Microporous polysaccharide multilayer coated BCP composite scaffolds with immobilized calcitriol promote osteoporotic bone regeneration both in vitro and in vivo

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SUPPLEMENTAL METHODS

Bone mesenchymal stem cells (BMSCs) and bone marrow monocytes (BMMs) cultures

In brief , the lower extremities of rats were aseptically cut after euthanasia and the bones were dissected free of soft tissues. Marrow cavities of both femur and tibia were flushed with DMEM medium supplemented with 10% FBS, 1% penicillin and streptomycin. About 1×105 cells were seeded in 25 cm2 culture flasks and grown in a humidified atmosphere of 5% CO2 at 37 °C. Non-adherent cells were removed by frequent medium change after 24 hours. The remaining adherent colonies were cultured for 14 days until reach 80% confluency and passaged after digestion with 0.25% trypsin for 3 minutes. The passage two cells were used for further experiments in the study. The differences of morphologic alteration and phenotype changes

between BMSCs derived from sham-rats and OVX-rats were determined by microscope and flow cytometry. (Figure S3)

BMMs were extracted from the long bone marrow of SD rats at 10 weeks of age using Ficoll plus lymphocyte separation medium gradient (ICN Biomedicals, Santa Ana, CA). The extracted cells were collected by centrifugation, cultured in a 75 cm^2 culture flask with α-MEM (adding 0% FBS, 1% penicillin and streptomycin and 50 ng/mL M-CSF). The culture medium was changed every 2 days, and cells were passaged when 90%-100% confluence were attained. BMMs from passages one to three were used in this study.

Flow cytometry analysis

To determine the phenotype of BMSCs derived from sham group and OVX one, cell surface markers were stained by incubating cells with each corresponding antibody for 30 min in dark according to the manufactory instructions. Flow cytometry was performed on a FACScan flow cytometer (Beckman Coulter, IN, USA). FITC conjugated monoclonal antibodies against CD44, CD45, CD29, CD90 and CD34 were used in this experiments.

Western blot

The total protein extracted from cells was isolated using RIPA lysis buffer with 1 mM PMSF (Phenylmethanesulfonyl fluoride) and on the ice for 10 min followed by 15 min centrifugation at 12000 rpm and 4°C, and then protein concentration was measured using the BCA protein assay kit (Beyotime). 40 μg of protein was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). After blocking with 5% nonfat milk for 2 h, the membranes were incubated with the primary antibody against collagen I (1:1000), OCN (1:250), Runx-2 (1:500), CaSR(1:500), β-actin (1:5000), PCNA (1:1000), CyclinD1 (1:1000), ICAM-1 (1:1000), c-Fos (1:1000) and NFATc1 (1:1000) overnight at 4 °C, and followed by subsequently incubation with respective secondary antibodies for 2 h at room temperature. After 3 times washing with TBST, the blots were visualized by electrochemiluminescence

plus reagent (Invitrogen). Finally, the intensities of these blots were quantified with Image Lab 3.0 software (Bio-Rad).

Alkaline phosphatase (ALP) activity

The ALP levels of the cells in different groups were examined by using an alkaline phosphatase colorimetric assay kit (Beyotime, shanghai, China) (n = 5) at day 7, 14 and 21. Quantitative measurements were normalized by the total protein contents which were determined by a BCA protein assay kit (Beyotime, shanghai, China). At day 14, the cells were washed with PBS and fixed in 4% paraformaldehyde. BCIP/NBT ALP color development kit (Beyotime, Shanghai, China) was used to perform the ALP staining.

Immunohistochemically examination

The sections embedded in paraffin were deparaffinized and rehydrated and then the endogenous peroxidase activity was need to be blocked by 3% hydrogen peroxide. After that, the sections were incubated with 0.4% pepsin (Sangon Biotech, Shanghai, China) in 5 mM HCl at 37 ℃ for 20 min for antigen retrieval. Nonspecific binding sites was blocked by 5% bovine serum albumin for 30 min at room temperature. The sections were then incubated with the primary antibody (anti-CaSR, 1:100; anti-OCN 1:50, anti-PCNA, 1:200; anti-ICAM-1 1:100) overnight at 4 ℃. Finally, the sections were incubated with an appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and counterstained with hematoxylin. Images were saved at 400 magnification and quantitated by using Image-Pro Plus software, version 6.0 (Media Cybernetics, Rockville, MD, USA). Five fields of each section were selected randomly and the integral absorbance values of each field were used as indicators of CaSR, OCN, PCNA and ICAM-1 levels. At least five sections from each specimen were used to analyze the expression of these proteins.

Immunofluorescences

For double labeled immunofluorescence staining, the OVX-rBMSCs were

seeded in glass plates in a six-well plate and then the cells were treated with Cal alone and Cal plus NPS-2143 as mentioned above. After treatments. the samples were rinsed three-times in PBS before fixation using 4% paraformaldehyde and followed by permeation using the 0.1% Triton X-100 diluted in PBS for 15 min. Then the cells were blocked with 5% bovine serum albumin for 1 h at 37 °C, rinsed with PBS and incubated with primary antibodies in cocktails of CaSR (1:100) and Runx-2 (1:400), which was diluted in PBS, in a humid chamber overnight at 4 °C**.** On the next day, the glass plates were washed and incubated with Alexa Fluor®488 labeled or Alexa Fluor®594 conjugated second antibodies (1:400) for 1 h at room temperature and labeled with DAPI for 5 min. Finally, three fields of each slