotic vertebral and non-vertebral fractures from randomized controlled studies and synthesized their result via meta-analysis. In the third part, we compared the transcription level of *DRG2* in osteoporosis and non-osteoporosis subjects, and furtherly fabricated *Drg2* knockout mice and analyzed the difference in bone phenotype of wild type and *Drg2* knockout mice. At the end of this part, we investigated the possible mechanisms and signals *Drg2* involved in osteoblastic differentiation. The results from the first part showed ErhBMP-2 could have comparable osteoinductivity with Chinese hamster ovary cell-derived BMP-2 while using the demineralized bone matrix as the carrier. In the second part, we found the medications could have a consistent effect on osteoporosis patients, regardless of their fracture history. And in the third part, we found osteoporosis patients had higher expression level of *Drg2* and knocking out of *Drg2* in mice significantly improved bone mass and mineral density even if mice were ovariectomized. The bone marrow-derived macrophage in *Drg2* knockout mice showed lower osteoclastogenesis while the bone marrow mesenchymal stem cell concurrently showed higher osteoblastogenesis than wild type mice. Furtherly, inhibition of *Drg2* expression in mouse MC3T3-E1 cells elevated its osteogenicity via canonical and non-canonical BMP pathway. In summary, we found the ErhBMP-2 might have the potential of being used as an anabolic

agent for osteoporosis fracture; currently available medications could have a significant effect on preventing secondary osteoporotic fracture; and *Drg2* as an important regulator in bone remodeling, which suggested *Drg2* inhibitor could be a potential anabolic for treating osteoporosis.

**Keywords:** bone morphogenetic protein 2; developmentally regulated GTP-binding protein 2; osteoporosis; bone remodeling; osteogenesis; osteoblast; osteoclast; animal experiment;

**Student Number:** 2016-35790

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# Introduction

With the increase of the public health burden of osteoporosis, the considerations of managing the debilitating condition increases. Osteoporosis fracture presents a unique clinical challenge because the osteoporotic bone is less likely to heal on its own and require surgery to repair the fracture.[1] Since obtaining optimal union in osteoporotic bone is of utmost importance, treating surgeons must be aware of the treatment algorithms to enhance clinical outcomes.[2] Because of the decreased expression of bone morphogenetic proteins (BMPs), an essential growth factor in fracture healing made the BMPs attractive for promoting bone healing in osteoporotic fracture patients.[3]

Two kinds recombinant human bone morphogenetic protein-2 were available currently, one is Chinese hamster ovary-derived rhBMP-2 (CrhBMP-2) and the other one is *Escherichia coli* (ErhBMP-2). ErhBMP-2 has larger yield and lower cost, but slightly lower osteoinductivity.[4-8] Also, the newly formed bone induced by ErhBMP-2 had more cyst-like structure and fatty tissue in the center, which was related to its expanding in the defect site.[6, 9] Therefore, an optimal delivery system was critical to optimize the efficacy of ErhBMP-2. Among the delivery systems,[10] demineralized bone matrix (DBM) was both osteoconductive and osteoinductive and has been as a scaffold for bone regeneration.[11-14] However, the effectiveness of DBM was inferior to autograft and might be different between batches because of the donor

variety.[12, 15, 16] Therefore, using DBM as a delivery system for rhBMP-2, which could provide a controlled release of the growth factor, could be appropriate to promote bone regeneration. Since the efficacy of DBM as a carrier of ErhBMP-2 was less investigated, we compared the osteoinductivity of ErhBMP-2 and CrhBMP-2 while both were loaded by it.

Other than promoting bone formation in the fusion region, controlling the osteoporosis itself was also an important part in managing osteoporotic fracture.[2] As a metabolic bone disease, osteoporosis increases the risk of low-trauma fracture in the wrist, hip, femur, and spine that leads to hospitalization.[17] Besides the pain and disability brought by the osteoporotic fracture, the bedridden after fracture also accompanies with complications like pressure ulcer, and deep vein thrombosis that could be life threating in elderly patients.[18, 19] Osteoporotic vertebral compression fracture (OVCF) is one of the most common fragile fractures, with a prevalence of 30% to 50% in people over 50 years of age.[20] It causes severe pain and disability, raises the risk of secondary fracture more than 4-fold [21, 22], and increases the risk of mortality [23]. Therefore, secondary prevention of OVCF was critical and should be emphasized to improve patients' quality of life. However, though the primary prevention efficacy of medications has been well summarized [24-29], only one systematic review targeted on their secondary prevention effects.[30]

Various medications, such as bisphosphonate, estrogen, receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitor, and teriparatide have been widely used to treat osteoporosis. They can be divided following their

mechanisms into antiresorptive and anabolic agents. The antiresorptive agents, like bisphosphonate and RANKL inhibitor, increase bone mass via suppressing bone resorption, but it cannot repair the bone structure that was already damaged.[31] Also, the medications have been shown to decrease the risk of secondary osteoporotic fracture, but the evidence in preventing both primary and secondary fracture was not as strong.[17] The anabolic agent, teriparatide, elevates both bone formation and resorption and therefore, increases bone mineral density (BMD) and reduces fracture risk in the vertebra and non-vertebra fracture. But it has not been reported to reduce fracture risk of hip and could cause side effects such as nausea, headache, and hypercalcemia.[32] Also, the previous study more focused on the primary prevention of osteoporotic fracture.[24-26, 29, 33, 34] The efficacy of the medications therapies on preventing the secondary fracture still need to be investigated.

Recently, a medication named romosozumab, a sclerostin inhibitor has been found to be both anti-resorptive and anabolic effects and presented a significant effect on treating osteoporosis.[35, 36] The medication indicated a potential of the medications possess both anti-resorptive and anabolic characters for reducing fracture risk, shorten rehabilitation time and reduce disability caused by the fracture.[37-39]

Developmentally regulated GTP binding protein 2 (DRG2), a member of small G protein family, increases the activity of osteoclast (OC) and also regulates the activity of mesenchymal stem cells, which indicated a potential role of DRG2 in regulating osteoblastic differentiation.[40] But as far as we know, no study

reported the effect of *Drg2* on osteoblastic differentiation. Therefore, we compared the transcription level of DRG2 in mesenchymal stem cells of osteoporosis and non-osteoporosis subjects. Furtherly, we fabricated the Drg2 knockout (KO) mice, investigated the effect of Drg2 on osteoclastic and osteoblastic differentiation and compared the BMD and bone architecture between wild type (WT) and KO mice under both biological and ovariectomized condition. In the end, we investigated the possible mechanisms of Drg2 regulating osteoblastic differentiation.

Therefore, this article was composed of three part: in the first part, we compared the effect of ErhBMP-2 with CrhBMP-2 in bone healing inducing bone formation. In the second part, we systematically reviewed the effect of current osteoporosis medications on preventing secondary osteoporotic vertebral and non-vertebral fracture. And in the third part, we investigated the function of DRG2 in bone homeostasis.

# Methods

## Osteoinductive treatment of human mesenchymal stem cells

Human mesenchymal stem cells (hMSCs) (Lonza, Walkersville, MD) were seeded in 100mm dishes with MSCBM kit (Lonza, USA) and were incubated in a humified, 5% CO2, 37 °C environment. For osteoinduction, the cells were seeded in 24-well plates with a density of $1 \times 10^5$/well and were cultured in the same environment as previously mentioned. After the coverage ratio reached 70%-80%, the cells were separated into basal medium group, osteoinduction medium group, ErhBMP-2 (Novosis, CG-bio) 10ng/ml, 50ng/ml, 100ng/ml, 250ng/ml and 500 ng/ml groups, and CHO-BMP-2 (Rafugen, Cellumed) 10ng/ml, 50ng/ml, 100ng/ml, 250ng/ml and 500 ng/ml groups. The osteoinduction medium was composed of $10^{-8}$M dexamethasone (Sigma, St, Louis, Mo, USA), 50μM ascorbic acid-2-phosphate (Sigma, St, Louis, Mo, USA), 10mM β-glycerophosphate (Sigma, St, Louis, Mo, USA) low glucose DMEM and 10% fetal bovine serum, and the medium was changed every 3 days.

## ALP staining and and ALP activity assay

For ALP staining, fast blue RR salt was dissolved in distilled water and Naphthol AS-MX phosphate alkaline solution was diluted in the water in a ratio

of 1:24. Cells were washed twice with DPBS. The fast blue RR salt/Naphthol AS-MX phosphate alkaline solution was then added followed by incubation at room temperature for 5 minutes. Staining was observed under optical microscopy (Leica).

ALP activity was measured with a spectrophotometer. Cells were washed twice with DPBS and lysed with 0.2% Triton X-100. Cell lysates were assayed for ALP activity using p-nitrophenyl phosphate as a substrate. ALP activity was defined as the amount of p-nitrophenol released after incubation for 30 min at room temperature. The color change was measured spectrophotometrically at 405 nm and the experiment was biologically repeated 3 times.

## Calcium staining and assay

Wells were washed twice with DPBS, added with 40nM alizarin red solution and incubated at room temperature for 5 minutes. The mineral deposition was observed with optical microscopy (Leica).

To quantify calcium formation with osteoblast differentiation, wells were washed with distilled water twice. Cells were lysed with 0.2 ml 0.5% HCl and 2.5 μl of the sample was transferred into a 96-well plate. An equal volume of reagent A and reagent B from QuantiChrom calcium assay kit (BioAssaySystems, Hayward, CA, USA) was mixed together, and 200 μL of the mixture was added into each well. After incubating at room temperature for 3 minutes, the optical density at 612 nm was measured with an ELISA reader. The experiment was biologically repeated 3 times.

## Real-time PCR

Expression levels of bone formation related genes were evaluated with real-time PCR. $1 \times 10^5$ cells were cultured in 96 well dishes and induced for 7 days. Total RNA was isolated with RNeasy mini kit (Qiagen, USA), and was reversely transcribed with High Capacity cDNA Reverse Transcription Kit (Intron Biotechnology, Korea). After cDNA synthesis, real-time PCR (LightCycler® instrument, Roche) was performed with SYBR Green PCR Mastermix and ABI StepOnePlus real-time PCR system (Applied Biosystems, USA). The expression levels of ALP, Runx-2, OCN, BSP, OPN, and OCN was calculated as $-2^{\Delta\Delta Ct}$.[41]

RNAs were extracted using Qiagen RNeasy mini prep kit(Life Technology) and reverse transcribed into cDNA using Maxime™ RT PreMix kit(Intron technology). Real-time PCR was performed with SYBR Green and LightCycler® 480 Instrument ( Roche Life Science) real-time PCR system. The expression level was calculated as $-2^{\Delta\Delta Ct}$, and the experiment was biologically repeated 3 times. The expression was corrected using GAPDH. [41] Primers used were listed in Table 4.

## Rat calvarial defect model

The procedures that involved the use of animals were approved by the international animal care and use committee (SNUH IACUC No.13-0348). Eight-week-old male Sprague-Dawley rats (200–220 g, total N = 156) were used for the animal experiment, all animals were kept in a 12:12 dark/light cycle,

specific-pathogen-free cage and were provided with abundant food and water. The experiments were performed after 1 week stabilization period and were randomly separated into ErhBMP-2 (Novosis, CGBio, Korea) 2.5 μg + DBM (Rafugen DBM gel, Cellmud, Korea) 0.05 ml group and CrhBMP-2 2.5 μg + DBM (Rafugen rhBMP-2 DBM gel, Cellumed, Korea) 0.05 ml group.

Animals were anesthetized with an intraperitoneal injection of 20 mg/kg Zoletil and 10 mg/kg xylazine. The scalp was shaved and sterilized, and an 8 mm calvarial defect was made. The composite from each group was implanted in the defect and the scalp was sutured.[42] All animals were injected 100 mg/kg cefazolin and housed in the environment as previously described.[43]

All animals were sacrificed with a $CO_2$ chamber under deep anesthesia on their $4^{th}$ or $8^{th}$ week after experiments. The implants were carefully harvested and were immediately fixed in 10% formalin for micro-CT evaluation and histological assessments.

## Micro-CT evaluation

The rat calvaria samples were scanned with a Skyscan 1172 micro-CT scanner (Bruker, Belgium). The samples were scanned with the following setting: 11.93 μm pixel size, 0.5 mm Al filter, 70 kV energy, 141 μA current, and 0.4° rotation step. The images were reconstructed analyzed with NRecon (Bruker, Belgium) and CT-An (Bruker, Belgium). The threshold values of newly-formed bone were referred to the native bone. Bone morphometric parameters, including percent bone volume (BV/TV), bone surface/volume ratio (BS/BV), trabecular

bone pattern factor (Tb.Pf), structure model index (SMI), trabecular bone thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and degree of anisotropy (DA) were analyzed with an 8-mm diameter region of interest.

The vertebra and femur samples from mice were scanned with the same 1172 micro-CT scanner under the following setting: pixel size, 9.86 μm; 0.5 mm Al filter; energy, 59 kV; current, 167 μA; and rotation step, 0.4˚. Cross-sectional images were reconstructed using the NRecon package (Bruker, Belgium) and analyzed with a CT Analyzer software (CT-An, Bruker, Belgium). Threshold values of newly-formed bone were compared with those of native bone. Trabecular bone thickness (Tb.Th), bone volume (BV), and percentage bone volume (BV/TV) were calculated within 8-mm diameter regions of interest (ROI), generated based on the defect site. In spine samples, the ROI was set as an elliptic cylinder (1.08 mm * 0.8mm * 0.61mm) located in the caudal region of a vertebra. (Fig. Supplementary 1 a) In femur samples, the trabecular bone was selected with semi-auto selecting program in CTAn femur head, proximal femur neck, and proximal femur. (Fig. Supplementary 1 b-d)

## Hematoxylin and eosin staining

The rat calvaria samples were fixed in 10% formalin, dehydrated in 80% to 100% ethyl alcohol, infiltrated and embedded with Technovit 7200 resin (EXAKT, Germany). Then resin was solidified with a polymerization system (EXAKT, Germany), and was sectioned to 200 μm thick slice; and the slice was

ground to a 50 μm thick with an EXAKT grinding system (Germany). The ground slices were stained with hematoxylin and eosin, and the bone formation was observed with an optical microscope.

The femur and vertebra of mice were fixed with formalin and were decalcified with 8% formic acid/8% HCl solution for 24 hours. After standard paraffin infiltration procedure,[44] samples were embedded in a paraffin block and sectioned at a thickness of 4 μm. After then, the slides were stained with hematoxylin and eosin and observed under an optic microscope.

## Search for studies

Four major electronic databases (MEDLINE, EMBASE, CENTRAL, and Web of Science) were searched with a developed search strategy that consisted of keywords “controlled trials”, “osteoporotic fracture”, “bisphosphonate”, “parathyroid hormone”, “denosumab” “calcitonin”, “Raloxifene”, “Bazedoxifene” “hormone replacement”, etc., and others (Supplementary Material 1). The search spanned the period from June 2015 to December 2017, with weekly alerts of updated published trials. Reference lists from other reviews and studies were also checked for relevant articles. The references were managed with Endnote X7 (Clarivate Analytics).

## Selection of studies

One author screened the titles and abstracts of studies and evaluated their relevance to our study. A study was included if it involved patients with

osteoporosis. A subsequent full-text assessment was done by two reviewers. Randomized controlled trials (RCTs) published in English that investigated the efficacy of currently approved medications for patients with OVCF were included. The studies that included osteoporosis patients without distinguishing their fracture history were included if the data of the participants with prevalent fractures was adequately presented. Studies that recruited patients with traumatic vertebral fracture, secondary osteoporosis, or did not report results in dichotomous data (i.e., patient-years, etc.), were excluded. Post hoc analyzed RCTs were also included, with taking care of duplicated data input. Disagreements between reviewers were resolved by discussion or, if unresolved, by consultation with librarians from Seoul National University Medical Library and a statistic professor from Seoul Metropolitan Government Seoul National University Boramae Medical Center.

## Data extraction and risk of bias

Basic characteristics of each study were extracted with a designed table that contains the number of participants, interventions, comparisons, and outcomes. The primary outcome of this study was the vertebral fracture ratio in the final visit, and the secondary outcomes were gastrointestinal (GI) complaints of bisphosphonates, discontinuation due to adverse events (AEs), and non-vertebral fracture ratio.

The risk of bias was measured with the tool recommended in updated guidelines of Cochrane Back and Neck Group [45]. The detection bias was

rated for the main result (vertebral fracture). The loss ratio was acceptable for a middle- or long-term trial (observational period > 1 year), if that was not exceeded by 30%. The risk of other sources of bias was rated as low risk if the article stated both conflicts of interest and sponsor of the trial and no other serious risk of bias was reported.

## Data analysis and quality of evidence

Relative risk (RR) and its 95% confidence intervals (CIs) were used to estimate the effect of interventions, with p-values < 0.05 considered significant. The overall effect size was calculated with a random effects model [45]. Heterogeneity between studies was identified and measured with p-value and $I^2$ value from Chi-squared test, p-value < 0.1 was identified as significant, and $I^2$ > 75% was identified as a considerable magnitude. In the studies that compared the effect of medications with placebo groups, the data from the medication groups were included in the intervention groups, and the data in the placebo groups were included in the control groups. In the studies with more than two arms, intervention groups were input into each subgroup and the data in the placebo groups were separated equally into control groups and were compared to their counterparts. Sensitivity analyses were used to explore the interference from a study by excluding it from syntheses and the impact from loss to follow-up population by compositing the missing events according to event ratio in control groups [46]. The data were analyzed with RevMan 5.3.3 (Cochrane)

We evaluated five factors of the results to determine the quality of evidence, including study limitation, imprecision, indirectness, inconsistent and publication bias, followed the GRADE approach. The criteria for downregulating the level referred to the handbook of GRADE and guidelines from Cochrane Back and Neck Group.[45, 46] In the case that an outcome included one trial with no unclear or high risk of bias, the study limitation item was rated as not serious if its result remained same direction and significance with the pooled result.

## Extraction of mesenchymal stem cells from human

Human ilium source mesenchymal stem cells were obtained during surgery for patients from the Seoul Metropolitan Government – Seoul National University Boramae Medical Center. All clinical procedures were approved by the Institutional Review Board of Seoul Metropolitan Government – Seoul National University Boramae Medical Center (IRB No. 06-2009-107). Informed consent was obtained from participants.

## Fabrication of DRG2 knock out mouse

*Drg2* KO founder mice were produced with C57BL/6N strain using CRISPR/Cas9 technique at Korea Research Institute of Bioscience and Biotechnology (KRIBB). For mutant lineage establishment, founder mouse was crossed with C57BL/6N mice to maintain a pure C57BL/6 background.

## Genomic typing and gender determination

For genomic typing, 2-3mm tail tissue was dissected under gaseous anesthesia. Tail tissue was lysed with Genomic DNA Extraction kit. (iNtRON Biotechnology, Inc, Korea) PCR amplification was conducted with Maxime PCR Premix (i-Star Taq) (iNtRON Biotechnology, Inc, Korea) under the following conditions: 95°C for 5 minutes, followed by 25 cycles of 95°C for 35 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. PCR products were finished with a final extension step at 72°C for 5 minutes. For determination of the gender of mouse newborn, the abdominal skin was dissected after euthanasia. Tissue was lysed with Genomic DNA Extraction kit. (iNtRON Biotechnology, Inc, Korea) PCR amplification was conducted under the following conditions: 95°C for 4.5 minutes, followed by 33 cycles of 95°C for 35 seconds, 58°C for 1 minute, and 72°C for 1 minute, then the PCR was finished with a final extension step at 72°C for 5 minutes. PCR products were loaded onto 1% agarose gel (USB Corporation, USA) and electrophoresed for 20 minutes. Images were taken under UV transillumination with molecular image software. (Kodak) Sequences of primers were listed in Table S1.

## Primary culture of BMMCs and bone marrow MSC

The mice were anesthetized with zoletil/xylazine (20 mg/kg and 10 mg/kg, respectively) mixture and were euthanized via $CO_2$ inhalation. The bone marrow inside tibia and femur was flushed out with basic DMEM medium

(DMEM low glucose, WELGENE, Cat. LM 001-12; 10% FBS and 1% antibiotic-antimycotic) and was collected in a 10 ml tube. For culturing BMMCs, the cells were cultured with basic DMEM medium containing macrophage-colony stimulating factor (M-CSF, ) at 25 ng/ml. For culturing MSCs, the cells were cultured with the basic medium. All the cells were cultured in a humidified chamber at 37 ℃ with 5% $CO_2$.

## TRAP staining

The BMMCs was seeded in 12-well plate with a density of $0.1 \times 10^6$. The control group was cultured with basic DMEM medium and 25 ng/ml M-CSF. The induce group was treated with basic DMEM medium, 25 ng/ml M-CSF and 50 ng/ml RANKL (PEPROTECH, Cat. No. 315-11). The medium was changed every 2 days. After 7 days after induction, the cells were stained following the instruction of the TRAP staining kit (SIGMA-ALDRICH, LOT, SLBP7748V)

## shRNA transfection of MC3T3-E1 cell

To diminish the transcription level of Drg2 in MC3T3-E1 cell line, shRNA (SANTA CRUZ BIOTECHNOLOGY, INC) was used following the manufacturer's instruction. For transfection, Lipofectamine (Thermo Fisher Scientific) containing 1 μg DRG2 shRNA Plasmid was mixed into opti-MEM medium, and the mixture was incubated at room temperature for 30 minutes.

The shRNA plasmid transfection medium was added in each well in a 6-well plate. Then the cells were incubated in a standard environment (5% CO2, 37 ºC ) for 5 hours. For selecting stably transfected cells, 10 μg/ml puromycin was added into the basic culture medium, i.e., alpha-MEM containing 10% FBS and 1% antibiotic-antimycotic (Thermo Fisher Scientific). The medium was changed every 2-3 days.

## Inducing osteoblastic differentiation in MC3T3-E1 cells

MC3T3-E1 cells were induced with α-MEM (containing 10% FBS and 1% antibiotic-antimycotic) based osteoinduction medium that contained $10^{-8}$ M dexamethasone, 100 μmol ascorbic acid, and 10 mM β-glycerophosphate.

## Western blot

Cells were harvested in RIPA buffer containing protease/phosphatase inhibitor (Thermo Fisher Scientific), and the protein was quantified using BCA assay. Primary antibodies used for immunoblotting include: beta-actin Rabbit mAb (Cell Signaling #8457) at a 1:2000 dilution, p38 MAPK Rabbit mAb (Cell Signaling, #8690) at a 1:2000 dilution, phospho-p38 MAPK Rabbit mAb (Cell Signalling #4511) at a 1:1000 dilution, phospho-Smad1 Rabbit mAb (Cell Signaling, #5753), phopho-Smad1/5 Rabbit mAb (Cell Signaling, #9516) at a 1:1000 dilution, Smad 1 Rabbit mAb (Cell Signaling, #6944) at a 1:1000 dilution, Smad 4

Rabbit mAb (Cell Signaling, #38454) at a 1:1000 dilution, Smad 5 Rabbit mAb (Cell Signaling, #12534) at a 1:1000 dilution. The secondary antibody used was Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling, #7074). Chemiluminescent signals were detected with ECL Select western blotting detection reagent (GE Healthcare, RNP2235) under Fujifilm LAS 4000.

## Semiquantitative RT-PCR

Expression level of *Drg2* was monitored with semi-quantitative RT-PCR, with primer kit from SANTA CRUZ BIOTECHNOLOGY, INC (product number DRG2 (m):sc-143171-PR).

## Feeding and maintaining

All procedures involved the use of animals were officially approved. (SSBMC-IACUC No. 2017-0007). Animals were kept in a specific-pathogen-free house. They were provided with abundant food and water and with a 12:12 dark/light cycle. Genotyping of tail tissue DNA was conducted with primers shown in Supplemental Table 1. These animals were maintained for more than nine generations by mating between heterozygous mice.

## Serum P1NP and CTX measurement

Eight weeks female mice were anesthetized with zoletil/xylazine (20 mg/kg and 10 mg/kg, respectively) mixture. After general anesthesia, blood was collected via cardiac puncture from each mouse. The blood was collected into serum separation tube.

Serum P1NP and CTX levels were measured with specific ELISA kits for mouse P1NP (MyBioSource, #X03147743) and CTX (MyBioSource, #32289442). Sensitivity was 0.6 ng/ml for CTX and 1.17 pg/ml for P1NP. Intra- and inter-assay variation coefficients were ≤ 8.0% and ≤ 12% for the CTX kit and < 8.0% and < 10% for the P1NP kit, respectively.

## Ovariectomy

Eight weeks old female mice were anesthetized with intraperitoneal injection of 20 mg/kg Zoletil mixed with 10 mg/kg xylazine. Surgical procedures were performed in semi-sterile conditions. The surgical site was shaven and sterilized with povidone iodine solution. After a lateral incision, the ovary in the OVX group was exposed and removed. It was put back to the abdominal cavity in the sham group.[47] All procedures involving the use of animals were approved by the IACUC (SSBMC-IACUC No. 2017-0007). Sixteen weeks after surgery (24 weeks of age), mice were euthanized and their spines (L1-L5) and femurs were harvested. Each vertebra and each femur was treated as individual sample.

## Calcein labeling

Calcein labeling followed a protocol published before.[44] Eight weeks male mice were injected with calcein solution at 7 days and 5 days prior to their scarification. Their vertebrae and hind limbs were harvested and kept in 10% formalin for 2 days and 5 days, respectively. After removing the muscle tissue of each the samples, femurs and tibia were incubated in 10% KOH for 8 days and vertebra were incubated for 4 days in 10% KOH. Bone was processed in a vacuum infiltrating tissue processor and embedded in paraffin. The bone was then sectioned with Leica rotatory microtome at a thickness of 4 μm. Two sections with 40 μm to 50 μm apart were obtained from each sample. These sections were observed under a fluorescence inverted microscope. Their mineralized surface and internal distance were measured following a previously published method.[48]

## Statistics

For in vivo data, each n value corresponds to a single sample. For in vitro data, each n value corresponds to an independent well. All data were presented as mean and standard deviation. Statistical analysis was performed with t-test and p-value (two-sided) less than 0.05 was deemed as statistically significant. All analyses were performed with SPSS (IBM).

# Results

## ALP assay

At the 3rd day, no significant difference was found between groups. At the 7th day, the cells treated with BMP-2 showed higher expression than control and induction group. In 10 ng/ml and 50 ng/ml groups, ErhBMP-2 groups showed a higher activity while CrhBMP-2 showed higher levels at the doses of 100 ng/ml, 250 ng/ml, and 500 ng/ml. At the 14th day, both BMP-2 had similar ALP activities at 10 ng/ml, 50 ng/ml and 500 ng/ml doses, while CrhBMP-2 had higher activity at 100 ng/ml and 250 ng/ml. (Fig. 1)

## ALP staining

No significant difference was observed between all groups on the 3rd day. At the 7th day, BMP-2 treated groups began to show higher expression level than control and induction groups, also showed a dose-dependent increase in stain density. In the 10 ng/ml group, ErhBMP-2 and CrhBMP-2 showed similar stain level, and in other groups, CrhBMP-2 had more intense color than ErhBMP-2. At the 14th day, all CrhBMP-2 groups showed higher ALP activity except the 10ng/ml group. (Fig. 2)

## Calcium assay

Accumulation of calcium was only detectable in 500ng/ml CrhBMP-2 group on the 7th day. At the 10th day, CrhBMP-2 had more calcium than ErhBMP-2 at

all doses except 10ng/ml. At the 14th day, except 10ng/ml, CrhBMP-2 showed higher calcium level than ErhBMP-2. (Fig. 3)

## Alizarin red staining

No calcium deposit could be observed on the 7th day, and at 10th day, the stained calcium deposits could be observed in 250ng/ml and 500ng/ml CrhBMP-2 group. At the 14th day, the accumulation of mineral could be observed in all groups. Though no significant difference was observed, CrhBMP-2 groups showed slightly more intense staining compare with their ErhBMP-2 counterparts. (Fig. 4)

## Real-time PCR

The expression levels of bone formation related genes were measured at 7th day after osteoblastic induction. The expression levels of ALP and BSP were higher in all CrhBMP-2 groups than ErhBMP-2 groups. The expression levels of OCN and OPN were higher in CrhBMP-2 at 10ng/ml, 50ng/ml, 100ng/ml and 250 ng/ml groups. The expression levels of Runx-2 were similar between ErhBMP-2 and CrhBMP-2 at doses of 250 and 500 ng/ml. In other doses, the CrhBMP-2 had higher levels than ErhBMP-2. (Fig. 5)

## Animal experiments

With DBM as the carrier, ErhBMP-2 performed significantly higher osteoinductivity than CrhBMP-2 on both weeks. On the 4th week, ErhBMP-2

group had 48% higher BV/TV and 38% higher coverage ratio and half of Tb.Sp than CrhBMP-2 group did, all with statistical significance. But the Tb.Pf in ErhBMP-2 group was significantly higher than that of CrhBMP-2, which indicated the bone in ErhBMP-2 group was less continued than the bone in CrhBMP-2. (Table 1) On the 8$^{th}$ week, the ErhBMP-2 group showed 4.26 times higher BV/TV, 1.86 times higher coverage ratio, 3.58 times narrower Tb.Sp and 2.94 times more Tb.N than CrhBMP-2 group, all with statistical significance. (Table 1) The significantly lower SMI in ErhBMP-2 group indicated it had more spherical cavities in bone; and the significantly lower Tb.Pf and DA indicated the bone in ErhBMP-2 was more continued and isotropic than CrhBMP-2.

The histomorphometric characters of new bone observed in histology sections were consistent with the results of micro-CT. On the 4$^{th}$ week, the bone volume in ErhBMP-2 group was significantly higher; and bridging of newly formed bone could only be observed in that group. Meanwhile, the newly formed bone in ErhBMP-2 had a cyst-like structure and more adipose tissue. On the 8$^{th}$ week, the ErhBMP-2 induced significantly more bone than CrhBMP-2 did, and the bone in ErhBMP-2 was more continued and isotropic than the bone in CrhBMP-2 group. (Fig. 6, Fig. 7)

## Characteristics of included studies and risk of bias

A total of 6157 articles were identified. Among them, 608 were subjected to full-text assessment. After full-text examination, 39 articles were finally

included in this study (Fig. 8). Among them, 34 compared the effects of medications with control groups. Bisphosphonates (BPs) were compared in 19 RCTs,[49-67] calcitonin in 3 [68-70], hormone replacement therapy (HRT) in 3,[71-73] parathyroid hormone (PTH) or teriparatide in 4,[74-77] denosumab in 2,[78, 79] and selective estrogen receptor modulators (SERMs) in 3.[80-82] Five trials compared between the effects of medications, risedronate vs. etidronate,[83] ibandronate vs. risedronate,[84] romosozumab vs. alendronate,[85] and teriparatide vs. risedronate.[86, 87] Follow-up duration in most trials was 2 to 3 years. Other basic characteristics of the included studies were summarized in Table 2.

Approximately half of the biases were rated as unclear risk (Supplementary Material 2). Risk of other sources of bias was rated as high in one study because the criteria used in its two clinical centers were different.[53] Performance bias was rated as high risk in 6 trials for significantly different compliance between groups [58, 77, 82] and the open-label study design used in 4 trials.[52, 59, 68, 71]

We treated a group in which participants accepted Teriparatide 1.2 μg/week delivered via subcutaneous injection as a control group, following the original study.[76] The robustness of the results was examined using a sensitivity analysis. Sorensen et al. reported a 2-year extension trial [55] of a 3-year original trial[54]; they treated the initial time point of extension trial as the baseline. Therefore, we deemed the data were not duplicated and pooled them of both original and extended trials. The risk of selection bias of the extended

trial was rated as high due to the significantly imbalanced medication history (Supplementary Material 2).

## Comparison with control group

### Antiresorptive medications

The result of antiresorptive medications, including BPs, HRT, SERMs, calcitonin, and denosumab, were pooled together to investigate the effects of the medications. Thirty-three studies involving 21,012 participants were included. The result indicated that the administration of antiresorptive medications could significantly reduce the risk of the secondary OVCF (RR, 0.59; 95% CI, 0.53-0.65, $p < 0.00001$) (Table 3, Supplementary Material 3a). Bisphosphonates did not significantly increase gastrointestinal (GI) complaints (RR, 1.02, $p = 0.45$; Supplementary Material 3b). The result was treated as a secondary outcome because of the heterogeneity in the comparison.

### Zoledronate

Moderate quality evidence proved that zoledronate could significantly decrease the risk of secondary OVCF (RR, 0.34; 95% CI, 0.17-0.69, $p = 0.003$; Fig. 9a, Table 3), without significant increase in discontinuation due to medication (RR, 1.99; 95% CI, 0.76-5.25, $p = 0.16$; Table 4, Supplementary Material 3c). Additionally, zoledronate could significantly decrease event ratio of non-vertebral fractures (RR, 0.54; 95% CI, 0.32-0.91; $p = 0.02$; Table 3, Supplementary Material 3d).

**Alendronate**

High quality evidence proved that administrating alendronate significantly reduced the proportion of participants who had subsequent vertebral fractures (RR, 0.54; 95% CI, 0.43-0.68; $p < 0.0001$; heterogeneity, $p = 0.63$, $I^2 = 0\%$; Fig. 9b, Table 3). No significant increase in GI complaints or discontinuation was observed in the alendronate group (GI complaints, RR, 1.03, $p = 0.55$; Discontinuation, RR, 0.88, $p = 0.46$; Table 4, Supplementary Material 3e and 3f). Alendronate had no significant effect on preventing non-vertebral fractures (RR, 0.81, $p = 0.07$; Table 3, Supplementary Material 3g).

**Risedronate**

Moderate quality evidence indicated that risedronate had a significant effect on preventing subsequent fractures (RR, 0.61; 95% CI, 0.51-0.73, $p < 0.0001$; Fig. 9c, Table 3). Risedronate administration did not significantly elevate GI complaints (RR, 1.09, $p = 0.18$) or discontinuation rate (RR, 0.88, $p = 0.28$) (Table 4, Supplementary Material 3h and 3i). Risedronate had a significant effect on preventing non-vertebral fractures (RR, 0.71, $p = 0.01$; Table 3, Supplementary Material 3j).

**Etidronate**

Moderate quality evidence showed that the administration of etidronate could significantly reduce the risk of new fractures (RR, 0.50; 95% CI, 0.29-0.87, $p < 0.01$; Fig. 9d, Table 3). The result consisted with that of sensitivity test, in which a study [61] with small sample size and big variance was excluded (Supplementary 3k). No significant difference was observed in GI complaints

(RR, 0.57, p = 0.12) or discontinuation (RR, 0.40, p < 0.50) between intervention and control groups (Table 4, Supplementary Material 3l and 3m). Etidronate did not have a significant effect on preventing non-vertebral fractures (RR, 0.95, p = 0.83; Table 3, Supplementary Material 3n).

**Ibandronate**

Moderate quality evidence proved that ibandronate administrated 2.5 mg daily or 20 mg intermittently could significantly reduce the fracture risk (RR, 0.52; 95% CI, 0.38-0.71, p < 0.0001; Fig. 9e, Table 3), while insufficient dosages (0.5 mg or 1 mg per 3 months) did not (RR, 0.87; 95% CI, 0.69-1.11, p = 0.27; Fig. 9f, Table 3). Ibandronate did not significantly raise the risk of discontinuation due to adverse events (sufficient dose: RR, 0.90, p = 0.45; insufficient dose: RR, 1.27, p = 0.07) (Supplementary material 3o and 3p, Table 4). Neither sufficient nor insufficient dosage of ibandronate had significant effect on preventing non-vertebral fractures (sufficient: RR, 1.10, p = 0.47; insufficient, only hip fracture: RR, 0.59, p = 0.19; Table 3, Supplementary Material 3q and 3r).

**Minodronate**

Low quality evidence proved minodronate had significant effect in reducing secondary fracture (RR, 0.44; 95% CI, 0.31-0.63; p < 0.001; Fig. 9g, Table 3). Minodronate did not have a significant effect on preventing non-vertebral fractures (RR, 0.80, p = 0.60; Table 3, Supplementary Material 3s).

**Pamidronate**

Very low quality evidence indicated significantly lower risk of secondary fracture due to pamidronate (RR, 0.33; 95% CI, 0.13-0.84, p = 0.02; Fig. 9h, Table 3). Pamidronate did not have significant effect on preventing non-vertebral fractures (RR, 0.33, p = 0.33; Table 3, Supplementary Material 3t).

**Calcitonin**

Very low-quality evidence proved calcitonin had no significant effect on preventing secondary fracture (RR, 1.02; 95% CI, 0.14-7.36, p = 0.98) (Table 3).

**HRT**

Low quality evidence proved HRT had no significant effect on prevention of secondary vertebral or non-vertebral fracture (vertebral: RR, 0.88; p = 0.78; non-vertebral: RR, 0.37; p = 0.36. Table 3. Supplementary 3u). HRT did not significantly elevate the risk of discontinuation (RR, 0.53, 95% CI, 0.17-1.61, p = 0.26; Table 4, Supplementary Material 3v).

**Parathyroid (PTH)**

Moderate quality evidence proved that the administration of teriparatide 28.2 μg/week or 56.5 μg/week, recombinant human (rh)PTH 20 μg/day or rhPTH 40 μg/day could significantly reduce the risk of secondary fracture (Table 3). The synthesized RR was 0.32 (95% CI, 0.24-0.43; p < 0.0001) and the heterogeneity between different doses was insignificant (p = 0.62, Fig. 10a). The result of the sensitive analysis that excluded the trial had teriparatide 1.4 μg/week group as its control group[76] showed no significant change (RR, 0.33,

95% CI, 0.24-0.44, $p < 0.00001$). The risk of discontinuation due to medication was significantly raised by PTH administration (RR, 1.54; 95% CI, 1.11-2.13, $p < 0.009$; Supplementary Material 3w). Forty μg/day rhPTH significantly elevated the risk of discontinuation, while 20 μg/day or 56.5 μg/week did not, but no significant heterogeneity was observed between groups. PTH had significant effect on preventing non-vertebral fractures (RR, 0.53; 95% CI, 0.36-0.78; $p = 0.005$; Table 3, Supplementary Material 3x)

**Denosumab**

Moderate quality evidence proved that the administration of denosumab significantly reduced the risk of secondary fracture (RR, 0.41; 95% CI, 0.29-0.57; $p < 0.0001$; Fig. 10b, Table 3). No significant increase in discontinuation due to medication was observed (RR, 0.75, $p = 0.29$; Table 4, Supplementary Material 3y). Denosumab did not have a significant effect on preventing non-vertebral fractures (RR, 0.45, $p = 0.06$; Table 3, Supplementary Material 3z).

**SERMs**

Both raloxifene (RLX) and bazedoxifene (BZA) could significantly reduce risk of secondary fracture (RLX: RR, 0.58; 95% CI, 0.44-0.76, $p < 0.0001$; BZA: RR, 0.66; 95%CI, 0.53-0.82, $p = 0.0002$; Fig. 10c and d). Heterogeneity between 60 μg/day and 120 μg/day of RLX was significant and substantial (test for subgroup differences, $p = 0.06$, $I^2 = 72.1\%$; Fig. 10c). The effect of BZA was proved by moderate quality evidence and the effect of RLX was supported by high quality evidence (Table 3).

## Comparison between interventions

### Comparison between BPs

Moderate quality evidence proved no significant difference in the effects on preventing vertebral fracture between risedronate and etidronate (RR, 1.12; p = 0.66; Supplementary Material 4a). High-quality evidence proved no significant difference between ibandronate and risedronate (RR, 1.01; p = 0.91; Supplementary Material 4b, Table 3).

### Hormone therapy vs. BPs

Very low-quality evidence indicated no significant difference between HRT and etidronate (RR, 0.63; p = 0.59; Supplementary Material 4c). Moderate quality evidence indicated teriparatide (20 μg/week) showed a significantly superior effect on preventing vertebral fracture and non-vertebral fracture than risedronate (vertebral fracture: RR, 1.98; 95% CI, 1.44-2.7, p < 0.0001, Supplementary Material 4d), without significantly increasing ratio of discontinuation (RR, 0.75, p = 0.05; Table 4, Supplementary Material 3aa). No significant difference in the effects of non-vertebral fracture was observed (Supplementary Material 3bb).

### Monoclonal antibody medication vs. BPs

Low-quality evidence proved the difference between the effects of alendronate and denosumab on preventing vertebral fracture was not significant (RR, 0.69; p = 0.17; Supplementary Material 4e). Moderate quality evidence proved romosozumab had a significantly better effect on preventing secondary vertebral fracture than alendronate (RR, 0.64, p = 0.001; Supplementary Material 4f).

Difference between the effects of alendronate and denosumab on preventing non-vertebral fracture was not statistically different (RR, 1.49; p = 0.46; Supplementary 3cc), neither was between romosozumab and alendronate (RR, 0.74; p = 0.05; Supplementary 3dd).

## Higher DRG2 expression correlates with lower BMD

We firstly compared the expression level of *DRG2* in bone marrow stem cells and BMD between osteoporosis and non-osteoporosis subjects. Six osteoporosis subjects and seven non-osteoporosis subjects were recruited. The osteoporosis group had a significantly higher age, lower BMD and T-score in lumbar and femur and markedly higher expression level of *DRG2* than the non-osteoporosis group. (Fig. 11, Fig. 12)

## Knocking out of Drg2 affects mice postnatal bone formation

To study the biological function of *Drg2* in vivo, *Drg2* knockout founder mice with C57BL/6N background was made at the Korean Research Institute of Bioscience and Biotechnology (KRIBB) with CRISPR/Cas9 technique.(Fig. 13) Three mutant founders were fabricated, since #2 founder (49 dp deletion in exon3) and #7 founder (31 dp deletion in exon 3) were sterile, #4 founder with 48 dp deletion in exon 3 were crossed with C57BL/6N mice in this study.(Fig. 14) No newborn mouse was viable in the mating between homozygous

knockout (KO) mice and therefore, heterozygous mice were crossed for homozygous mice.

On P1, KO newborn mice had a phenotypically normal skeleton compared with that of WT mice.(Fig. 15) On the 4th week, body length and weight of KO mice were both significantly smaller than those of WT mice in both genders.(Fig. 16) Staining results indicated that male KO mouse had a smaller but phenotypically normal skeletal structure in calvaria, mandibular, forelimb, and hindlimb.(Fig. 17) On the 8th week, serum P1NP level was significantly higher in the KO group than that in WT. Serum level of CTX was lower in the KO group without showing statistical significance. (Fig. 18) In the trabecula of vertebra and femur head, KO mice showed longer mineralization surface and internal distance in their 8th week.(Fig. 19)

## Inhibition of DRG2 improves bone architecture and BMD even in ovariectomized mice

To examine whether knocking out of DRG2 could recover bone loss caused by postmenopausal, we sham-operated or ovariectomized 8 weeks old female mice and analyzed their bone histomorphometric of the vertebra and proximal femur in their 24th week. In the sham-operated group, KO mice had significantly higher percent bone volume (BV/TV), trabecular bone number (Tb.N) and bone mineral density (BMD) and significantly lower trabecular bone thickness (Tb.Th) and trabecular bone separation (Tb.Sp). Similarly, in the OVX group, the KO group had higher bone formation related to

histomorphometric parameters than WT group. Additionally, no significant difference was observed in BV/TV or BMD between the WT-sham and KO-OVX groups. These results indicated KO mice had more bone mass and bone mineral content with a relatively stronger structure in vertebra than WT mice did.(Fig.20-24)

In the femur head, the significant bone architecture change and bone mass decrease caused by OVX were not evidently affected by knocking out of the gene.(Fig. 25-27) However, in the femur neck region, KO-sham mice showed significantly lower bone volume but significantly higher bone thickness, outer.Pm/inner.Pm ratio and BMD compared to WT-sham mice. The same phenomena were also observed in OVX mice. When the WT-sham group and KO-OVX group were compared, Tb.Th, and outer.Pm/inner.Pm ratio were comparable between the two groups. Additionally, the BMD in the KO-OVX group was significantly higher than that in the WT-sham group.(Fig. 28, Fig. 29). In the proximal femur region, the BMD of KO mice was significantly higher than that of WT mice in both sham and OVX groups.(Fig. 30)

Combining these results, bone mass and architecture were impaired by OVX in both WT and KO mice. But the bone loss was compensated, at least partially, by knocking out of *Drg2* in vertebra and femur neck.

## Results of the GO enrichment and KEGG pathway analysis

To further predict the function and mechanisms related, functional and pathway enrichment analyses, including GO and KEGG, were performed using Affymetrix® Expression Console™ Software. The top 10 significant terms in biological processes, molecular function, and cellular component, and the top 20 terms in enrichment test were listed Fig. 31.

## Inhibiting the expression of DRG2 inhibits the osteoclastic differentiation of BMMCs and elevates osteoblastic differentiation of bone marrow MSCs

The previous study presented overexpression of the *DRG2* in mice elevated the osteoclastic differentiation of BMMCs.[40] Therefore, we evaluated whether the knockout of the gene could affect the osteoclastic differentiation of BMMCs. Consistent with the previous study, the BMMCs from *Drg2* mice showed lower differentiation capacity than the WT mice. After treating with RANKL for 7 days, the WT BMMCs showed several large and multinucleated osteoclast indicated sufficient osteoclastic differentiation. (Fig. 32) But no large OCs was observed in the KO BMMCs group and the number of OCs was also less than that in WT group. Also, the real-time PCR (RT-PCR) results showed the WT group had higher transcription levels of NFATc1 and CathepsinK than the KO group. (Fig. 33)

In the osteoblastic lineage, the MSC in KO group showed obviously denser ALP staining than that in WT group from the 3rd day after osteoinduction. (Fig. 34) Also, the MSC in the KO group began to show mineral deposition on the 7th day after induction with osteoinductive medium (OM), while the WT-MSC showed mineral deposits from the 14th day. On both 14th and 21st day, the KO-MSC showed obvious calcium stain than the WT-MSC.(Fig. 35) In the ALP and calcium assay, KO-MSC began to show significantly higher ALP activity from the 7th day after induction, and significantly higher calcium level from the 14th day after induction. (Fig. 36) The real-time PCR result indicated higher transcription levels of Runx2, Ocn, Col1, Bsp and Alp in KO-MSC 14 days after treating with OM. (Fig. 37)

## Inhibiting the expression of DRG2 elevates osteogenicity of MC3T3-E1 cells

To furtherly investigate the function of *Drg2* in osteoblastic differentiation, we inhibited the expression of *Drg2* in mouse preosteoblast MC3T3-E1 cells with shRNA.(Fig. 38) Functionally, shRNA-*Drg2* E1 cells showed denser ALP staining than control E1 cells after inducing with OM for 7 d, 14 d, and 21 d.(Fig. 39). Alizarin red staining results also showed more evidently mineral deposit compared with control E1 cells after treatment with OM for 14 d and 21 d. (Fig. 40). The result of ALP and calcium assay also showed higher levels in the shRNA-*Drg2* E1 cell group than the control E1 cells when both cells were induced with the osteoinductive medium for 14 and 21 days. (Fig. 41)

Expression levels of osteoblast phenotypic marker genes, including bone sialoprotein (*Bsp*), collagen 1 (*Col1*) osteopontin (*Opn*) and osteocalcin (*Ocn*) were markedly elevated after induced by OM. The shRNA-*Drg2* E1 cell group had significantly higher expression levels in *Bsp*, *Col1*, and *Alp* compared with control E1 cell group while both were treated with OM. (Fig. 42)

## Inhibition of DRG2 elevates OB differentiation via canonical and non-canonical BMP signaling

To gain insight into the mechanism of Drg2 regulating osteoblast differentiation, we screened major transcription factors involved in osteoblast differentiation.[88] Among candidate transcription factors, expression levels of osterix (*Osx*), runt-related transcription factor (*Runx2*), distal-less homeobox 5 (*Dlx5*) and special AT-rich sequence binding protein 2 (*Satb2*) were all higher in shRNA E1 cells. (Fig. 43)

Since an increase of *Runx2* expression could be caused by an activated BMP signaling, we furtherly investigated the changes in Smad-dependent canonical signaling and Smad-independent non-canonical signaling.[89, 90] Real-time PCR results showed that shRNA-*Drg2* E1 cells already had a significant increase in expression of *Smad1/5/8/4/6* before they were treated with OM. When osteoblastic differentiation was induced, shRNA-*Drg2* E1 cells showed significantly higher expression levels of *Smad1/8/4/6*.(Fig. 44)

In non-canonical signaling, when cells were treated with basic medium, expression levels of *p38-alpha*, *ERK1*, and *ERK2* were already elevated with inhibition of *Drg2*. After cells were treated with OM, expression levels of *p38-alpha*, *ERK1*, and *ERK2* were significantly higher in shRNA-*Drg2* cells on the 7$^{th}$ and the 14$^{th}$ day.(Fig. 45) These results indicated that *Drg2* influenced osteoblast differentiation via canonical and non-canonical BMP pathway.

The western blot results showed a thicker band of p-Smad1/5 in shRNA-*Drg2* E1 cells after treated with OM. The level of p-Smad1 in shRNA-*Drg2* E1 cells was lower on the 3$^{rd}$ day but was higher on the 7$^{th}$ and 14$^{th}$ day after induced. After treated with OM, the level of p-p38 was slightly lower in shRNA-*Drg2* E1 cells in the 3$^{rd}$ day but became higher in both 7$^{th}$ and 14$^{th}$ day.(Fig. 46)

# Discussion

Osteoporosis often accompanies with iatrogenic instability and fracture following surgery, which remains a clinical challenge for the spine surgeon. Multidisciplinary approaches are encouraged and the preoperative plan is critical for obtaining successful fusion.[2] Since osteoporosis is caused by negative bone remodeling, antiresorptive and anabolic agents have been developed to promote bone formation. As a widely used growth factor that promotes osteogenesis, the effect of BMP-2 was less investigated that was mainly due to the lacking data of the use of BMPs in osteoporotic patients.[1] The theoretical advantages of using BMPs include the potential of inducing a rapid increase in bone strength and bone. Several preclinical studies have investigated the effect of BMPs in osteoporotic animal models [3] and Park et al. have presented early spinal fusion in the rat OVX osteoporosis model with BMP-2 treatment.[91] Despite the theoretical but potential benefits from BMP-2, using rhBMP-2 comes with a high initial price that impedes its use in the clinic. Between the widely used rhBMP-2, ErhBMP-2 could be produced in large scale at a lower cost than CrhBMP-2 but had relatively less osteoinductivity than the latter one.[6] In the study, the authors reported more fatty marrow and cyst-like bone in ErhBMP-2.[6] Therefore, in the first part, of this series study, we compared whether using DBM as a carrier could elevate osteoinductivity of ErhBMP-2. [13, 92] Also, in reviewing current medications of osteoporosis, we noticed most of the studies focused on the primary prevention effect of the medications. Therefore, in the second part we furtherly

reviewed the effect of the medications on preventing secondary osteoporotic fracture. Recently, a sclerostin inhibitor, romosozumab had been reported to have a significant effect on treating osteoporosis via both anti-resorptive and anabolic function and indicated a great potential of new medications development.[93] Overexpression of DRG2 was reported to increase osteoclastogenesis and also regulate the activity of MSC, it indicated the potential of DRG2 in both osteoclastogenesis and osteoblastogenesis. But the effect of DRG2 on osteoblastic differentiation was less investigated. Therefore, in the third part of this study, we investigate the effect of knocking out of DRG2 in bone remodeling.

## The first section

In vitro results from this study showed CrhBMP-2 had slightly higher osteoinductivity than ErhBMP-2 at 100 ng/ml and 200 ng/ml doses and had similar osteoinductivity at the dose of 500 ng/ml which was consistent with results from previous studies.[8, 94, 95] Additionally, the difference between two rhBMP-2 in ALP and Alizarin red staining was not significant. Therefore, the ErhBMP-2 might have slightly lower osteoinductivity that was related to its non-glycosylation structure, but it might be not critical for inducing bone formation.[8]

Animal experiment results showed ErhBMP-2 induced significantly higher bone formation on both $4^{th}$ week and $8^{th}$ week. On the $4^{th}$ week, the newly formed bone in ErhBMP-2 had significantly higher BV/TV, higher coverage

ratio, less surface complexity, and narrower bone separation space. On the 8th week, the difference became more obvious. The significant advantage of ErhBMP-2/DBM composites not only showed in the main parameters like BV/TV, Tb.N, Tb.Sp, and coverage ratio, also in Tb.Pf, BS/BV and DA. The parameters consistently indicated the bone in ErhBMP-2 group had significantly more volume, number, continuity, and isotropic, and less separation and surface complexity, and therefore, the data could be interpreted as the bone in ErhBMP-2 had the advantage in both volume and quality. Previous studies have shown ErhBMP-2 had compatible osteoinductivity with CrhBMP-2 in rat calvarial defect model,[8] dog supraalveolar peri-implant defect,[7] and ectopic ossification model.[6, 8, 94]

Combined the results from cell and animal experiments, it might be appropriate to believe the CrhBMP-2 had advantage at lower doses in the cell level, but the advantage became insignificant at a dose that was sufficient for inducing bone formation in vivo. Also, though the bone structure is different in ErhBMP-2 group due to lacking heparin-binding sites, the total efficiency might not be affected.

The animal experiment results indicated the newly formed bone in CrhBMP-2 on the 8th week was lower than that on the 4th week. The time-dependent bone decrease was reported to be related to bone maturation or the BMP-2 carrier.[96, 97] But in this study, it might be more attributed to the quality of the product we used. As the effect of rhBMP-2 was affected by various factors, the result might be different from other CrhBMP-2 products.[98] Therefore, it might be

appropriate to believe ErhBMP-2 have compatible osteoinductivity with CrhBMP-2 while loaded with DBM.

In the histologic sections, ErhBMP-2 induced relatively more adipose tissue and cyst-like bone than CrhBMP-2. It was reported to be related with lack of heparin-binding sites in ErhBMP-2,[6] and heparin-conjugated carrier system was expected to reduce the fatty tissue formation and increase the density of newly formed bone.[9] The system reduced fatty tissue formation while loaded with low concentration ErhBMP-2 but did not elevate the new bone formation in mouse calvaria defect.

Previous studies have reported other carriers for delivering ErhBMP-2, including hydroxyapatite,[99] biphasic calcium phosphate,[100] collagenated biphasic calcium phosphate,[101] and absorbable collagen sponge.[6-8] However, the efficacy of the ErhBMP and CrhBMP were only compared with collagen as a carrier. In this study, we proved ErhBMP-2 might have compatible osteoinductivity with CrhBMP-2 while carried by DBM.

One limitation in this study is that the DBM itself might induce bone regeneration that could interference the comparison between two kinds of rhBMP-2. Also, since the DBM comes in a powder form that lacks the mechanical strength, it was not an ideal scaffold that could be used standalone for all bone defects.

In this study, we showed ErhBMP-2 had slightly lower osteoinductivity than CrhBMP-2 in vitro at lower doses but could induce more bone formation in rat

calvarial defect model. Therefore, ErhBMP-2 might have non-inferior osteoinductivity with CrhBMP-2 while both were carried with DBM.

## The second section

In this study, we focused on osteoporosis patients with a history of OVCF. We collected related RCTs, synthesized their results, and finally estimated the secondary prevention effects of the medications on OVCF. We found zoledronic acid, alendronate, risedronate, etidronate, ibandronate, minodronate, pamidronate, PTH, denosumab, romosozumab, and SERMs had significant secondary prevention effect on OVCF. In the comparisons between the medications, teriparatide had a significantly superior effect to risedronate, and the quality of evidence was high. The effects of risedronate, ibandronate, PTH, and SERMs were supported by moderate quality evidence and the effects of alendronate, denosumab was supported by high-quality evidence.

In the result of discontinuation due to adverse events, PTH was the only intervention that significantly elevated the ratio. None of the bisphosphonates increased the risk of GI complaints. Zoledronic acid, risedronate, and PTH had a significant effect on preventing non-vertebral fracture in patients with prevalent OVCF.

Most of widely used BPs, include zoledronic, alendronate, risedronate, etidronate, and ibandronate, had a significant effect, which were supported by moderate quality evidences. Among the medications, risedronate and ALN are first line osteoporosis medications, whose effects have been proved by

substantial evidence [24, 26]. Ibandronate is a nitrogen-containing BPs and IV injection of it allows for a dosing interval even longer than 2 months [102]. Zoledronic acid is another nitrogen-containing BPs that has the highest potency among clinical use BPs [103]. According to our result, 5mg/year iv injection of zoledronate could significantly reduce the risk of secondary OVCF. The extremely low medication frequency could be its another advantage that might improve patients' compliance rate. Significantly elevated adverse events ratio or rare adverse events caused by BPs (e.g. osteonecrosis of the jaw or atypical fracture, etc.) was not reported in any trial. Insignificant difference in GI complaints between BPs and control group indicated properly administrated BPs might help avoiding the risk of GI complaints, which was consistent with previous studies [104].

PTH is a bone anabolic medication that has significant efficacy against OVCF [105]. Presently, moderate quality evidence proved that the injection of PTH or teriparatide significantly reduces the risk of secondary OVCF. Even the lowest dosage (28.2 μg/week) showed a significant effect. However, treatment was also associated with a series of adverse events that increased the risk of discontinuation, which was unique to the interventions included in our study. The most frequent adverse event was nausea. Other complaints included vomiting, headache, dizziness, and leg cramps.[74, 77] Nonetheless, teriparatide had a significantly better effect than risedronate and was the only medication that showed significant superiority compared to the others in this study.

SERMs included in the study were raloxifene and bazedoxifene. Both showed a significant effect in preventing secondary fracture. Raloxifene seemed to have a better effect when prescribed at a higher dosage, which was indicated by the significant and substantial heterogeneity between the two groups. Besides beneficial skeletal effects, SERMs reduce the risk of breast cancer [106]. However, an elevated risk of venous thromboembolic events due to raloxifene and bazedoxifene has been described [82]. Additionally, raloxifene significantly raises the risk of discontinuation.[80] Therefore, SERMs should be prescribed with an awareness of their risk of side effects.

Denosumab is a RANKL inhibitor that was proved to possess a significant effect on preventing secondary OVCF. Side effects of it include skin rashes, infections, and osteonecrosis of the jaw,[105] but presently, there was no significant difference in adverse events compared with the control group. Additionally, Boonen et al. reported a significant reduction of fatal adverse events ratio with denosumab in patients with prevalent vertebral fracture.[79] One advantage of denosumab is its low dosing frequency, which might elevate compliance. Romosozumab is a sclerostin inhibitor that has been proved to have a better effect on preventing secondary OVCF than alendronate. However, it should be noticed that the cardiac ischemic events and cerebrovascular events ratio were higher in the romosozumab group. The role of sclerostin in vessels remains unclear, and the results from basic studies were controversial.[107-109] Therefore, further evaluation of the safety profile of romosozumab is needed.

Unlike the superior effects on OVCF of most medications, only zoledronic acid, risedronate, and PTH had a significant effect on preventing non-vertebral fractures in patients with prevalent OVCF. Combined with the effects of medications on OVCF, the findings might indicate zoledronic acid, risedronate, and PTH is better options for patients with prevalent OVCF. Additionally, denosumab and alendronate showed marginally significant effects. The results might have less credibility than the main outcome because of missed information concerning the non-vertebral fracture status of the participants. But, the patients included in this study could still be considered as having a high risk of non-vertebral fracture because prevalent vertebral fracture and low bone mineral density are potential risk factors of non-vertebral fractures.[110, 111] Therefore, the data might be instructive for clinical usage of the medications.

It must be noted that many phase 3 studies were excluded from this meta-analysis because the data of patients with prevalent fractures were not reported. The exclusion might cause an underestimation of the effects of some newly developed medications like denosumab and zoledronic acid. Other limitations of this study include the absence of searching the gray literature, which might increase the risk of publication bias that might lead to an overestimation of the effect of newly developed medications like romosozumab and bazedoxifene. Additionally, our criteria for assessing the risk of bias might be too stringent, which might underestimate the quality of evidence. The generalizability of results of GI complaints was limited because most of the trials excluded patients with upper GI disease at baseline.

Most systematic reviews and meta-analyses included osteoporosis patients, regardless of their fracture history that introduces indirectness in the results.[24-29] The results might be overestimated on patients had fracture history, and for optimized treatment, accurate analyses of OVCF patients are urged. However, only one systematic review satisfied the demand.[112] Compared with that, we included 12 more RCTs and a new medicine (romosozumab) that allowed for a more comprehensive review and allowed comparisons between different medications. Also, our results included vertebral fracture, non-vertebral fracture, GI complaints of BPs and discontinuation due to AEs. In the end, we evaluated the quality of evidence. The updated information could offer more practical evidence for clinical use.

Our results are consistent with those from other systematic reviews about primary prevention of OVCF[24-26, 28, 33, 113, 114]. This could indicate that the medications have a consistent effect on osteoporosis patients, regardless of their OVCF history. Also, medications used to prevent osteoporotic fracture had a low risk of severe adverse events in most of the 2-3 years follow-ups. Therefore, the benefits of reducing the risk of fracture, disability, and mortality very likely outweigh the disadvantages. But, careful evaluation of risk factors and arrangement of drug holidays are also necessary to minimize the risk of adverse events.[115]

Lack of RCTs that compared interventions of secondary prevention effect limited our assessment of differences between interventions. Although indirect

comparisons could be conducted through statistical analyses, high-quality RCTs that provide direct evidence are necessary for a solid conclusion.

## The third section

GTP binding proteins have been found in all living organisms thus far, and play crucial roles in the regulation of fundamental cellular processes. Small monomeric GTP-binding proteins are involved in a number of essential processes, like signal transduction, protein synthesis and translocation, and cell cycle regulation.[116, 117] According to the classification of small GTP-binding protein, a subfamily of proteins named DRG has been distinguished. Homologous DRG proteins have been identified in species including *Xenopus, Drosophila, Caenorhabditis elegans,* fission yeast, and halobacterium. All the proteins harbor the five characteristic motifs G1-G5 that are believed to interact with GTP. But the exact function of the DRGs is not yet known, but their striking conservation throughout the major kingdoms suggests an essential role in a fundamental pathway.[118] DRG2 regulates cell proliferation via regulates cell cycle proteins that involve p21 and Cdk1.[119] Also, DRG2 is a direct target of miR-1915-3p, and furtherly regulates cell apoptosis.[120] In regulating cell activity, DRG2 is associated with phosphatidylinositol 3-phosphate–containing endosomes and depletion of DRG2 impaired the interaction between Rab5 and Rab-GAP5, Rab5 deactivation on endosomes and Tfn recycling.[121] Depletion of DRG2 significantly decreased the level of Drp1 and induced mitochondrial swelling,[122] and also impaired the stability

of membrane tubules.[123] In a previous study, overexpression of *Drg2* regulates the ratio of RANKL/OPG expressed by bone marrow stromal cells/osteoblast.[40] This indicates that *Drg2* regulates biological function of the osteoblastic lineage. In this study, we identified *Drg2* in osteoblast as an inhibitor of bone formation and a potential therapeutic target for osteoporosis. Osteoporosis subjects were found to have a higher expression level of *DRG2* than non-osteoporosis subjects. *Drg2* knockout mice showed higher BMD and stronger bone architecture than WT mice under both biological and OVX situation. Surprisingly, inhibiting *Drg2* simultaneously suppressed osteoclastogenesis and elevated osteoblastogenesis. The increased osteoblastogenesis was found to be related with both canonical and non-canonical BMP pathways.

BMP signaling is one of the major signal pathways that regulate osteoblastic differentiation, which had been reported to have crosstalk with endosome.[124, 125] Since *DRG2* localizes at the endosome where it interacts with Rab5 and regulates its activity,[121] it is rational to assume that *Drg2* affects the activity of endosome and further regulates the osteogenicity via both canonical and non-canonical BMP pathways.

In this study, we revealed the direct effects of *Drg2* on osteoblast and osteoclast, but it should be noticed that the genetic modified animals used were general knockout animal, therefore the possible changes in other organs could play a role in regulating the bone homeostasis.[126] Therefore, a conditional knockout technique (e.g., Cre-lox recombination system) could be used to

illuminate the specific efficacy of *Drg2* in local regulation of bone remodeling.[127, 128] Also, the function of *Drg2* in other organs should be further investigated before it could be used as an agent. Another point should be noticed is the significantly different age of subjects between non-osteoporosis and osteoporosis groups recruited from the clinic. Though it did not alter the observation that *Drg2* directly regulated osteogenicity of preosteoblast, the relation between senescence and DRG2 still should be investigated.

One limitation in this study is the in-frame deletion mutation mice we used. Because of the sterility of the frameshift mutation mice, only the in-frame deletion subjects were available in this study. The sterility of the mice indicated a vital role of *Drg2* in the reproductive system, which should be furtherly investigated. Also, in current stage of the study, the histomorphometric analysis was performed via calcein labeling and micro-CT analysis, the quantifying of active OB and OC in histology sections was missed due to the expenditure and limited number of available samples. In further studies, the histological analysis via various staining technology, such as Van Kossa stain, TRAP staining should be performed for a direct measurement of the biological activity of the OB and OC. Additionally, monoclonal antibody of DRG2 could be furtherly developed for further study of the biologic function of the gene.

With the progressive aging of the population, osteoporosis is becoming a clinical and public health concern.[39] Various osteoporosis medications have been developed. They can be classified as antiresorptive and anabolic

agents.[129] Antiresorptive medications increase BMD via suppressing bone turnover [130, 131] but the impaired bone structure might not be restored with antiresorptive agents.[31] Since bone strength depends on bone microstructure like bone connectivity and architecture, a mere increase in BMD might not always reduce fracture risk.[132] Therefore, mere antiresorptive agents might not be an optimal strategy for treating osteoporosis. Among the anabolic medications, PTH has been proved to be effective monotherapy for treating osteoporosis, but neither teriparatide or intact PTH showed to be able to reduce hip fracture risk.[39] Compared with the medications, inhibiting *Drg2* could suppress osteoclast activity and promote osteogenesis simultaneously, which showed a great potential of Drg2 inhibitor as an osteoporosis therapy candidate. This character of *Drg2* finally resulted in significantly elevated BV/TV, Tb.N, and BMD and lower Tb.Sp in vertebrae. Additionally, among current medications, only potent antiresorptive medications such as alendronate, risedronate, zoledronate and denosumab can reduce the risk of hip fracture.[39] Since inhibiting *Drg2* showed significantly more bone formation in both vertebra and femur, the *Drg2* inhibitor could be a potent osteoporosis medication for preventing both vertebra and femur osteoporotic fracture. Therefore, it is rational to estimate *DRG2* inhibitor could be an osteoporosis medication with both antiresorptive and anabolic effects.

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# Figures

Fig. 1. ALP assay result. At the 3rd day, the absorbance of CrhBMP-2 250 ng/ml was significantly but mildly higher than that of ErhBMP-2 250 ng/ml. At the 7th day, CrhBMP-2 showed significantly lower ALP activity at 10 ng/ml than ErhBMP-2 but significantly higher ALP activity at 100 ng/ml, 250 ng/ml and 500 ng/ml. At the 14th day, CrhBMP-2 had significantly higher ALP activity at the dose of 100 ng/ml and 250 ng/ml. The experiment was biologically repeated 5 times, and the data was presented as mean and standard deviation. *, $p < 0.05$.

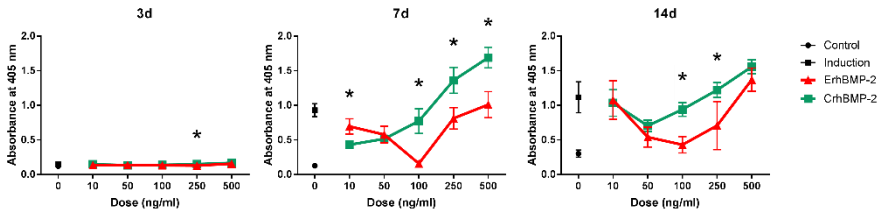


Fig. 2. ALP staining result. No significant difference was observed at the 3rd day. At the 7th day, BMP-2 treated groups showed a dose dependent increase in stain density. In the 10 ng/ml group, ErhBMP-2 and CrhBMP-2 showed similar stain level, and in other groups, CrhBMP-2 had more intense color than ErhBMP-2. At the 14th day, all CrhBMP-2 groups showed slightly higher ALP activity except the 10 ng/ml group.

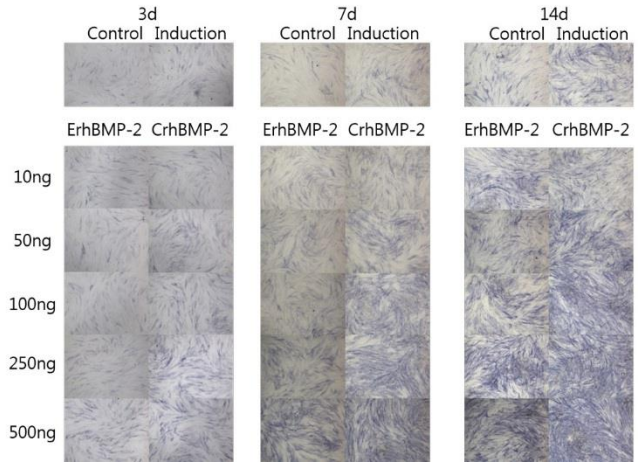


Fig. 3. Calcium assay result. Accumulation of calcium was only detectable in 500 ng/ml CrhBMP-2 group at the 7th day, and it was significantly higher than that of ErhBMP-2 500 ng/ml group. At the 10th day, CrhBMP-2 had more calcium than ErhBMP-2 at doses of 50 ng/ml, 100 ng/ml, 250 ng/ml and 500 ng/ml, the difference was statistically significant at the dose of 250 ng/ml. At the 14th day, CrhBMP-2 showed higher calcium level than ErhBMP-2 at all doses except 10 ng/ml, and the differences were statistically significant at doses of 50 ng/ml, 250 ng/ml and 500 ng/ml. The experiment was biologically repeated 5 times, and the data was presented as mean and standard deviation. *, $p < 0.05$.

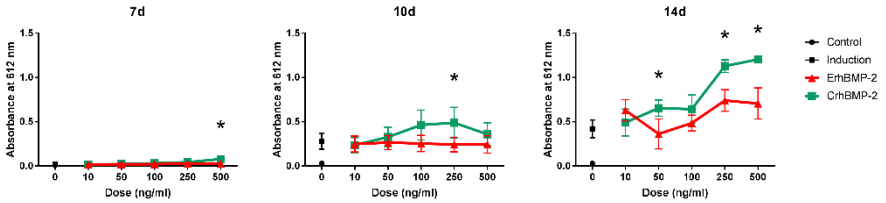


Fig. 4. Alizarin red staining result. Calcium deposit could be observed in 250 ng/ml and 500 ng/ml CrhBMP-2 group at the 10th day. At the 14th day, mineral deposit could be observed in all groups, but no significant difference was observed between two kinds of rhBMP-2.

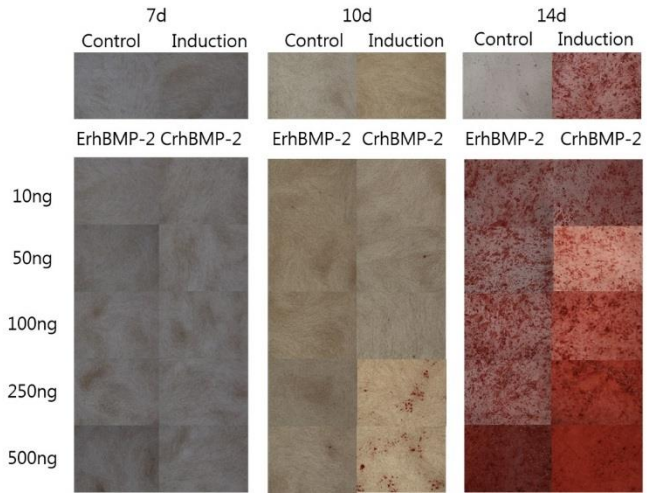


Fig. 5. Real-time PCR result. The expression levels of *ALP* were significantly higher in CrhBMP-2 than that in ErhBMP-2 groups at doses of 250 ng/ml and 500 ng/ml. The expression levels of *BSP* were significantly higher in CrhBMP-2 groups than ErhBMP-2 groups at doses of 100 ng/ml and 250 ng/ml. The expression level of *OCN* was significantly higher in CrhBMP-2 at the dose of 100ng/ml. The expression levels of *OPN* was significantly higher in the ErhBMP-2 at the dose of 500 ng/ml. The expression levels of *RUNX2* were higher in CrhBMP-2 at all doses, but no statistical significance was observed. The experiment was biologically repeated 3 times, and the data was presented as mean and standard deviation. *, $p < 0.05$.

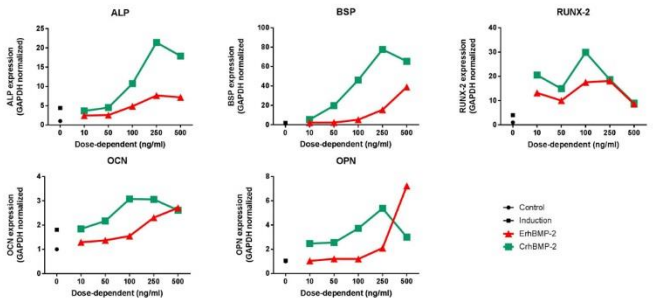


Fig. 6. Micro-CT images of newly formed bone. The coverage ratio of newly formed bone in ErhBMP-2 group was higher than that in CrhBMP-2 group at both time points.

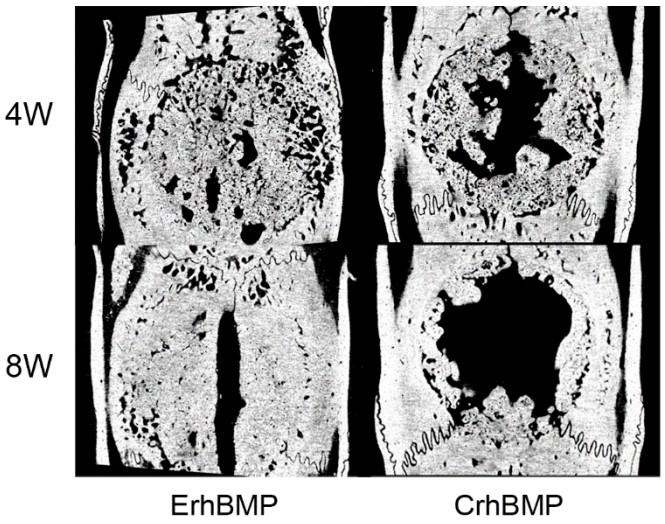


Fig. 7. Histology sections of newly formed bone. The bridging of new bone was only observed in ErhBMP-2 group. More adipose tissue and cyst-like structure bone was observed in ErhBMP-2 group.

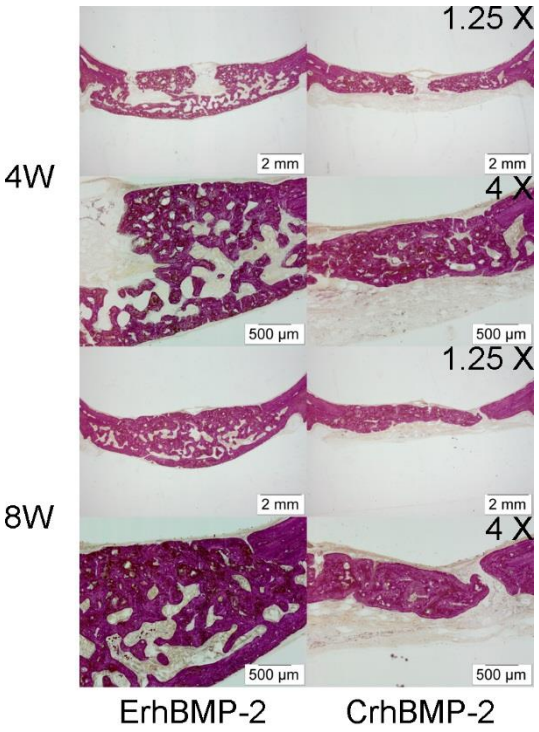


Fig. 8. Flow chart of selected studies.

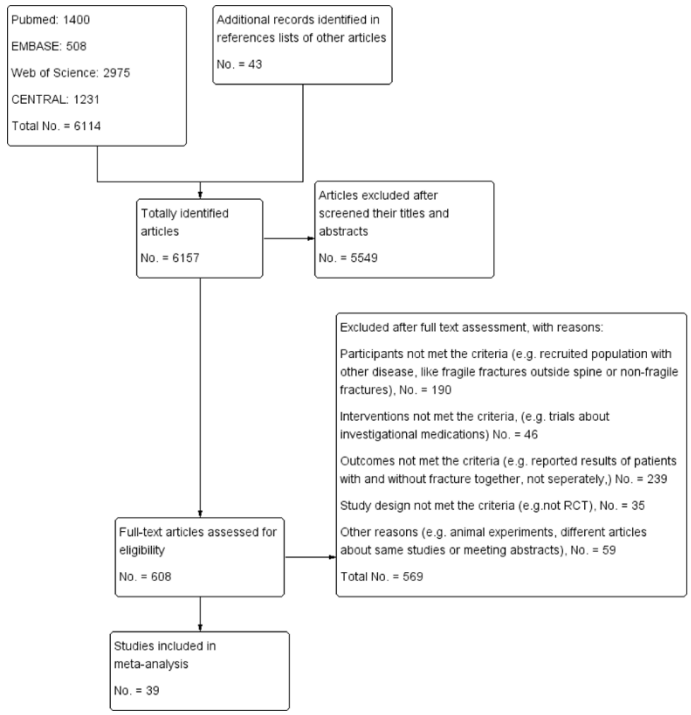


Fig. 9. Forest plot of the secondary prevention effects of bisphosphonates. a, Zoledronate; b, Alendronate; c, Risedronate; d, Etidronate; e, Ibandronate (sufficient dose); f, Ibandronate (insufficient dose); g, Minodronate; h, Pamidronate.

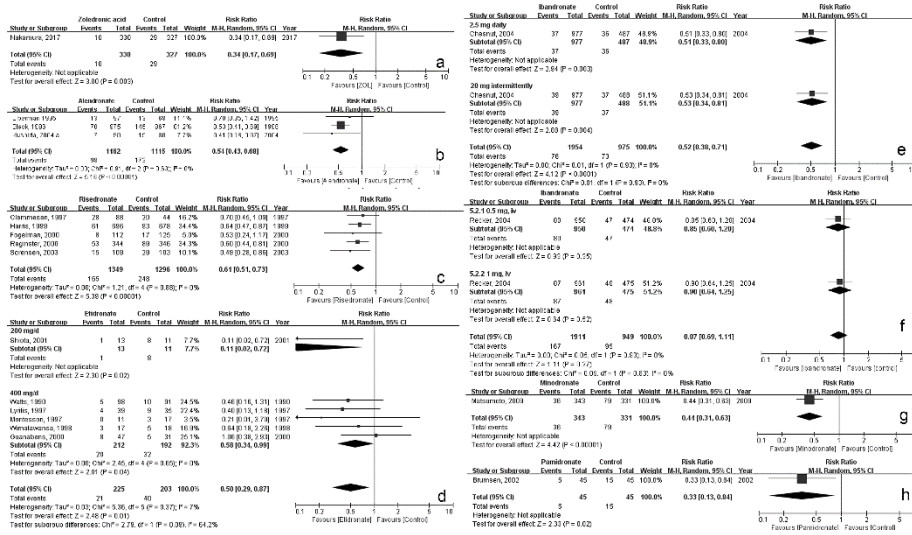


Fig. 10. Forest plot of the secondary prevention effects of: a, Parathyroid; b, Denosumab; c, Raloxifene; and d, Bazedoxifene.

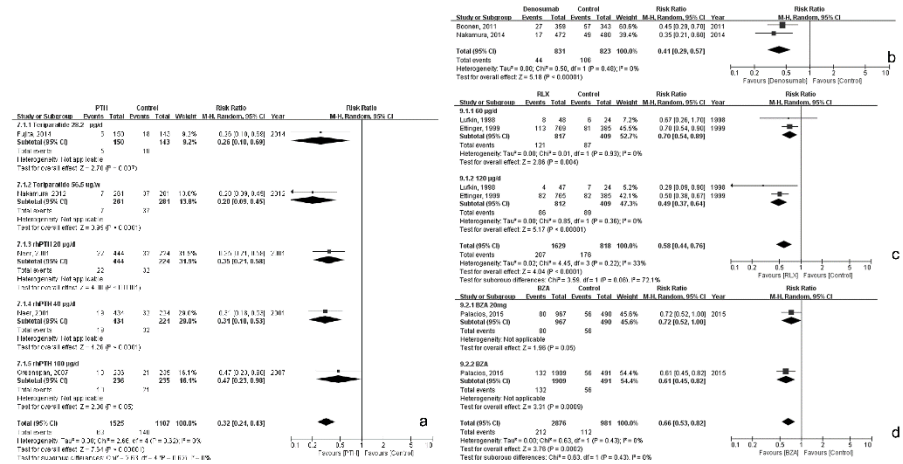


Fig. 11. Age, bone mineral density (DBM) and T-score of recruited osteoporosis subjects and non-osteoporosis subjects.

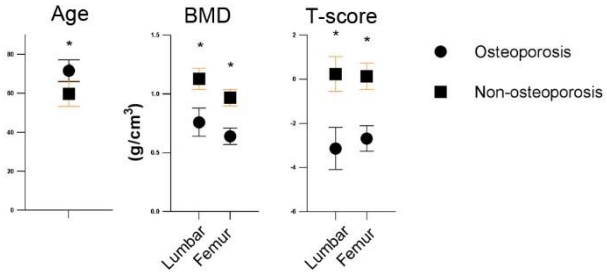



Fig. 12. Expression level of DRG2 in osteoporosis and non-osteoporosis subjects.

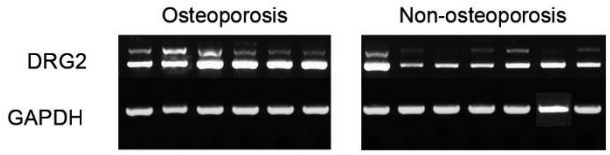



Fig. 13. Knocking out Drg2 with sgRNA and genomic typing of wild type (WT) and knockout (KO) mice.

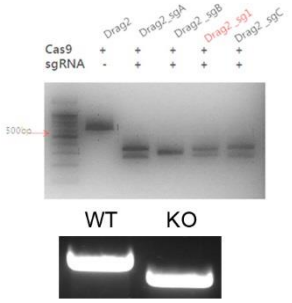


Fig. 14. Schematic diagram of experiment design of transgenic mice.

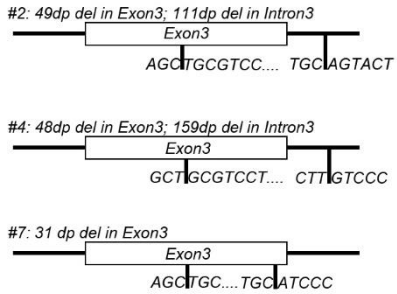



Fig. 15. Alizarin red and alcian blue staining of WT and KO newborn on their P1.


Fig. 16. Length and weight of KO and WT mice on P28. n = 12 for each gender and genotype.

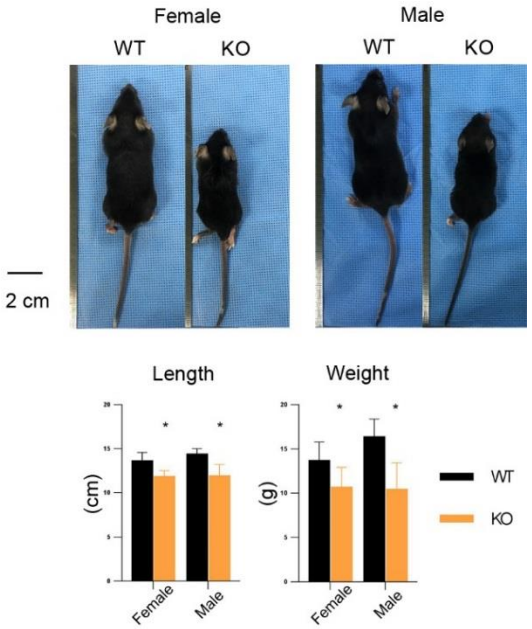


Fig. 17. Alizarin red and alcian blue staining of WT and KO mouse on their P28

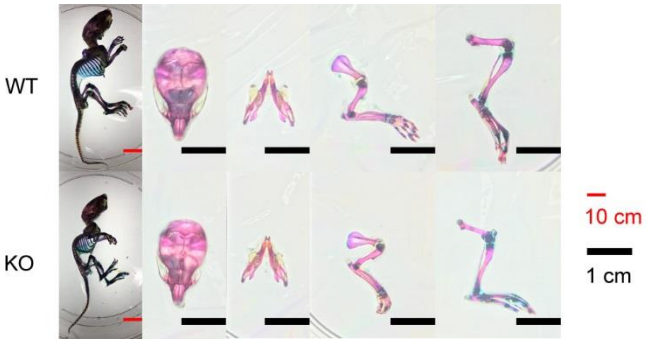



Fig. 18. Serum level of total procollagen type 1 N-terminal propeptide (P1NP) and C-terminal telopeptides type 1 collagen (CTX). n = 6. *, $p < 0.05$.

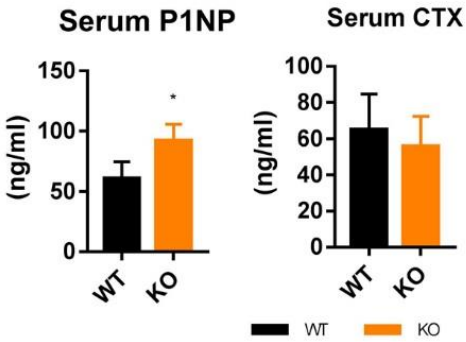


Fig. 19. Mineralization surface and internal distance of vertebra and femur head ( n = 3 images/mouse).

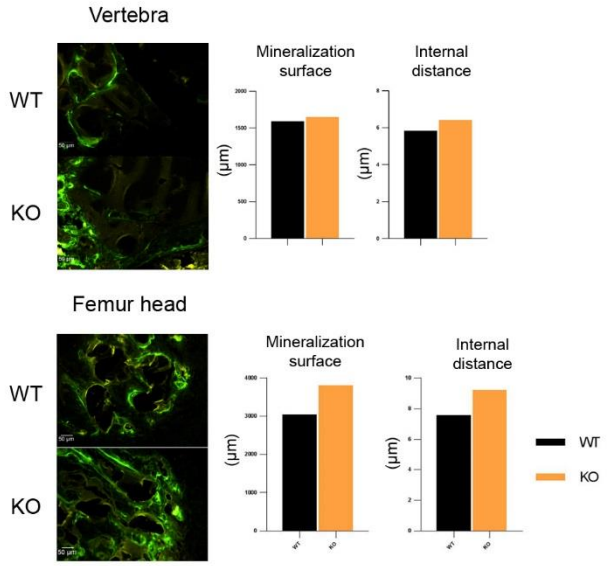



Fig. 20. Micro-CT result of vertebra. n = 25 vertebra samples in WT-sham group, n = 30 vertebra samples each group in WT-OVX, KO-sham and KO-OVX group. *, $p < 0.05$

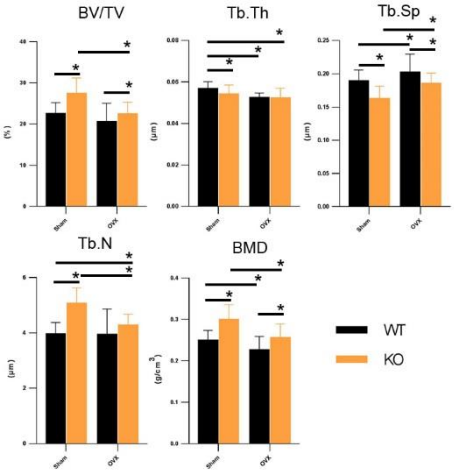


Fig. 21. Hematoxylin and eosin (H&E) staining of vertebra.

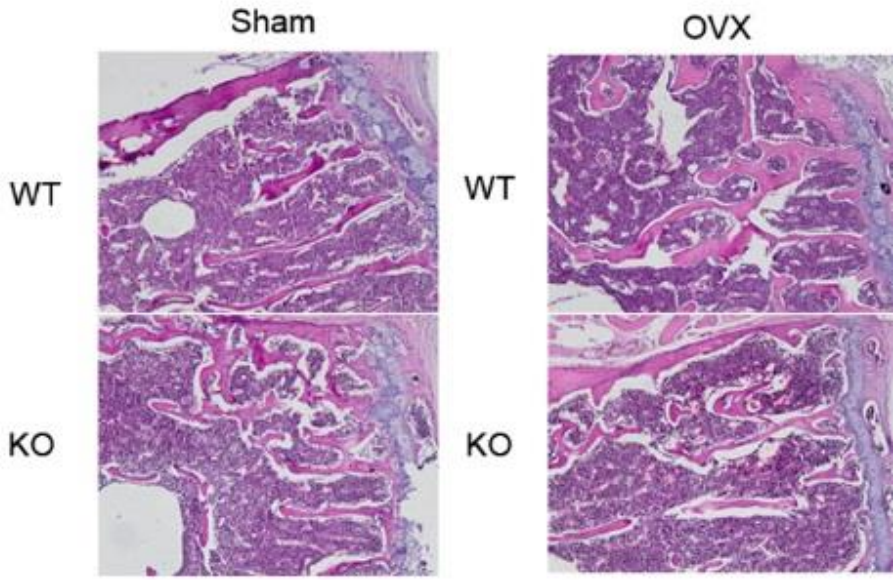



Fig. 22. Representative 3D image of vertebra.

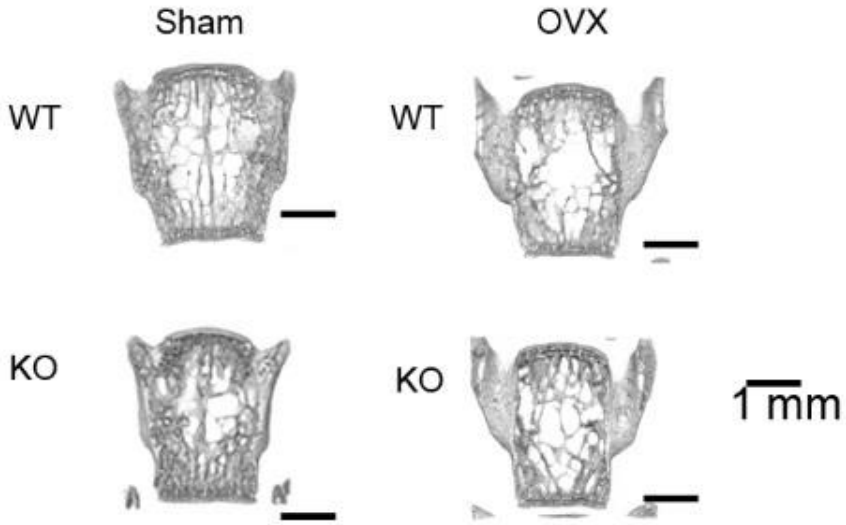


Fig. 23. Representative images of trabecular bone separation in vertebra.

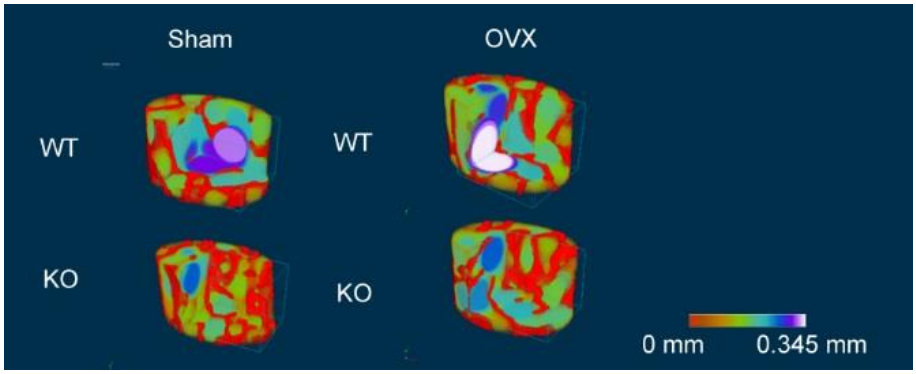



Fig. 24. Representative images of trabecular bone thickness in vertebra.

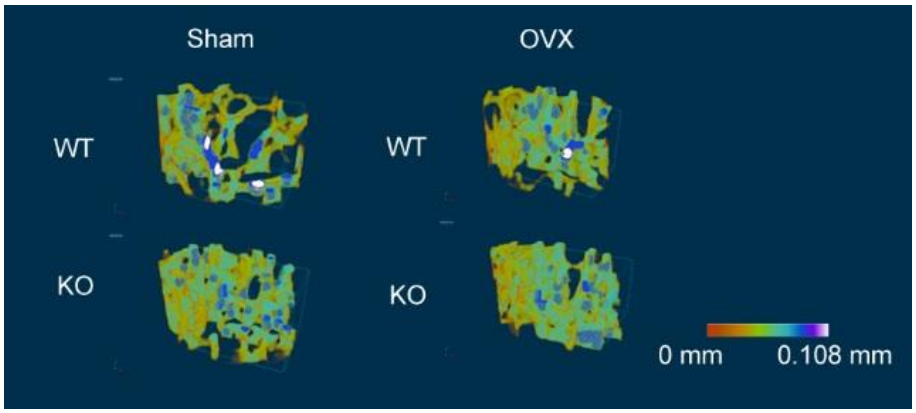


Fig. 25. Micro-CT result of femur head. n = 11 samples in WT-sham group, n = 12 samples each group in WT-OVX, KO-sham and KO-OVX group. *, $p < 0.05$.

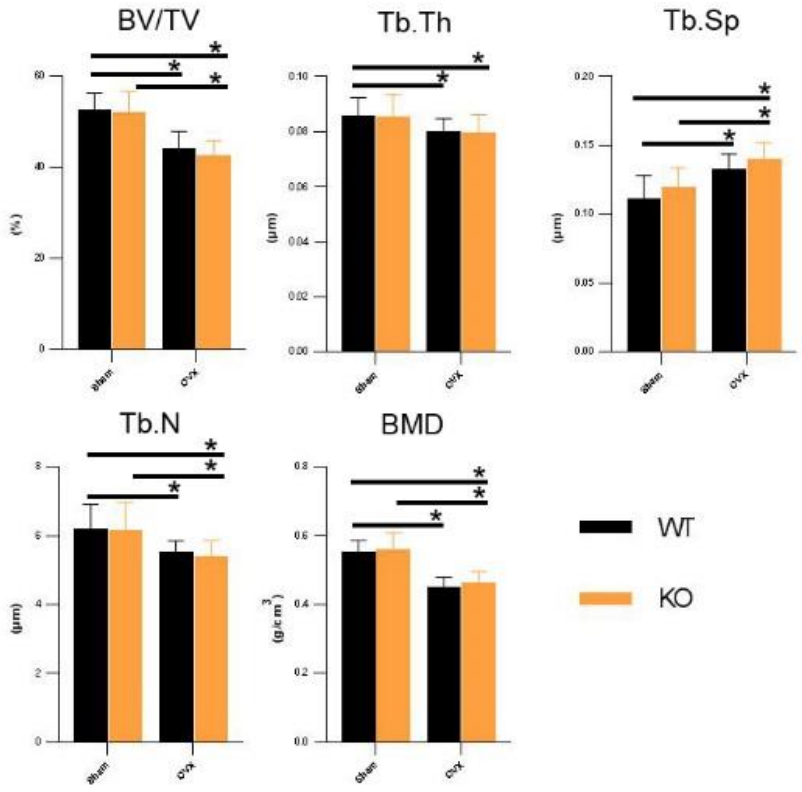



Fig. 26. H&E staining of femur head.

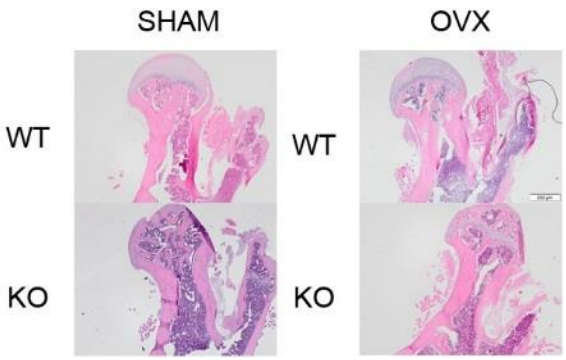


Fig. 27. Representative images of 3D images of femur head.

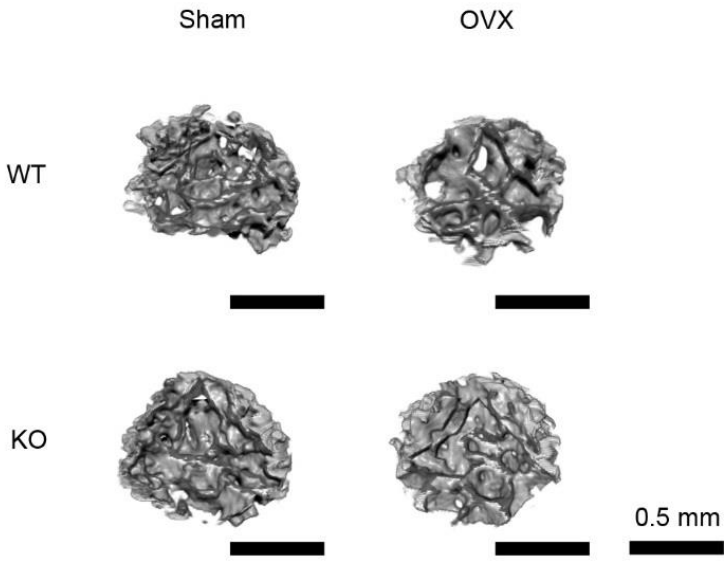



Fig. 28. Micro-CT result of femur neck. n = 11 samples in WT-sham group, n = 12 samples each group in WT-OVX, KO-sham and KO-OVX group. *, $p < 0.05$.

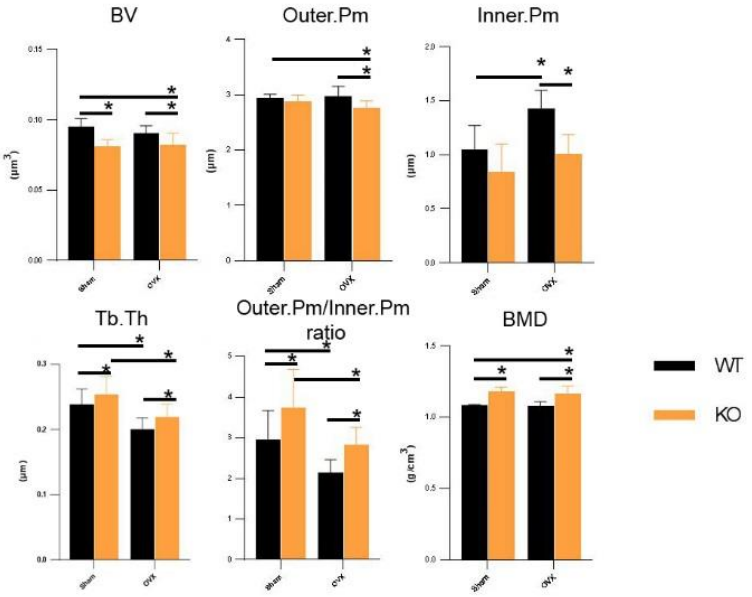


Fig. 29. Representative images of femur neck.

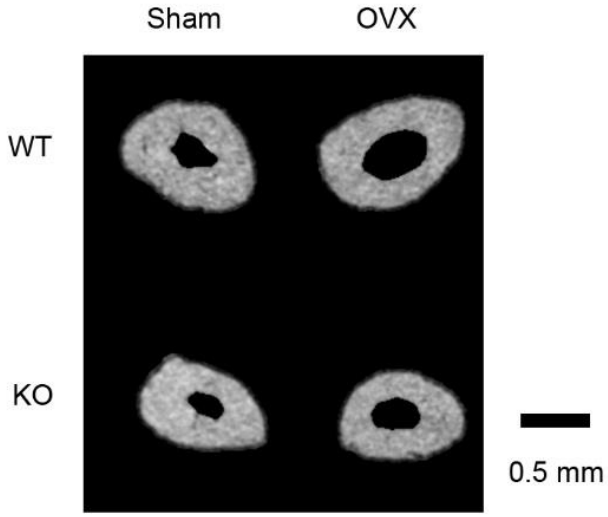



Fig. 30. Bone mineral density (BMD) of proximal femur. n = 11 samples in WT-sham group, n = 12 samples for each group (WT-OVX, or KO-sham and KO-OVX). *, $p < 0.05$.

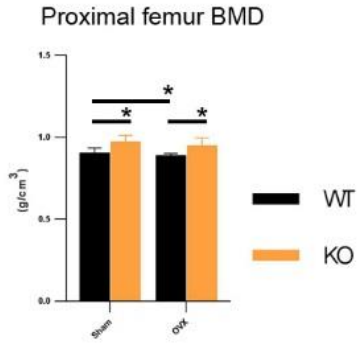


Fig. 31. Microarray result.

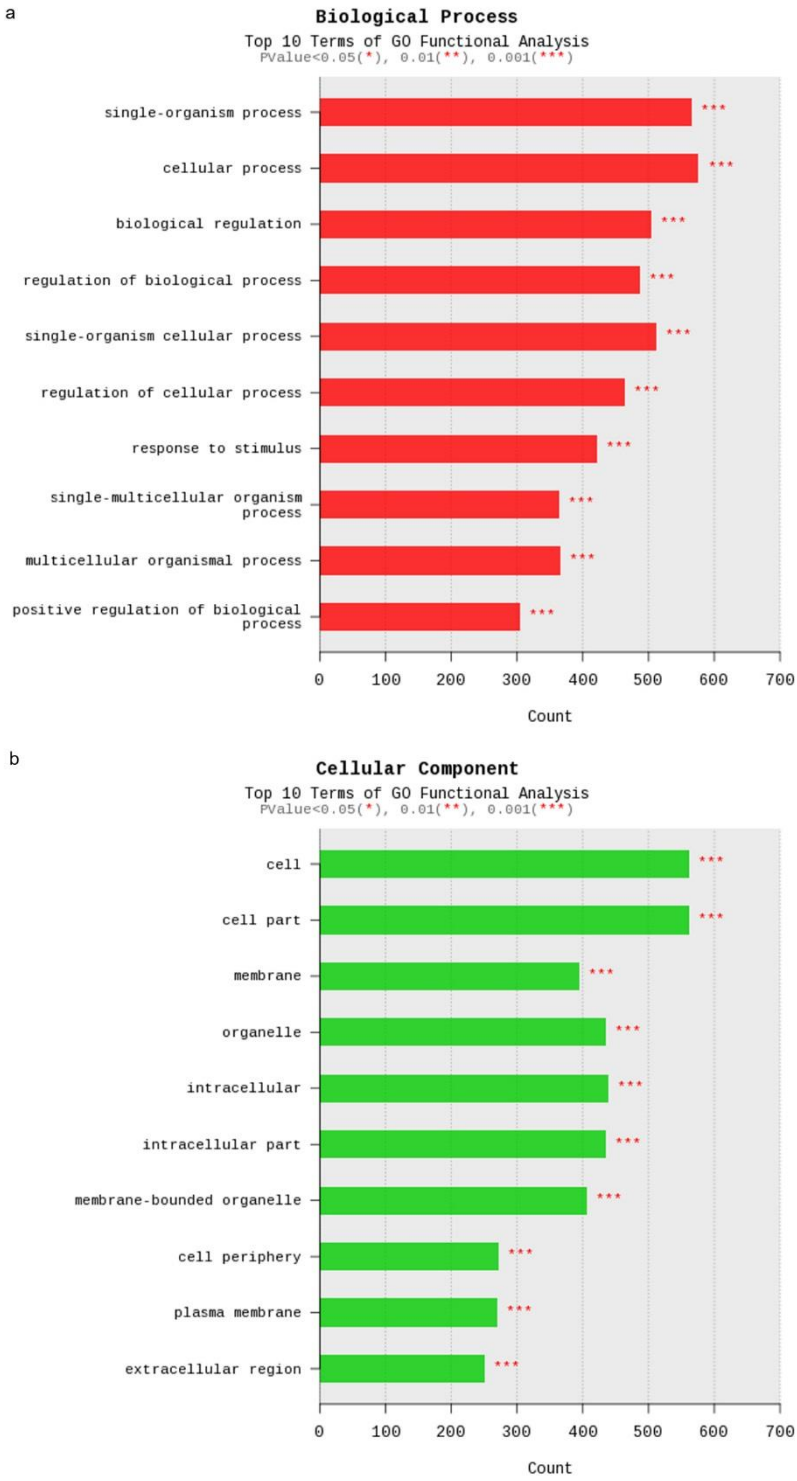


Fig. 32. Fig. 31 Microarray result (continue)

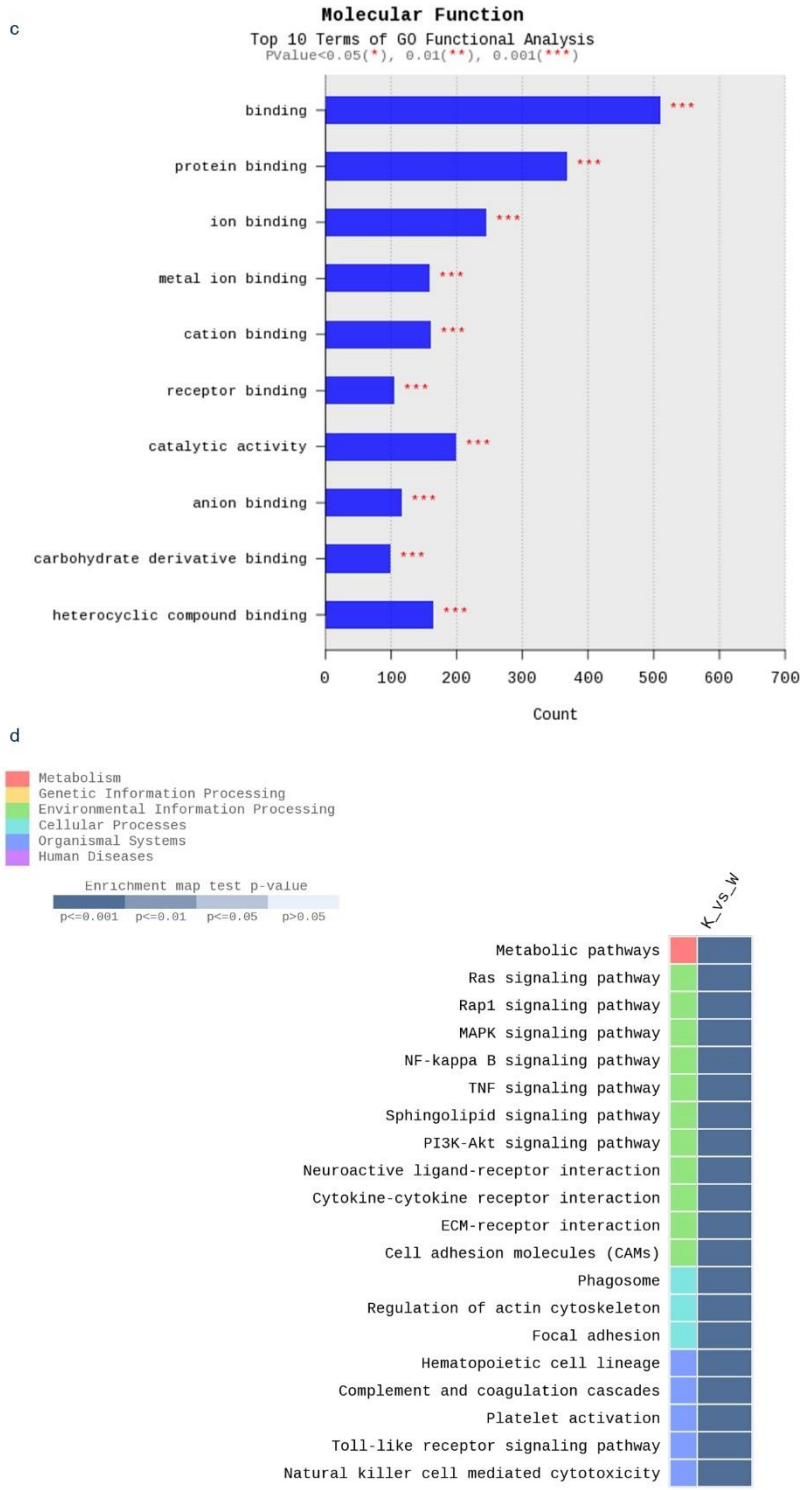


Fig. 33. TRAP staining of BMMCs. After induce with RANKL for 7 days, the BMMCs form WT mice had differentiated into multinucleated osteoclast. But the BMMCs from KO mice showed less number of differentiated osteoclast and the size of osteoclast was smaller than that of WT mice.

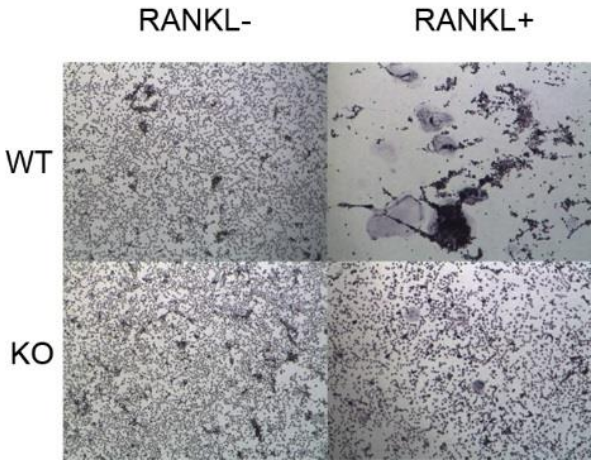



Fig. 34. Real-time PCR results. The transcription levels of NFATc1 and CathepsinK in KO cells were lower than that in WT cells when both were induced with RANKL. The experiment was biologically repeated twice and the mean value was presented.

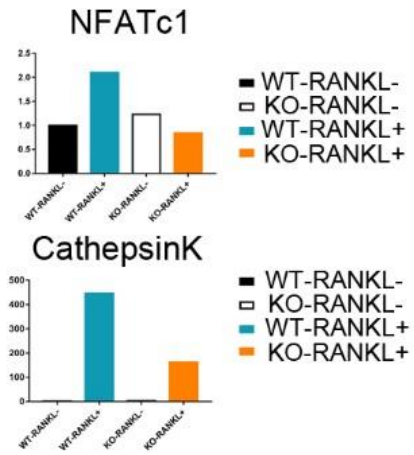


Fig. 35. ALP staining of bone marrow MSC. The KO MSC showed obviously denser staining than its WT MSC counterparts when treated with basic medium or OM.

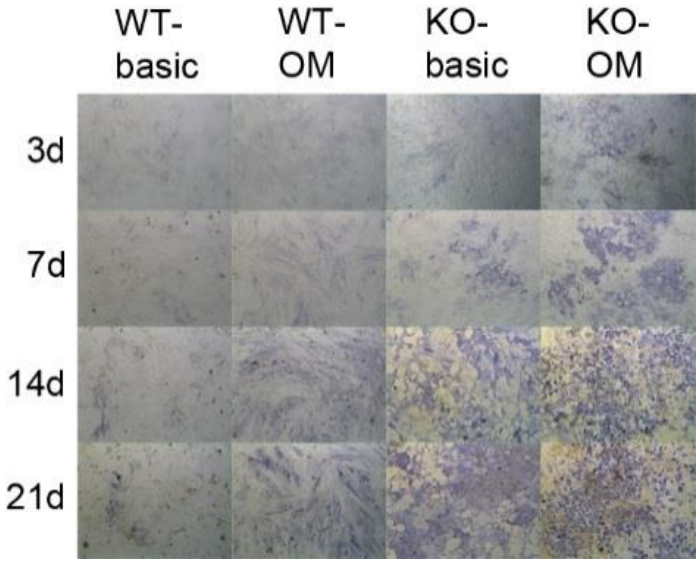



Fig. 36. Calcium staining of bone marrow MSC. The KO MSC showed denser staining than WT MSC when both were treated with OM. The KO MSCs began to show mineral deposition from the 7th day. On 14th and 21st day the KO MSCs showed larger and denser staining then WT MCSs.

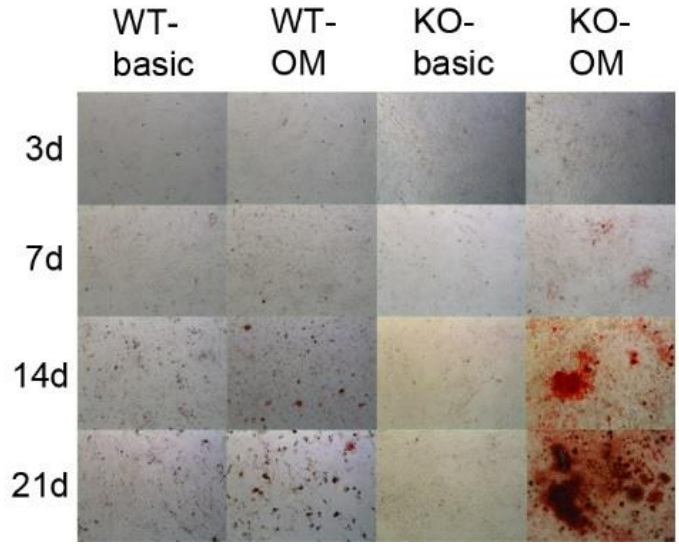


Fig. 37. ALP and calcium assay. The KO group had significantly higher ALP activity on 7th day and 14th day compared with WT MSC counterpart when treated with basic medium or OM. Additionally, KO MSC had higher calcium level than WT MSC on both 14th day and 21st day after OM treatment. The experiment was biologically repeated 3 times. The data was presented as mean and standard deviation.

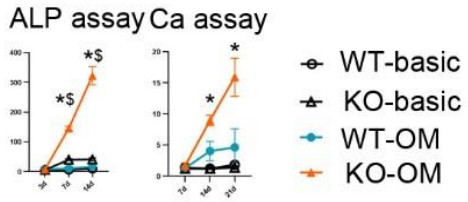



Fig. 38. The transcription level of bone markers. The KO MSC showed higher transcription level of Runx2, Ocn, Col1, Bsp and Alp than the WT MSC when both were treated with osteoinductive medium. *, $p < 0.05$, between differentiated control and shRNA MC3T3-E1 cells. $, $p < 0.05$, between undifferentiated control and shRNA MC3T3-E1 cells.

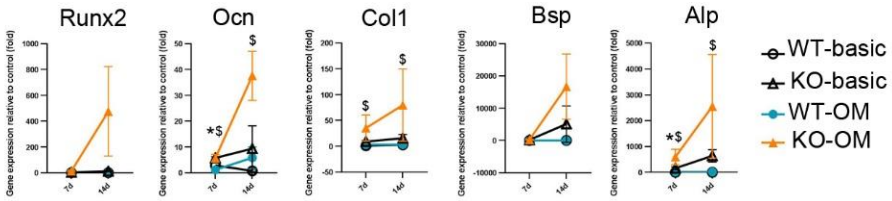



Fig. 39. Semi-quantitative RT-PCR results showing knocking down of Drg2 expression in MC3T3-E1 cells. The experiment was repeated twice.

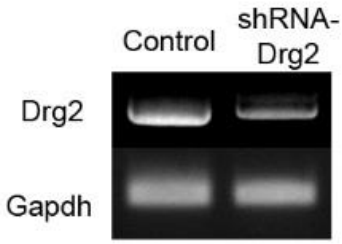


Fig. 40. Representative images of ALP staining of control and shRNA treated MC3T3-E1 cells after treatment with osteoinductive medium (OM) on 3d, 7d,14d and 21d. The shRNA MC3T3-E1 had denser staining than the MC3T3-E1 cells couterparts when both were treated with basic or OM.

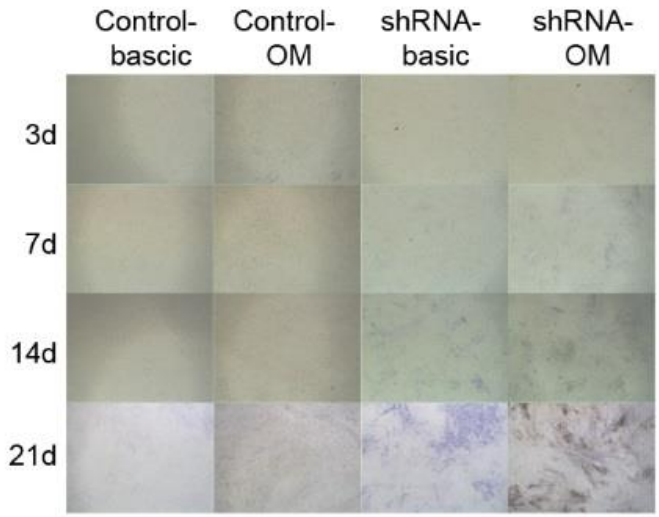



Fig. 41. Representative images of alizarin red staining of control and shRNA treated MC3T3-E1 cells after basic or OM treatment. The shRNA MC3T3-E1 cells showed obviously denser staining than the MC3T3-E1 on both 14th and 21st day.

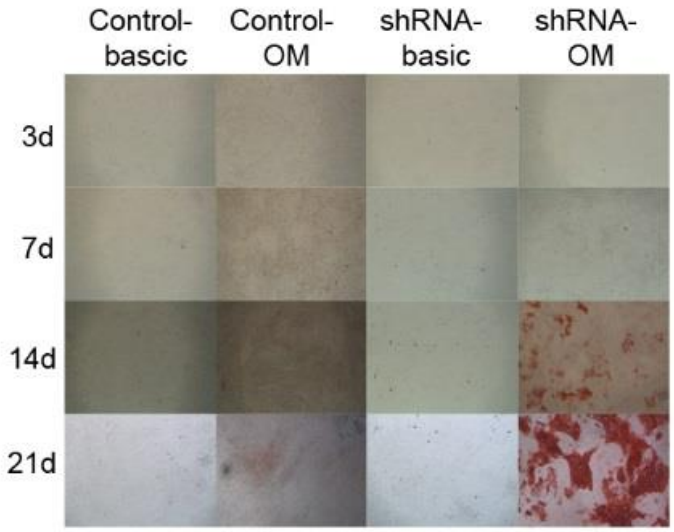


Fig. 42. ALP and calcium assay of control and shRNA MC3T3-E1 cells after basic or OM treatment. The experiment was biologically repeated three times, the data was presented as mean and standard deviation. $, $p < 0.05$, between undifferentiated control and shRNA MC3T3-E1 cells. *, $p < 0.05$, between differentiated control and shRNA MC3T3-E1 cells.

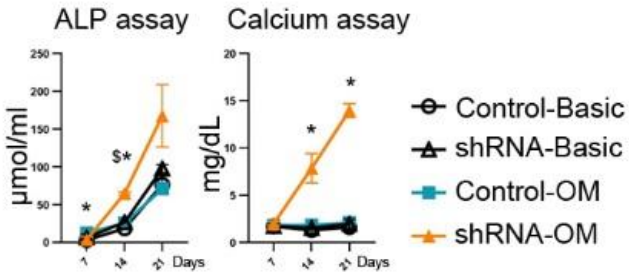



Fig. 43. Real-time PCR analysis of bone markers of control and shRNA treated cells after basic or OM treatment for 3, 7 and 14 days. The experiment was biologically repeated three times, the data was presented as mean and standard deviation. $, $p < 0.05$ between undifferentiated control and shRNA MC3T3-E1 cells. *, $p < 0.05$ between differentiated control and shRNA MC3T3-E1 cells.

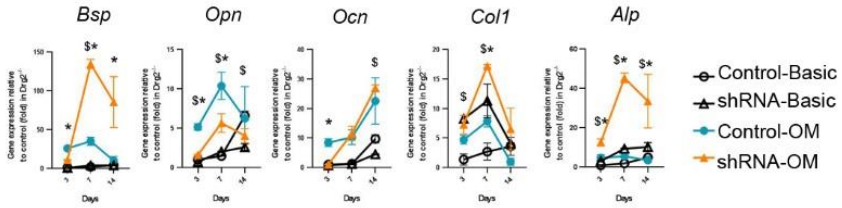


Fig. 44. Real-time PCR analysis of expression levels of bone formation related transcription factors in control and shRNA treated MC3T3-E1 cells with or without osteoinduction medium (OM) treatment for 3d, 7d and 14d. The experiment was repeated three times, the data was presented as mean and standard deviation. $, $p < 0.05$, between undifferentiated control and shRNA MC3T3-E1 cells. *, $p < 0.05$, between differentiated control and shRNA MC3T3-E1 cells.

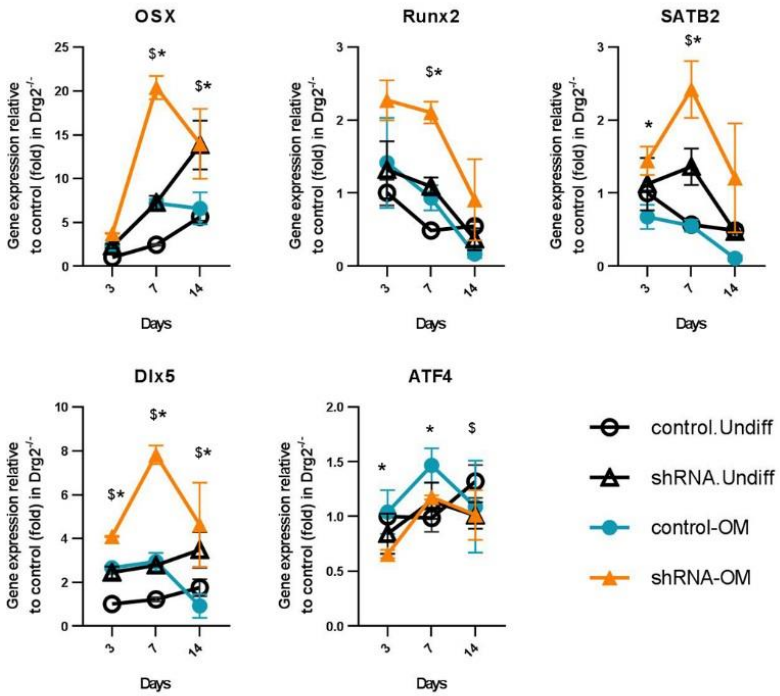


Fig. 45. Real-time PCR analysis of expression level of Smad composites involved in BMP pathway. The experiment was repeated three times, the data was presented as mean and standard deviation. $, $p < 0.05$ between undifferentiated control and shRNA MC3T3-E1 cells. *, $p < 0.05$ between differentiated control and shRNA MC3T3-E1 cells.

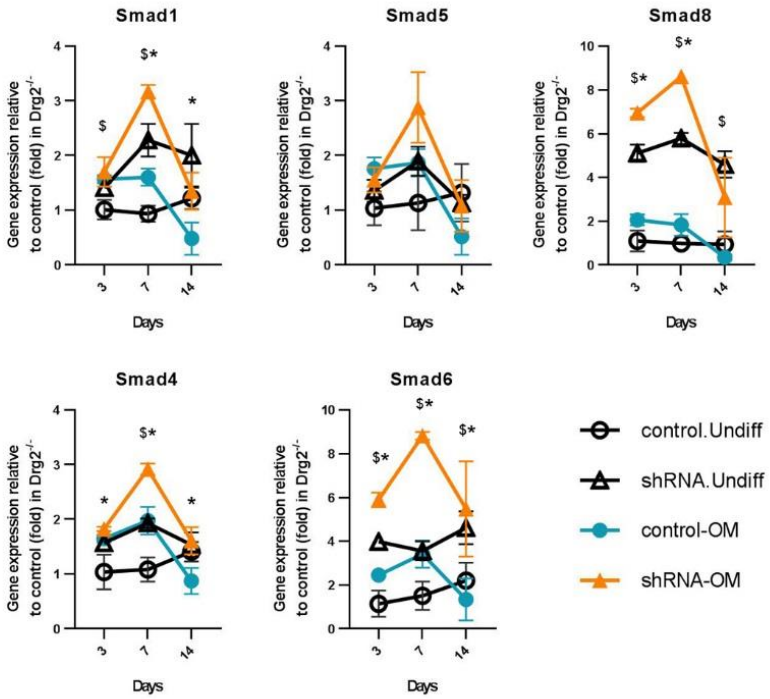


Fig. 46. Real-time PCR analysis of expression level of genes involved in non-canonical BMP pathway. The experiment was repeated 3 times and the data was presented as mean and standard deviation. $, $p < 0.05$ between undifferentiated control and shRNA MC3T3-E1 cells. *, $p < 0.05$ between differentiated control and shRNA MC3T3-E1 cells.

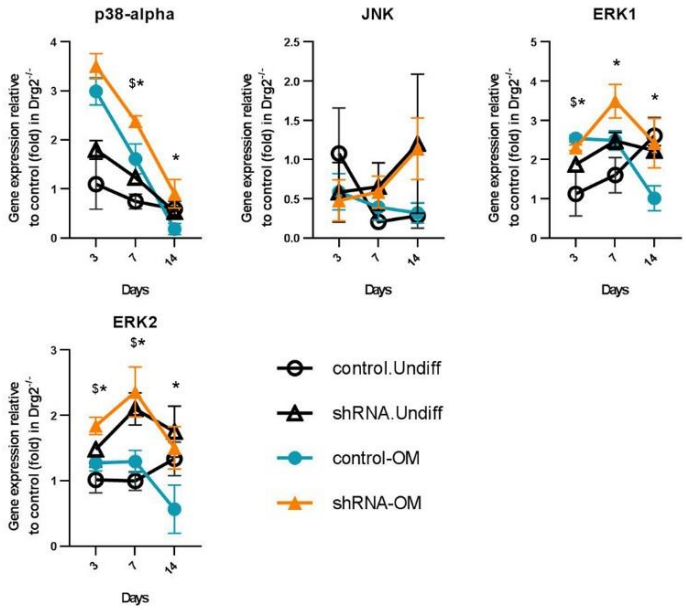



Fig. 47. Representative western blots of MC3T3-E1 cells treated with shRNA and basic medium/OM.

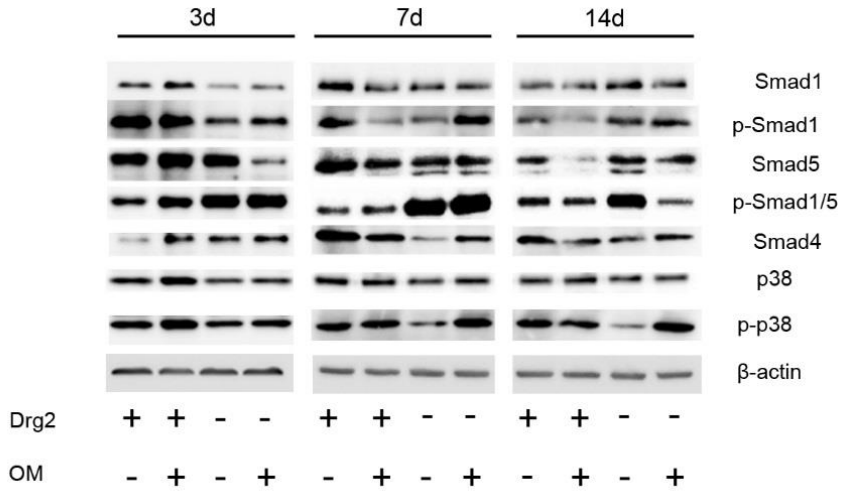
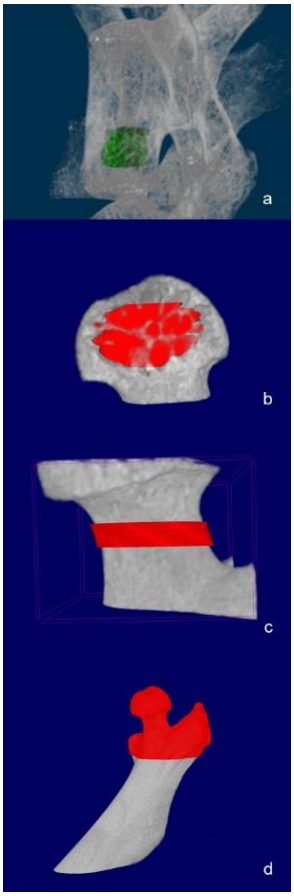


Fig. 48.  Fig. Supplementary 1. Region of interest in micro-CT analysis.



# Table

Table 1. Micro-CT result.

| | ErhBMP-2 (13) | CrhBMP-2 (13) | p |
|---|---|---|---|
| 4W | | | |
| BV/TV* | 46.07 (10.71) | 31.12 (8.16) | 0.001 |
| BS/BV* | 22.4 (12.66) | 36.32 (13.04) | 0.011 |
| Tb.Pf* | -22.76 (12.19) | -35.22 (10.04) | 0.009 |
| SMI | -2.93 (0.89) | -3.31 (1.22) | |
| Tb.Th | 0.2 (0.01) | 0.2 (0.01) | |
| Tb.N | 3.18 (0.94) | 2.83 (0.71) | |
| Tb.Sp | 0.4 (0.08) | 0.8 (0.12) | 0 |
| DA | 0.39 (0.15) | 0.45 (0.19) | |
| Coverage ratio* | 0.83 (0.26) | 0.6 (0.14) | 0.012 |
| 8W | | | |
| BV/TV* | 58.96 (12) | 11.21 (4.77) | 0 |
| BS/BV* | 19.59 (14.95) | 39.92 (23.37) | 0.014 |
| Tb.Pf* | -25.75 (14.5) | -13.12 (5.02) | 0.01 |
| SMI* | -5.12 (2.6) | -1.24 (1.44) | 0 |
| Tb.Th | 0.23 (0.04) | 0.21 (0.04) | |
| Tb.N* | 3.91 (1.82) | 0.99 (0.39) | 0 |
| Tb.Sp* | 0.33 (0.11) | 1.26 (0.18) | 0 |
| DA* | 0.41 (0.12) | 0.59 (0.14) | 0.002 |
| Coverage ratio* | 1 (0.01) | 0.35 (0.22) | 0 |

* $p < 0.05$

Table 2. Characteristics of included studies.

| Study ID | Number of participants had prevalent fractures | Proportion of participants had prevalent fractures | Mean age | Intervention & Comparison | Calcium | Vitamin D | Observation period (year) | Lost to follow up |
|---|---|---|---|---|---|---|---|---|
| **Compare with control group** | | | | | | | | |
| *Zoledronate* | | | | | | | | |
| Nakamura, 2017 | 661 | 100% | 74.15 | G1: Zoledronic Acid 5mg/year, intravenous infusion;<br>G2: PLC | Both groups | Both groups | 2 | 0.6% |
| *Alendronate* | | | | | | | | |
| Black, 1996 | 1942 | 100% | 71 | G1: Alendronate 5 mg/d on the first 2 years, 10mg/d on the third year<br>G2: PLC | Selectively offer | Selectively offer | 3 | 9% |
| Kushida, 2004 | 170 | 100% | 72 | G1: Alendronate 5 mg/d<br>G2: Alfacalcidol 1 μg/d | 1.5g/d | Alfacalcidol | 3 | 30% |
| Liberman, 1995 * | 165 | 18.72% | 64 | G1: Alendronate 5-10 mg/d<br>G2: PLC | All groups | Not reported | 3 | 16% |
| *Risedronate* | | | | | | | | |
| Clemmesen, 1997 | 132 | 100% | 68 | G1: Risedronate 2.5mg/d continuously<br>G2: Risedronate 2.5mg/ cyclically<br>G3: PLC | All groups | Not reported | 3 | 30% |
| Reginster, 2000 | 690 | 100% | 71 | G1: Risedronate 5 mg/d;<br>G2: Risedronate 2.5 mg/d;<br>G3: PLC | All groups | All groups | 3 | 42% |
| Sorensen, 2003 | 212 | 100% | 72 | G1: Risedronate 5 mg/d<br>G2: PLC | Both groups | Both groups | 2 | 17% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Fogelman, 2000 * | 237 | 43.81% | 64 | G1: Risedronate 2.5 mg/d<br>G2: Risedronate 5 mg/d<br>G3: PLC | All groups | Not reported | 2 | 21% |
| Harris, 1999 | 1374 | 100% | 69 | G1: Risedronate 5 mg/d;<br>G2: Risedronate 2.5 mg/d;<br>G3: PLC | All groups | All groups | 3 | 42% |
| *Etidronate* | | | | | | | | |
| Guanabens, 2000 | 118 | 100% | 65 | G1: Etidronate 400mg/d for 14 days in a cyclic of 90 days<br>G2: Sodium fluoride 50mg/d | Selectively offer | Not reported | 3 | 34% |
| Lyritis, 1997 | 100 | 100% | 72 | G1: Etidronate 400mg/d for 20 days in a cyclic of 90 days<br>G2: 5 days' vitamin D+85 days calcium | Both groups | Both groups | 4 | 26% |
| Montessori, 1997 * | 28 | 35% | 62.5 | G1: Etidronate 400mg/d for 14 days in a cyclic of 90 days<br>G2: Calcium 500mg/d | Selectively offer | Not reported | 3 | 20% |
| Shiota, 2001 * | 24 | 60% | 61.7 | G1: Etidronate 200mg/d for 14 days in a cyclic of 84 days<br>G2:2 g/d calcium and 0.5 µg/d alphacalcidol for 2 years | Selectively offer | Selectively offer | 2 | Not reported |
| Harris, 1993 | 423 | 100% | | G1: PLC and PLC<br>G2: Phosphate and PLC<br>G3: PLC and Etidronate 400mg/daily for 14 in a cycle of 91 days<br>G4: Phosphate and Etidronate | All groups | Not mentioned | 4[$] | 20% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Watts, 1990 | 423 | 100% | 65 | G1: PLC for 17 days in a cyclic of 91 days<br>G2: Phosphonate 2g/d for 3 days in a cyclic of 91 days<br>G3: Etidronate 400mg/d for 14 days in a cyclic of 91 days<br>G4: Phosphonate 2g/d for 3 days + Etidronate 400mg/d for next 14 days in a cyclic of 91 days | All groups | Not reported | 2 | 14% |
| *Ibandronate* | | | | | | | | |
| Chesnut, 2004 | 2929 | 100% | 69 | G1: Ibandronate 2.5 mg/d, oral<br>G2: Ibandronate 20mg alternate day for 12 doses every 3 months, oral<br>G3: PLC | All arms | All arms | 3 | 34% |
| Recker, 2004 | 2860 | 100% | 67 | G1: Ibandronate 0.5mg injection, every 3 months<br>G2: Ibandronate 1mg injection, every 3 months<br>G2: PLC | All arms | All arms | 3 | 18% |
| *Minodronate* | | | | | | | | |
| Matsumoto, 2009 | 704 | 100% | 72 | G1: Minodronate 1mg/d<br>G2: PLC | Both groups | Both groups | 2 | 31% |
| *Pamidronate* | | | | | | | | |
| Reid, 1994 | 61 | 100% | 66 | G1: Pamidronate 150mg/d<br>G2: PLC | Both groups | Not reported | 2 | 79% |
| Brumsen, 2002 | 101 | 100% | 65 | G1: Pamidronate 150mg/d<br>G2: PLC | Both groups | Both groups | 3 | 10% |
| *Calcitonin* | | | | | | | | |
| Peichl, 1999 | 42 | 100% | 62 | G1: Nasal salmon calcitonin 100IU twice daily for 2 months with a pause of 2 months<br>G2: Control group | Both groups | Only control groups | 1 | Not reported |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Hodsman, 1997 | 30 | 100% | 67 | G1: PTH sc injections 800 IU/d for 28 days in a cyclic of 90 days<br>G2: PTH sc injections 800 IU/d for 28 days + salmon calcitonin 75 U/d for 42 days in a cyclic of 90 days | Both groups | Not prescribed | 2 | 23% |
| Chesnut, 2005 | 91 | 100% | 67.4 | G1: Calcitonin nasal spray 200 IU/d<br>G2: Placebo nasal spray | Both groups | Not reported | 2 | 78% |
| *Hormone replace therapy* | | | | | | | | |
| Gutteridge, 2002 | 99 | 100% | 69 | G1: Fluoride<br>G2: Control group<br>G3: Fluoride + Estrogen 0.625 mg/d<br>G4: Estrogen 0.625 mg/d | All groups | All groups | 2.25 | 24% |
| Wimalawansa, 1998 | 72 | 100% | 65 | G1: HRT group, Permarin 0.625mg/d + norgestril 150µg for 12days each month<br>G2: Etidronate group, Etidronate 400mg/d for 14 days each 12 week<br>G3: Combined therapy, combination of G1 and G2 with same dose<br>G4: control group | All groups | All groups | 4 | 17% |
| Lufkin, 1992 | 75 | 100% | 65 | G1: Estrogen group, Estradiol 0.1mg/d on the first 21 days + medroxyprogesterone acetate for the days 11 to 21 in a 28 days' cycle<br>G2: Placebo | Both | Not reported | 1 | |
| *Parathyroid hormone* | | | | | | | | |
| Neer, 2001 | 1637 | 100% | 71.0 | G1: rhPTH 20 µg/d<br>G2: rhPTH 40 µg/d<br>G3: PLC | All groups | All groups | 2 | 6% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Nakamura, 2012; | 578 | 100% | 75.3 | G1: Teriparatide 56.5 µg/w, sc injection<br>G2: PLC, sc injection | Both groups | Both groups<br>400IU/d | 1.5 | 26% |
| Greenspan, 2001* | 471 | 18.6% | 64.4 | G1: Teriparatide 100 µg/d, sc injection<br>G2: PLC | Both groups | Both groups | 1.5 | 33% |
| Fujita, 2014 | 316 | 100% | 71 | G1: Teriparatide 28.2 µg/w, injection<br>G2: Teriparatide 1.4 µg/w, injection | Both groups | Not prescribed | 3 | 17% |
| *Denosumab* | | | | | | | | |
| Nakamura, 2014 | 1262 | 100% | 69.6 | G1: Denosumab 60mg/6 months, sc injection<br>G2: PLC<br>G3: Alendronate 35mg/w | All groups | All groups | 3 | 13% |
| Boonen, 2011 # | 759 | 100% | 73.7 | G1: Denosumab 60mg/6 months, sc<br>G2: PLC | Both groups | Both groups | 3 | 18% |
| *Romozumab* | | | | | | | | |
| Saag, 2017 | 4093 | 100% | 74.3 | G1: Alendronate: 70 mg/w<br>G2: Romosozumab: 210 mg/m sc injection | Both groups | Both groups | 3 | 11% |
| *Raloxifene* | | | | | | | | |
| Ettinger, 1999 * | 2304 | 33.74% | 68 | G1: Raloxifene 60mg/d<br>G2: Raloxifene 120mg/d<br>G3: PLC | All groups | All groups | 3 | 23% |
| Lufkin, 1998 | 143 | 100% | 68 | G1: Raloxifene 60mg/d<br>G2: Raloxifene 120mg/d<br>G3: PLC | All groups | All groups | 1 | 9% |
| *Bazedoxifene* | | | | | | | | |
| Palacios, 2015 * | 3857 | 49.40% | 67 | G1: Bazedoxifene 60mg/d<br>G2: Bazedoxifene 40mg/d<br>G3: Bazedoxifene 20mg/d<br>G4: PLC | All groups | All groups | 7 | 74% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Compare between medications** | | | | | | | | |
| Kushida, 2004 b | 547 | 100% | 72 | G1: Risedronate 2.5 mg/d<br>G2: Etidronate 200 mg/d cyclically | Both groups | Not reported | 2 | 21% |
| Nakamura, 2013 | 1265 | 100% | 72.7 | G1: Ibandronate 0.5mg injection per month<br>G2: Ibandronate 1mg iv injection per month<br>G3: Risedronate 2.5 mg/d | All arms | All arms | 3 | 10% |
| Hadji, 2012 | 710 | 100% | 71 | G1: Risedronate: 35 mg/w<br>G2: Teriparatide: 20 µg/w subcutaneous injection | Both groups | Both groups | 1.5 | 26% |
| Kendler, 2017 | 1360 | 100% | 72.1 | G1: Risedronate 35mg/w<br>G2: Teriparatite: 20 µg/d subcutaneous injection | Both groups | Both groups | 2 | 26% |

Table 3. Summary of findings of osteoporotic vertebral fracture and non-vertebral fracture.

| Comparison | RR (95% CI) | No. of participants (studies) | Quality of the evidence |
|---|---|---|---|
| **Vertebral fracture** | | | |
| Antiresorptive medication vs. Control | 0.59 (0.53 to 0.65) | 21012 (30 RCTs) | - |
| ZOL vs. Control | 0.34 (0.17 to 0.69) | 657 (1 RCT) [1] | MODERATE [2,] |
| ALN vs. Control | 0.54 (0.43 to 0.68) | 2277 (3 RCTs) [3] | HIGH |
| RISE vs. Control | 0.61 (0.51 to 0.73) | 2645 (5 RCTs) [4] | MODERATE [5] |
| Etidronate vs. Control | 0.60 (0.39 to 0.92) | 618 (7 RCTs) [6] | MODERATE [7,] |
| Ibandronate (sufficient) vs. Control | 0.52 (0.38 to 0.71) | 2929 (1 RCT) [8] | MODERATE [9], |
| Ibandronate (insufficient) vs. Control | 0.87 (0.69 to 1.11) | 2860 (1 RCT) [10] | MODERATE [11] |
| Minodronate vs. Control | 0.44 (0.31 to 0.63) | 674 (1 RCT) [12] | LOW [13] |
| Pamidronate vs. Control | 0.33 (0.13 to 0.84) | 90 (1 RCT) [14] | VERY LOW [15] |

[1] Nakamura, 2017
[2] Study limitations: the trial included had unclear risk of performance bias..
[3] Liberman, 1995; Black, 1996; Kushida, 2004
[4] Clemmesen, 1997; Harris, 1999; Reginster, 2000; Fogelman, 2000; Sorensen, 2003
[5] Study limitations: four trials were included, with unclear risk of selection bias, performance bias and attribution bias.
[6] Shiota, 2001; Montessori, 1997; Lyritis, 1997; Watts, 1990; Harris, 1993; Wimalawansa, 1998; Guanabens, 2000
[7] Study limitations: seven trials were included, with unclear to high risk of selection bias, attribution bias, other bias, and performance bias.
[8] Chesnut, 2004
[9] Study limitations: one trial was included, with unclear risk of performance bias and attribution bias.
[10] Recker, 2004
[11] One trial included, with unclear risk of performance bias and other bias.
[12] Matsumoto, 2009
[13] One trial was included, with unclear risk of performance bias, attribution bias and other source of bias. Imprecision: the number of events was 115 and OIS was not met.
[14] Brumsen, 2002
[15] Study limitation: one trial included, with unclear risk of selection bias. Imprecision (rating down two levels): 20 events and CIs included appreciable benefit.

| | | | |
|---|---|---|---|
| Calcitonin vs. Control | 1.02 (0.14 to 7.36) | 157 (3 RCTs) [16] | VERY LOW [17] |
| HRT vs. Control | 0.86 (0.29 to 2.52) | 147 (3 RCTs) [18] | LOW [19] |
| PTH vs. Control | 0.32 (0.24 to 0.43) | 2632 (4 RCTs) [20] | MODERATE [21] |
| Denosumab vs. Control | 0.41 (0.29 to 0.57) | 1654 (2 RCTs) [22] | MODERATE [23] |
| RLX vs. Control | 0.58 (0.44 to 0.76) | 2447 (2 RCTs) [24] | HIGH |
| BZA vs. Control | 0.66 (0.53 to 0.82) | 3857 (1 RCT) [25] | MODERATE [26] |
| ALN vs. Denosumab | 0.69 (0.41 to 1.17) | 722 (1 RCT) [27] | LOW [28] |
| RISE vs. Etidronate | 1.12 (0.69 to 1.81) | 433 (1 RCT) [29] | MODERATE [30] |
| Ibandronate vs. RISE | 1.01 (0.79 to 1.31) | 1228 (1 RCT)[31] | HIGH |
| RISE vs. Teriparatide | 1.98 (1.44 to 2.70) | 2070 (2 RCTs) [32] | HIGH |

[16] Hodsman, 1997; Peichl, 1999; Chesnut, 2005

[17] Study limitation: two trials had unclear to high risk of selection bias, performance bias, attribution bias and other bias. Imprecision (rating down two levels): 15 events and CIs included appreciable benefit and harm.

[18] Lufkin, 1992; Wimalawansa, 1998; Gutteridge, 2002

[19] Study limitation: two trials had unclear risk of selection bias. Two trials had unclear to high risk of performance bias. Three trials had unclear risk of attribution bias. Three trials had unclear risk of other bias. Imprecision (rating down two levels): 34 events and CIs included appreciable benefit and harm.

[20] Nakamura, 2012, Neer, 2001, Greenspan, 2007, Fujita, 2014

[21] One trial had unclear risk of selection bias, performance bias and attribution bias. One trial had high risk of performance bias.

[22] Boonen, 2011; Nakamura, 2014.

[23] Study limitation: two trials had unclear risk of selection bias and performance bias. One trial had had unclear risk of other bias.

[24] Ettinger, 1999, Lufkin, 1998

[25] Palacios, 2015

[26] Study limitation: one trial had high risk of performance bias and unclear risk of attribution bias and other bias.

[27] Nakamura, 2014

[28] Study limitation: one study included, with unclear risk of selection bias, performance bias, and other bias. Imprecision: the number of events was 66, and OIS was not met.

[29] Kushida, 2004

[30] Study limitation: one trial was included, with unclear risk of selection bias, attribution bias and other bias.

[31] Nakamura, 2013

[32] Hadji, 2012; Kendler, 2017

| | | | |
|---|---|---|---|
| HRT vs. Etidronate | 0.63 (0.12 to 3.32) | 35 (1 RCT) [33] | VERY LOW [34] |
| **Non-vertebral fracture** | | | |
| ZOL vs. Control | 0.54 (0.32 to 0.91) | 661 (1 RCT)[35] | - |
| ALN vs. Control | 0.81 (0.65 to 1.01) | 2027 (1 RCT)[36] | - |
| RISE vs. Control | 0.71 (0.54 to 0.92) | 2836 (4 RCTs)[37] | - |
| Etidronate vs. Control | 0.95 (0.59 to 1.53) | 395 (4 RCTs)[38] | - |
| Ibandronate (sufficient) vs. Control | 1.10 (0.85 to 1.41) | 2929 (1 RCT)[39] | - |
| Ibandronate (insufficient) vs. Control (only Hip fracture) | 0.59 (0.26 to 1.31) | 2860 (1 RCT)[40] | - |
| Minodronate vs. Control | 0.80 (0.35 to 1.84) | 674 (1 RCT)[41] | - |
| Pamidronate vs. Control | 0.33 (0.04 to 3.10) | 100 (1 RCT)[42] | - |
| PTH vs. Control | 0.53 (0.36 to 0.78) | 2454 (3 RCTs)[43] | - |
| Denosumab vs. Control | 0.45 (0.20 to 1.03) | 952 (1 RCT)[44] | - |
| Romosozumab vs. ALN | 0.74 (0.54 to 1.00) | 4093 (1 RCT)[45] | |
| ALN vs. Dmab | 1.49 (0.52 to 4.24) | 722 (1 RCT)[46] | - |

[33] Wimalawansa, 1998

[34] Study limitation: one trial was included, with unclear risk of performance bias, attribution bias and other bias. Imprecision (rating down two levels): few events and CIs included appreciable benefit and harm.

[35] Nakamura, 2017

[36] Black, 1996

[37] Clemmesen, 1997; Harris, 1999; Reginster, 2000; Sorensen, 2003

[38] Watts, 1990; Lyritis, 1997; Montessori, 1997; Guanabens, 2000

[39] Chesnut, 2004

[40] Recker, 2004

[41] Matsumoto, 2009

[42] Brumsen, 2002

[43] Nakamura, 2012; Neer, 2001, Fujita, 2014

[44] Nakamura, 2014

[45] Saag, 2017

[46] Nakamura, 2014

| | | | |
|---|---|---|---|
| RISE vs. Teriparatide | 1.28 (0.94 to 1.73) | 2070 (2 RCTs)[47] | - |
| HRT vs. Etidronate | 0.94 (0.06 to 13.93) | 35 (1 RCT)[48] | - |

RR, Relative Risk; ZOL, Zoledronic acid; ALN, Alendronate; RISE, Risedronate; PTH, Pamidronate; RLX, Raloxifene; BZA, Bazedoxifene; HRT, Hormone replace therapy.

[47] Hadji, 2012; Kendler, 2017
[48] Wimalawansa, 1998

Table 4. Discontinuation due to medication.

| Comparison | No. of participants (studies) | RR (95% CI) |
|---|---|---|
| Zoledronate vs. Control | 665 (1 RCT)[1] | 1.99 (0.76, 5.25) |
| Alendronate vs. Control | 2750 (2 RCTs)[2] | 0.88 (0.64, 1.22) |
| RISE vs. Control | 2707 (3 RCTs)[3] | 0.88 (0.69, 1.12) |
| Etidronate vs. Control | 322 (3 RCTs)[4] | 0.40 (0.03, 5.48) |
| Ibandronate vs. Control | | |
| 2.5 mg/d & 20 mg alternatively | 2929 (1 RCT)[5] | 0.90 (0.69, 1.18) |
| 0.5 mg & 1 mg per 3 months | 2860 (1 RCT)[6] | 1.27 (0.97, 1.66) |
| PTH vs. Control | 2215 (2 RCTs)[7] | 1.54 (1.11, 2.13) |
| 56.5 μg/w | 578 (1 RCT)[8] | 1.80 (1.00, 3.24) |
| 20 μg/d | 813 (1 RCT)[9] | 1.10 (0.62, 1.95) |
| 40 μg/d | 824 (1 RCT)[10] | 1.82 (1.07, 3.10) |
| Dmab vs. Control | 956 (1 RCT)[11] | 0.75 (0.44, 1.27) |
| HRT vs. Control | 79 (2 RCTs)[12] | 0.53 (0.17, 1.61) |
| Alendronate vs. Denosumab | 717 (1 RCT)[13] | 0.79 (0.15, 4.02) |
| Romosozumab vs. Alendronate | 4093 (1 RCT)[14] | 1.00 (0.58, 1.74) |
| RISE vs. Teriparatide | 2070 (2 RCT)[15] | 0.75 (0.57, 1.00) |
| HRT vs. Etidronate | 35 (1 RCT)[16] | 2.83 (0.33, 24.66) |

[1] Nakamura, 2017
[2] Black, 1996; Nakamura, 2014
[3] Harris, 1999; Reginster, 2000; Sorensen, 2003
[4] Watts, 1990; Wimalawansa, 1998; Guanabens, 2000
[5] Chesnut, 2004
[6] Recker, 2004
[7] Nakamura, 2012; Neer, 2001
[8] Nakamura, 2012
[9] Neer, 2001
[10] Neer, 2001
[11] Nakamura, 2014
[12] Wimalawansa, 1998; Gutteridge, 2002
[13] Nakamura, 2014
[14] Saag, 2017
[15] Hadji, 2012; Kendler, 2017
[16] Wimalawansa, 1998

Table 4. Primers used in this study.

| | |
|---|---|
| mouse Drg2 | F-AGGCAGGTCCCTTACCTGAG |
| | R-AGATGTGCAGAGGGCAAAGA |
| human Drg2 | F-ACATTCTTGAGTCTGATGACCT |
| | R-AGTAGATGTTAGGCTTGTGCTT |
| Sry | F-TGGGACTGGTGACAATTGTC |
| | R-GAGTACAGGTGTGCAGCTCT |
| IL3 | F-GGGACTCCAAGCTTCAATCA |
| | R-TGGAGGAGGAAGAAAAGCAA |
| mDlx5 | F-CCGCTTTACAGAGAAGGTTTCA |
| | R-TCTTCTTGATCTTGGATCTTTTGTT |
| Smad1 | F-TGAAAACACCAGGCGACATA |
| | R-TGAGGCATTCCGCATACAC |
| Smad5 | F-GCAGTAACATGATTCCTCAGACC |
| | R-GCGACAGGCTGAACATCTC |
| Smad8 | R-CGGATGAGCTTTGTGAAGG |
| | F-GGGTGCTCGTGACATCCT |
| Smad4 | R-AAGCTGCCCTGTTGTGACTGT |
| | F-GGAGAGTTGACCCAAGCAAAAG |
| Smad6 | F-GTTGCAACCCCTACCACTTC |
| | R-GGAGGAGACAGCCGAGAATA |
| OPN | F-GATGATGATGACGATGGAGACC |
| | R-CGACTGTAGGGACGATTGGAG |
| Collagen I | F-ATCTCCTGGTGCTGATGGAC |
| | R-ACCTTGTTTGCCAGGTTCAC |
| BSP | F-GAGACGGCGATAGTTCC |
| | R-AGTGCCGCTAACTCAA |
| p38 | F-CCCAGCAACCTAGCTGTG |
| | R-GCTCGGTACCACCTGGTAG |
| JNK | F-TCCCAGCTGACTCAGAGCAT |
| | R-GCTTCATCTACGGAGATCCTT |
| ERK1 | F-CCTGCTGGACCGGATGTTA |
| | R-TGAGCCAGCGCTTCCTCTAC |
| ERK2 | F-GGAGCAGTATTATGACCCAAGTGA |
| | R-TCGTCCACTCCATGTCAAACT |

# Supplementary material

## Supplementary material 1. Searching strategy.

PubMed:

1. randomized controlled trial[pt]
2. controlled clinical trial[pt]
3. randomized controlled trials[mh]
4. random allocation[mh]
5. double-blind method[mh]
6. single blind method[mh]
7. clinical trial[pt]
8. clinical trials[mh])
9. clinical trial"[tw]
10. latin square[tw])
11. placebos[mh]
12. placebo*[tw]
13. random*[tw]
14. research design[mh:noexp]
15. placebos[mh]
16. control*[tw]
17. prospective*[tw]
18. volunteer*[tw]) NOT (animal[mh] NOT human[mh])
19. #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19
20. Osteoporosis compression fracture
21. osteoporotic fracture
22. #20 OR #21
23. spine
24. spinal
25. vertebral
26. vertebrae
27. #23 OR #24 OR #25 OR #26
28. medication[tiab]

29. medicine[tiab]
30. drug*[tiab]
31. bisphosphonate[tiab]
32. bisphosphonates[tiab]
33. alendronate[tiab]
34. risedronate[tiab]
35. ibandronate[tiab]
36. clodronate[tiab]
37. zoledronate[tiab]
38. pamidronate[tiab]
39. parathyroid hormone[tiab]
40. parathyroid hormone[tw]
41. teriparatide[tiab]
42. teriparatide[tw]
43. denosumab[tiab])
44. denosumab[tw]
45. calcium[tiab]
46. vitamine D[tiab]
47. calcitonin[tiab]
48. calcitonin[tw]
49. serm[tiab]
50. raloxifene[tiab])
51. bazedoxifene[tiab]
52. HRT[tiab]
53. HRT[tw]
54. ERT[tiab]
55. ERT[tw]
56. hormone replacement[tiab]
57. hormone replacement[tw]
58. estrogen replacement[tiab]
59. estrogen replacement[tw]
60. estradiol[tiab]
61. estradiol[tw]
62. estrone[tiab]
63. estrone[tw]

64. dien estrol[tiab]
65. dien estrol[tw]
66. pamidronate[tiab]
67. #28 OR #29 OR #30 OR #31 OR #32 OR #33 OR #34 OR #35 OR #36 OR #37 OR #38 OR #39 OR #40 OR #41 OR #42 OR #43 OR #44 OR #45 OR #46 OR #47 OR #48 OR #49 OR #50 OR #51 OR #52 OR #53 OR #54 OR #55 OR #56 OR #57 OR #58 OR #59 OR #60 OR #61 OR #62 OR #63 OR #64 OR #65 OR #66
68. OR ##19 AND #22 AND #27 AND #67

**Supplementary material 2. Risk of bias table.**

| Article ID | Year | a | b | c | d | e | f | g | h | i | j | k | l | m |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Zoledronic acid** | | | | | | | | | | | | | | |
| Nakamura | 2017 | + | + | + | ? | ? | + | ? | + | + | + | + | + | + |
| **Alendronate** | | | | | | | | | | | | | | |
| Black | 1996 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Kushida | 2004a | ? | ? | + | ? | ? | + | ? | + | + | + | + | + | ? |
| Liberman | 1995 | ? | ? | + | - | - | + | ? | + | + | + | + | + | ? |
| **Risedronate** | | | | | | | | | | | | | | |
| Clemmesen | 1997 | ? | ? | + | ? | ? | + | + | + | + | ? | + | + | - |
| Fogelman | 2000 | ? | ? | + | ? | ? | + | ? | + | + | + | + | + | + |
| Harris | 1999 | + | + | + | + | + | + | + | + | + | ? | + | + | + |
| Reginster | 2000 | ? | ? | + | ? | ? | + | + | + | + | + | ? | + | ? |
| Sorensen | 2003 | ? | ? | - | ? | ? | ? | ? | + | + | + | + | + | ? |
| **Etidronate** | | | | | | | | | | | | | | |
| Guanabens | 2000 | ? | ? | + | ? | ? | ? | - | + | + | ? | ? | + | ? |
| Harris | 1993 | + | ? | + | ? | ? | + | ? | + | + | + | + | + | ? |
| Lyritis | 1997 | ? | ? | + | - | - | + | ? | + | + | + | ? | + | ? |
| Montenssori | 1997 | + | ? | + | ? | ? | ? | ? | + | + | + | + | + | ? |
| Shiota | 2001 | ? | ? | + | ? | ? | ? | ? | + | + | + | ? | ? | ? |
| Watts | 2014 | + | ? | + | ? | ? | + | ? | + | + | + | ? | + | ? |
| Wimalawansa | 1998 | + | ? | + | ? | ? | + | ? | + | + | + | ? | + | ? |
| **Ibandronate** | | | | | | | | | | | | | | |
| Chesnut | 2004 | + | ? | + | ? | ? | + | ? | + | + | ? | + | + | + |
| Recker | 2004 | + | + | + | ? | ? | + | + | + | + | + | + | + | ? |
| **Minodronate** | | | | | | | | | | | | | | |
| Matsumoto | 2009 | + | + | + | ? | ? | + | + | + | + | ? | + | + | ? |
| **Pamidronate** | | | | | | | | | | | | | | |
| Brumsen | 2002 | ? | ? | + | + | + | + | + | + | + | + | + | + | + |
| **Calcitonin** | | | | | | | | | | | | | | |
| Chesnut | 2005 | + | ? | + | ? | ? | ? | + | + | + | + | + | + | + |
| Hodsman | 1997 | ? | ? | + | ? | ? | + | ? | + | + | + | ? | + | ? |
| Peichl | 1999 | ? | ? | ? | - | - | ? | ? | + | + | ? | ? | + | ? |
| **HRT** | | | | | | | | | | | | | | |
| Gutteridge | 2002 | + | ? | ? | - | - | + | ? | + | + | + | ? | + | ? |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lufkin | 1992 | ? | ? | + | + | + | + | ? | + | + | + | ? | ? | ? |
| **PTH** | | | | | | | | | | | | | | |
| Fujita | 2014 | + | + | + | ? | ? | + | ? | + | + | + | + | + | + |
| Greenspan | 2007 | + | + | + | ? | ? | + | - | + | + | ? | + | + | + |
| Nakamura | 2012 | + | + | + | + | + | + | ? | + | + | + | + | + | + |
| Neer | 2001 | ? | ? | + | ? | ? | + | + | + | + | + | ? | + | + |
| **Denosumab** | | | | | | | | | | | | | | |
| Boonen | 2011 | ? | ? | + | ? | ? | + | ? | + | + | + | + | + | + |
| Nakamura | 2014 | ? | ? | + | ? | ? | + | ? | + | + | + | + | + | ? |
| **SERMs** | | | | | | | | | | | | | | |
| Palacios | 2015 | + | + | + | ? | ? | - | + | + | + | ? | + | + | + |
| Ettinger | 1999 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Lufkin | 1998 | + | ? | + | ? | ? | + | ? | + | + | + | + | + | ? |
| **Between medication** | | | | | | | | | | | | | | |
| **Ibandronate vs. Risedronate** | | | | | | | | | | | | | | |
| Nakamura | 2013 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **Risedronate vs. Etidronate** | | | | | | | | | | | | | | |
| Kushida | 2004 b | ? | ? | + | + | + | + | ? | + | + | + | ? | + | ? |
| **Risedronate vs. Teriparatide** | | | | | | | | | | | | | | |
| Hadji | 2012 | ? | ? | ? | + | ? | + | ? | + | + | + | + | + | ? |
| Kendler | 2017 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **Monoclonal antibody vs. Alendronate** | | | | | | | | | | | | | | |
| Nakamura | 2014 | ? | ? | + | ? | ? | + | ? | + | + | + | + | + | ? |
| Saag | 2017 | + | + | + | ? | ? | + | ? | + | + | + | + | + | + |
| **Etidronate vs. HRT** | | | | | | | | | | | | | | |
| Wimalawansa | 1998 | + | ? | + | ? | ? | + | ? | + | + | + | ? | + | ? |

a, random sequence generation (selection bias)
b, allocation concealment (selection bias)
c, group similarity at baseline (selection bias)
d, blinding to patients (performance bias)
e, blinding to care providers (performance bias)
f. influence of co-interventions (performance bias)
g, compliance with interventions (performance bias)
h, blinding to outcome assessors (detection bias) - Fracture
i, timing of outcome assessments (detection bias)
j, incompleter outcome data (attribution bias) - lost ratio
k, incomplete outcome data (attribution bias) - ITT or modified ITT
l, selective reporting (reporting bias)

m, other source of bias

Reasons of being rated as unclear or high risk of bias.

| Article ID | Year | Reasons |
|---|---|---|
| **Zoledronic acid** | | |
| Nakamura | 2017 | d, Not reported; e, Not reported; g, Not reported; |
| **Alendronate** | | |
| Black | 1996 | |
| Kushida | 2004a | a, Not reported; b, Not reported; d, Not reported; e, Not reported; g, Not reported; m, Conflict of interest was not stated. |
| Liberman | 1995 | a, Not reorted; b, Not reported; d, Open label; e, Open label; g, Not reported; m, Conflict of interest was not stated. |
| **Risedronate** | | |
| Clemmesen | 1997 | a, Not reported; b, Not reported; d, No description of the appreance of geltain capsules; e, Not reported; j: 32% i n3 years; m, the criteria of fracture were different in different centers. |
| Fogelman | 2000 | a, Not reported; b, Not reported; d, Not reported; e, Not reported; g, Not reported; j, 21% in 2 years; n, no conlifct of interest stated. |
| Harris | 1999 | j, 42% in 3 years. |
| Reginster | 2000 | a, Not reported; b, Not reported; d, Not reported; e, Not reported;, k, Not reported; m, Not reported. |
| Sorensen | 2003 | a, Not reported; b, Not reported; c, Participants in risedronate group already had 3 years risedronate; d, Not reported; e, Not reported; g, Not eported; m, Conflict of interest was not stated. |
| **Etidronate** | | |
| Guanabens | 2000 | a, Not reported; b, Not reported; d: Not reported; e, Not reported; f, Participants in etidronate group did not receive calcium on the day they receive etidronate; g, Significant (p=0.01) difference in complaince of medications between groups; j, 34% in 3 years; k, no report of adverse events; m, no conflict of interest reported. |
| Harris | 1993 | b, Not reported; d, Not reported; e, Not reported; g, Not reported; m, Conflict of interest was not stated. |
| Lyritis | 1997 | a, Not reported; b, Not reported; d, Open label design; e, Open label design; g, Not reported; k, Not reported; m, Conflict of interest is not stated. |

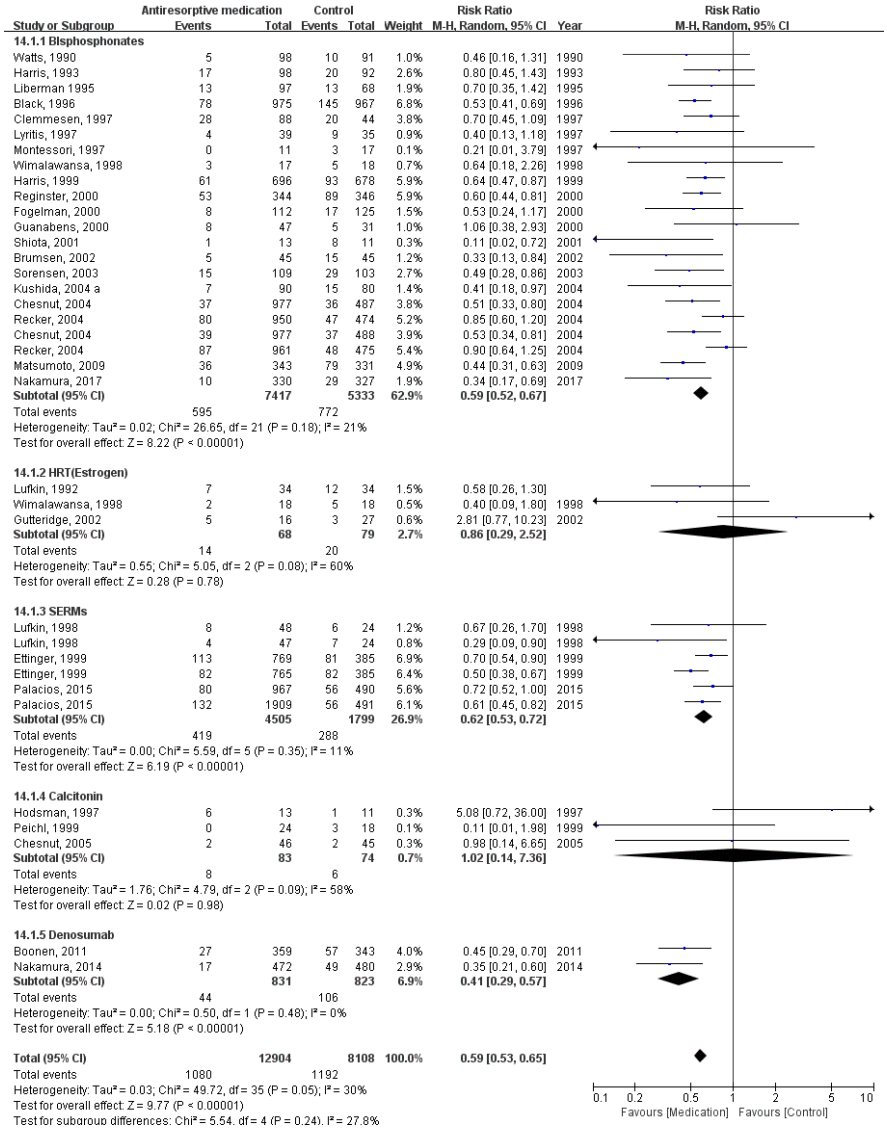
| | | |
|---|---|---|
| Montenssori | 1997 | b, Not reported; d, Not reported; e, Not reported; f, Calcium intake is different between groups; g, Not reported; m, Not reported |
| Shiota | 2001 | a, Not reported; b, Not reported; d, Not reported; e, Not reported; f, Calcium and alfacalcidol supplement were different between groups; g, Not reported; k, Not reported;l, Safety data was not reported; m, Conflict of interest was not stated. |
| Watts | 2014 | b, Not reported; d, Not reported; e, Not reported; g, Not reported; k, Not reported; m Conflict of interest was not stated. |
| Wimalawansa | 1998 | b, Not reported; d, Not reported; e, Not reported; g, Not reported; k, Not reported; m, Conflict of interest was not stated. |
| **Ibandronate** | | |
| Chesnut | 2004 | b, Not reported; d, Not reported; e, Not reported; j, 34% in 3 years. |
| Recker | 2004 | d, Not reported; e, Not reported; g, Not reported; e, Not reported; j, Not reported. |
| **Minodronate** | | |
| Matsumoto | 2009 | d, Not reported; e, Not reported; j, 30.11% in 3 years. |
| **Pamidronate** | | |
| Brumsen | 2002 | a, Not reported; b, Not reported. |
| **Calcitonin** | | |
| Chesnut | 2005 | b, Not reported; d, Not reported; e, Not reported. |
| Hodsman | 1997 | a, Not reported; b, Not reported; d, Not reported; e, Not reported; g, Not reported; j, 23% in 2 years; k, Not reported; m,, Conflict of interest was not stated. |
| Peichl | 1999 | a, Not reported; b, Not reported; c, Concommitant medication history is different between participants; d, Open design; e, Open design; f, Vitamin D is only offered for control group; g, Not reported; j, Not reported; k, Not reported; m, Conflict of interest is not stated. |
| **HRT** | | |
| Gutteridge | 2002 | b, Not reported; b, Not reported; c, Age and CaE at the baseline is different between groups; d, Open design; e, Open design; g, Not reported; j, 24% in 2 years; k, Not reported; m, Conflict of interest is not stated. |

| | | |
|---|---|---|
| Lufkin | 1992 | b, Not reported; b, Not reported; g, Not reported; j, Not reported; m, Conflict of interest was not stated. |
| **PTH** | | |
| Fujita | 2014 | d, No specific description; e, No specific description; g, Not reported. |
| Greenspan | 2007 | c, Not reported; d, Not reported; g; Compliance was different among different groups.j, 32.82% in 18 months. |
| Nakamura | 2012 | g, Not reported. |
| Neer | 2001 | a, Not reported; b, Not reported; d, Not reported; e, Not reporeted; j, Not reported. |
| **Denosumab** | | |
| Boonen | 2011 | a, Not reported; b, Not reported; d, Not reported; e, Not reported; g, 5979 (76% received all injections (Cummings, 2009). |
| Nakamura | 2014 | a, Not reported; b, Not reported; d, Not reported; e, Not reported; g, Not reported; m, Sponsors were resposible for data collection and analysis.. |
| **SERMs** | | |
| Palacios | 2015 | d, Not reported; e, Not reported; f, Significantly ($p<0.01$) higher proportion of Placebo-treated women used concomitant bone-active nonstudy mdications; j, 25% in extension 2; 77% in whole trial ( 7 years). |
| Ettinger | 1999 | |
| Lufkin | 1998 | b, Not reported; d, Not reported; e, Not reported; g, Not reported; j, Not reported; m, Conflict of interest is not stated. |
| **Between medication** | | |
| **Ibandronate vs. Risedronate** | | |
| Nakamura | 2013 | |
| **Risedronate vs. Etidronate** | | |
| Kushida | 2004b | a, Not reported; b, Not reported; g, Not reported; k, 23% in 2 years; k, per protocol set was used; m, Conflict of interest was not stated. |
| **Risedronate vs. Teriparatide** | | |
| Hadji | 2012 | a, Not reported; b, Not reported; c, BMD of femoral neck is different between groups; f, Not reported; g, Not reported; j, 26% in 18 months; n, Data was collected and analyzed by sponsor. |

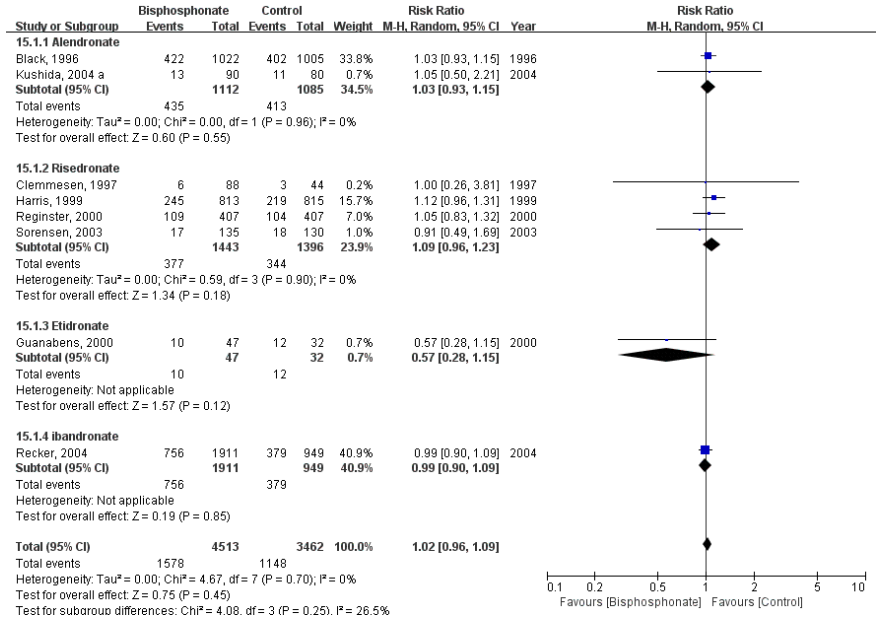
| | | |
|---|---|---|
| Kendler | 2017 | |
| **Monoclonal antibody vs. Alendronate** | | |
| Nakamura | 2014 | a, Not reported; b, Not reported; d, Not reported; e, Not reported; g, Not reported; m, Sponsors were resposible for data collection and analysis.. |
| Saag | 2017 | d, Not reported; e, Not reported; g, Not reported; h, Not reported. |
| **Etidronate vs. HRT** | | |
| Wimalawansa | 1998 | b, Not reported; d, Not reported; e, Not reported; g, Not reported; k, Not reported; m, Conflict of interest was not stated. |

## Supplementary material 3. Forest plot of secondary outcomes.

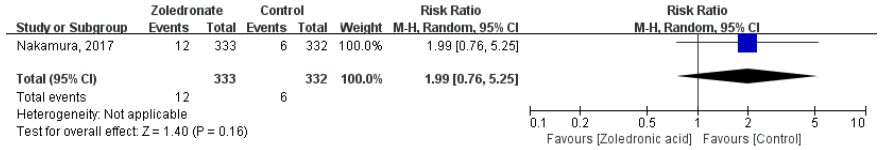
a. Forest plot. Effect of antiresorptive medications preventing secondary vertebral fracture.



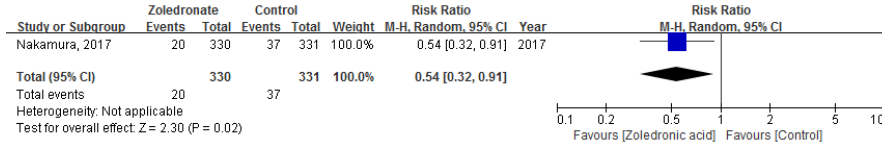
b. Forest plot. GI complaints of bisphosphonates.



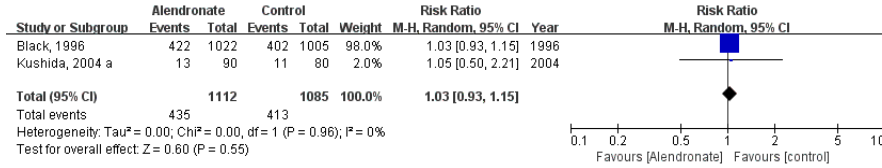

c. Forest plot. Discontinuation due to AEs – Zoledronate



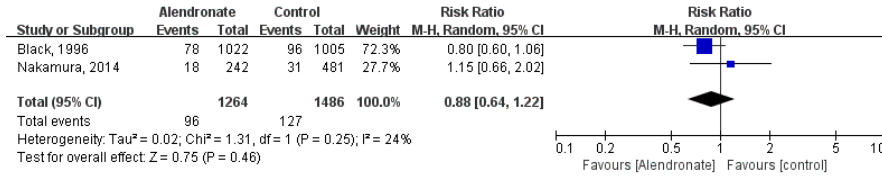

d. Forest plot. Preventing non-vertebral fracture – Zoledronate



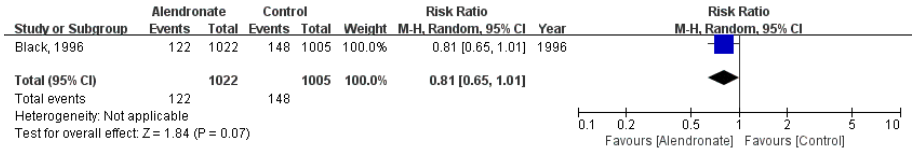

e. Forest plot. GI complaints - Alendronate



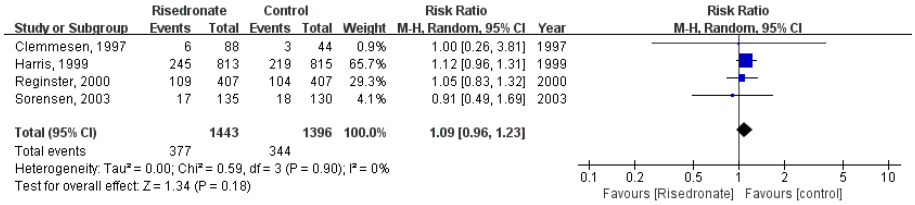

f. Forest plot. Discontinuation due to AEs – Alendronate



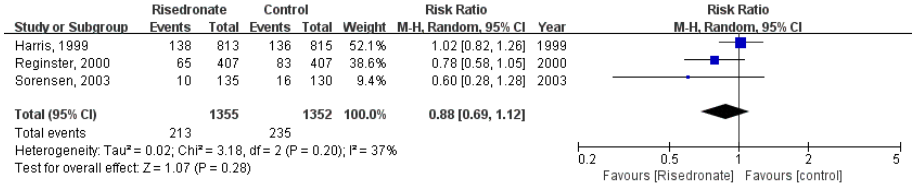

g. Forest plot. Non-vertebral fracture – Alendronate



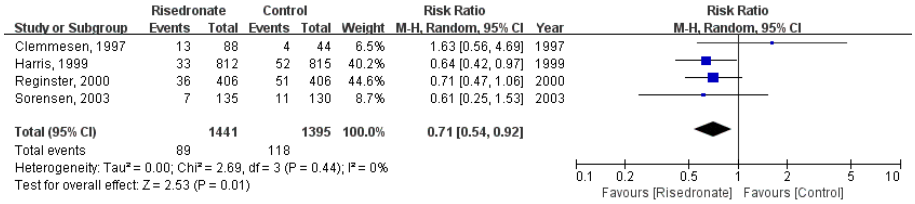

h. Forest plot. GI complaints – Risedronate



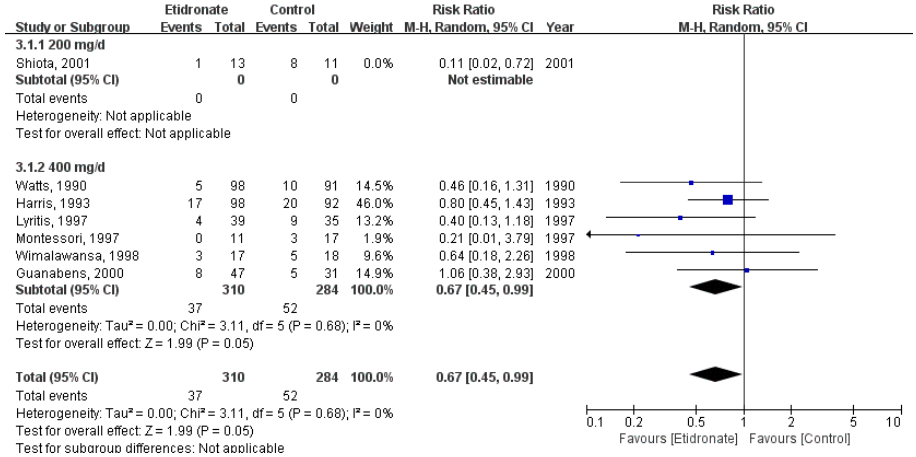

i. Forest plot. Discontinuation due to AEs - Risedronate



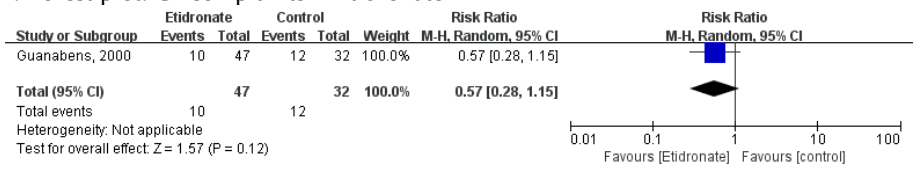

j. Forest plot. Non-vertebral fracture – Risedronate



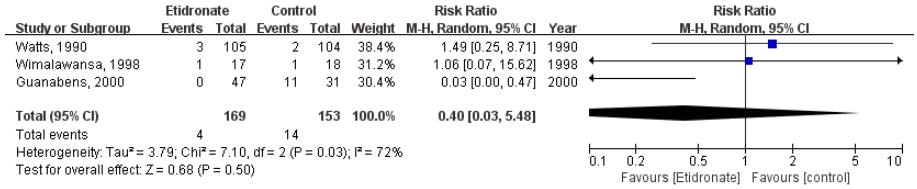

k. Forest plot. Sensitivity test. Excluding a study with a small sample size and big variance with other studies.



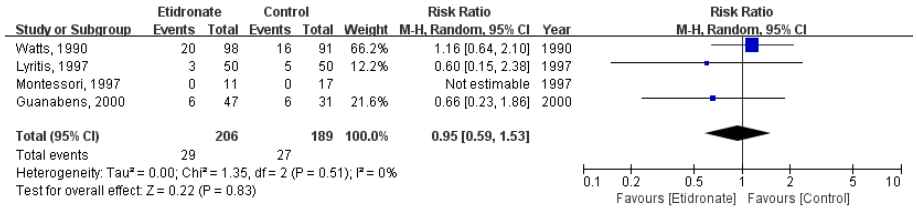

l. Forest plot. GI complaints – Etidronate



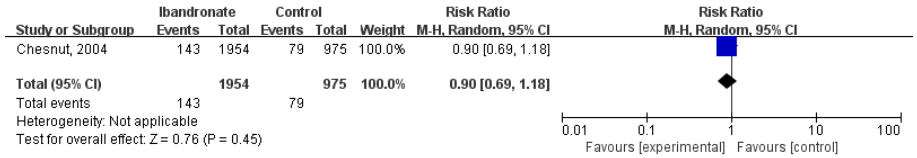
m. Forest plot. Discontinuation – Etidronate



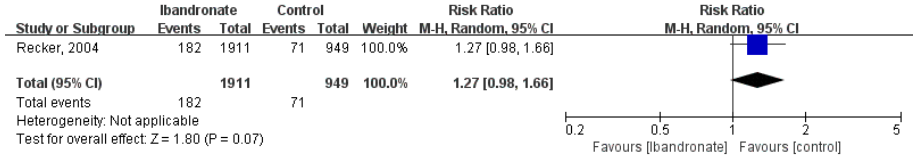

n. Forest plot. Non-vertebral fracture – Etidronate



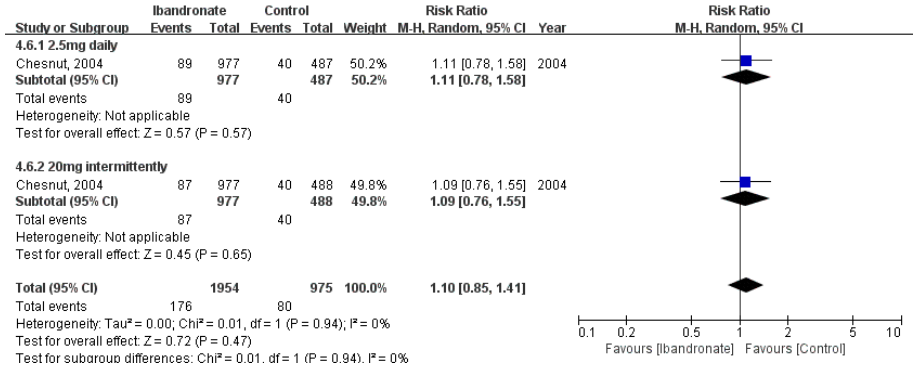

o. Forest plot. Discontinuation due to AEs – Ibandronate (sufficient dose)



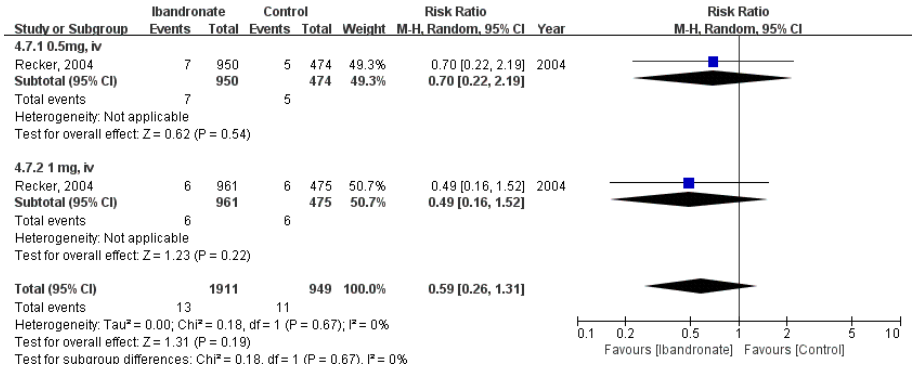

p. Forest plot. Discontinuation due to AEs – Ibandronate (insufficient dose)



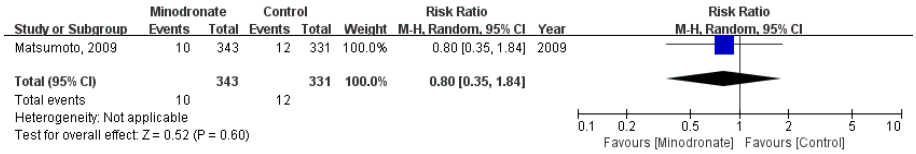

q. Forest plot. Non-vertebral fracture – Ibandronate (sufficient dose)



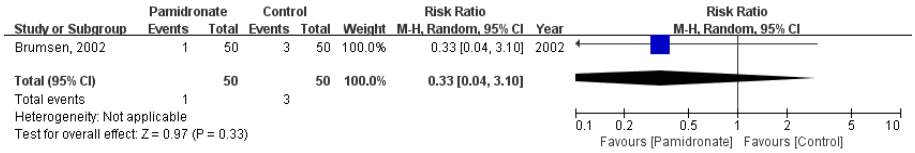

r. Forest plot. Non-vertebral fracture – Ibandronate (insufficient dose)



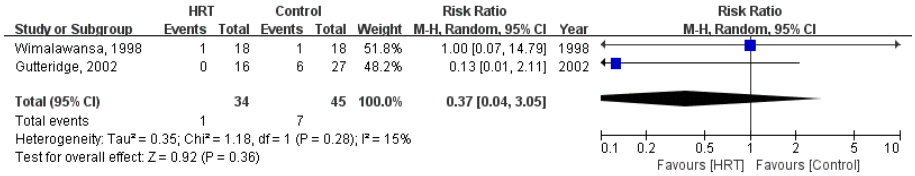

s. Forest plot. Non-vertebral fracture – Minodronate



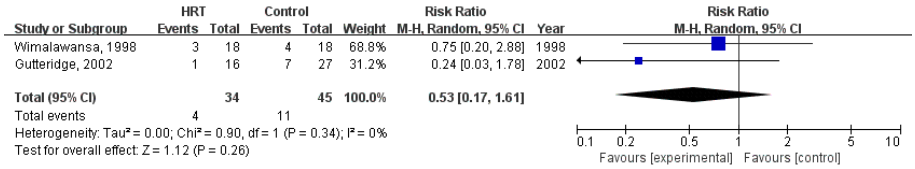

t. Forest plot. Non-vertebral fracture – Pamidronate



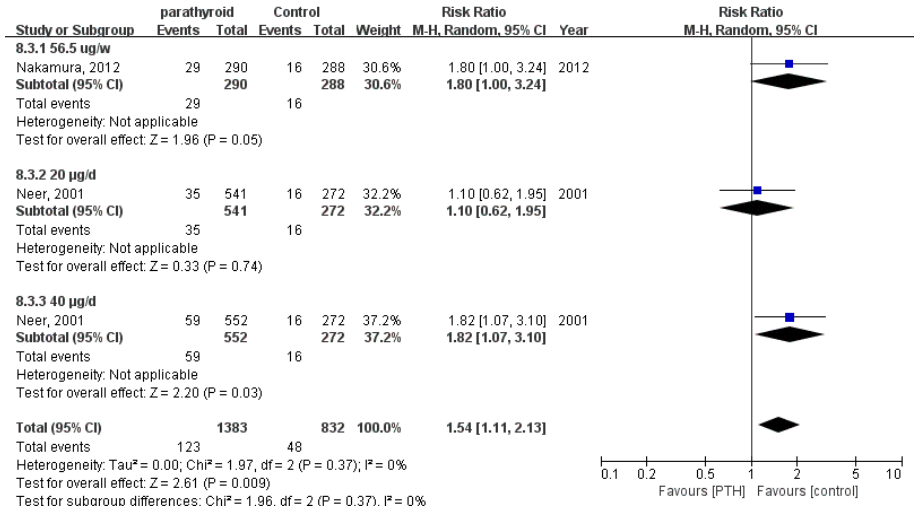

u. Forest plot. Non-vertebral fracture – HRT



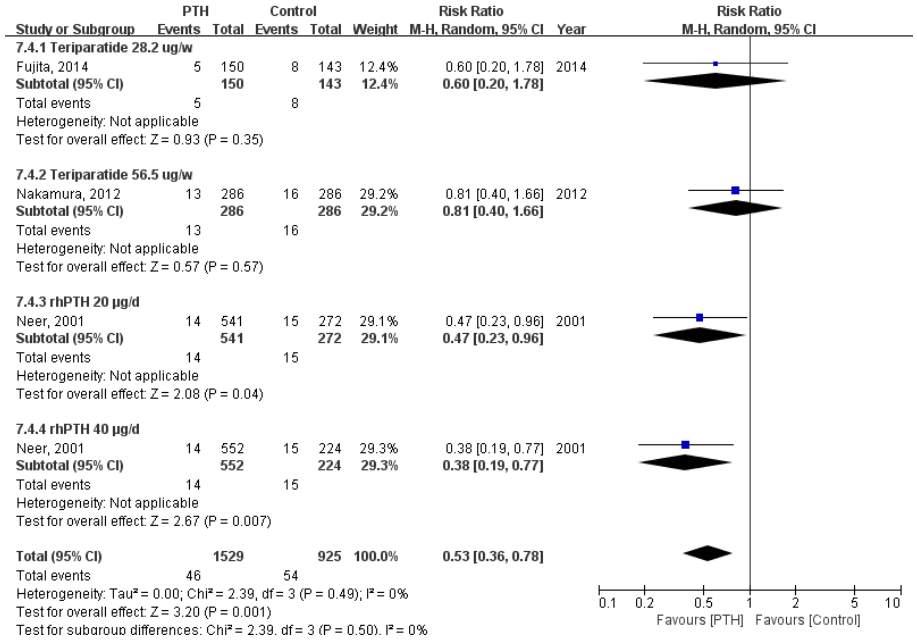

v. Forest plot. Discontinuation due to AEs – HRT



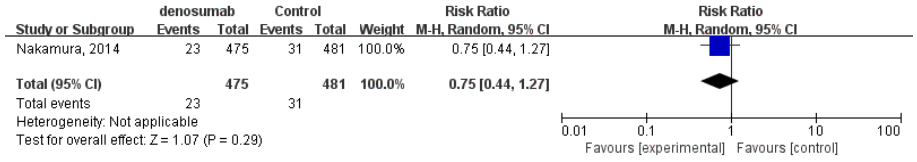

w. Forest plot. Discontinuation due to AEs – PTH




x. Forest plot. Non-vertebral fracture – PTH

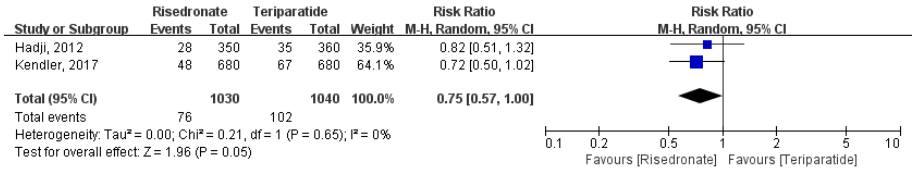



y. Forest plot. Discontinuation due to AEs – Denosumab

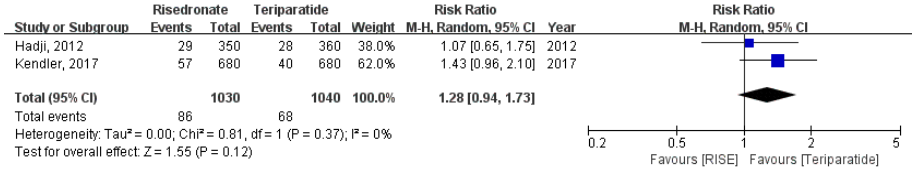



z. Forest plot. Non-vertebral fracture – Denosumab

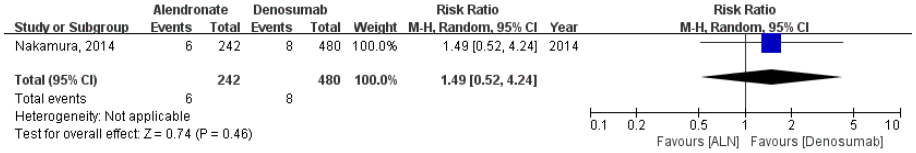

aa. Forest plot. Discontinuation – Risedronate vs. PTH



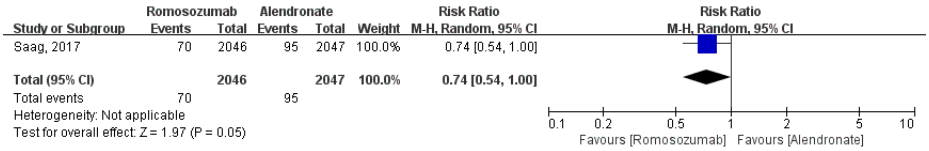

bb. Forest plot. Non-vertebral fracture – Teriparatide vs. Risedronate




cc. Forest plot. Non-vertebral fracture – Alendronate vs. Denosumab




dd. Forest plot. Non-vertebral fracture – Romosozumab vs. Alendronate



**Author contributions**:

Y-Z J: carried out the experiment, collected and analyzed data, and wrote the manuscript.

JHL: conceived and designed the study and analysis, verified the analytical methods, interpreted data, applied critical revision of the manuscript for important intellectual content , obtained funding, and supervised the whole study.

# 논 문 초 록

# DRG2 유전자의 골대사에 대한 영향

골다공증은 골량 감소와 골 미세구조 이상을 야기하는 질환으로 골형성과 골흡수간 불균형에 의해 발생하며, 저손상 골절의 위험을 증가시키는 질환이다. 새로운 골다공증 치료법을 개발하기 위하여 다음 3 단계의 연구를 진행하였다. 골다공증 환자는 골대체제가 필요하기 때문에 재조합 골형성단백질 제 2 형(rhBMP-2) 의 골형성 효능 연구, 현재 사용되는 골다공증 치료제의 2 차 골절예방 효능에 관한 메타 분석 연구와 함께, developmentally regulated GTP binding protein 2 (Drg2) 의 골 항상성에 미치는 영향을 연구하였다. 첫번째 연구에서는, 대장균 유래 골형성 단백질 제 2 형(ErhBMP-2)과 동물세포 유래 골 형성 단백질 제 2 형(CrhBMP-2)의 골유도성을 인간 간엽줄기세포 및 랫드 두개골 결손모델에서 비교하였고 두번째 연구에서는, 기존 골다공증 치료제가 골다공증성 척추 및 비척추 골절을 예방하는 효과에 대하여 메타분석을 시행하였으며 세번째 연구에서는, 골다공증이 있는 환자군과 정상 대조군의 골수 유래 간엽줄기세포 에서

*DRG2*의 발현을 비교하였고, *Drg2* 결손 마우스를 제작하여 대조군과 골 표현형의 차이를 분석하였다. 또한 *Drg2* 가 조골세포의 분화에 관여하는 기전과 신호전달 연구를 수행하였다. 첫번째 연구에서 ErhBMP-2 가 탈회골기질을 담체로 사용할 때 CrhBMP-2 와 유사한 골 유도능력을 가질 수도 있음을 확인하였으며 두번째 연구에서는 골다공증 환자의 골절 병력과 무관하게 약물치료가 지속적인 영향이 있을 수 있다는 것을 알 수 있었습니다. 세번째 연구에서 골다공증 환자는 *Drg2* mRNA 발현 정도가 정상 대조군보다 더 높았고, Drg2 결손마우스는 난소절제술을 시행하였을 때 골량 보호 효과가 관찰되었다. *Drg2* 결손 마우스에서 얻은 골수유래 대식세포를 대조군과 비교했을 때, 파골세포 분화력이 낮았으며 골수유래 줄기세포의 조골세포 분화력은 높았다. 또한 마우스 MC3T3-E1 세포에서의 *Drg2* 발현을 억제시키면 정식 및 비정식 BMP 경로를 통하여 조골세포의 분화가 증가하였다. 결론적으로, 본 연구에서는 ErhBMP-2 가 골다공증성 골절에서 동화제제로서 사용 될 수 있는 가능성과 *Drg2* 유전자가 골 재형성에 있어 중요한 조절 인자임을 확인하였다.

**주요어:** 골형성단백질 2; developmentally regulated GTP-binding protein 2; 골다공증; 골재형성; 골형성; 조골세포; 파골세포;

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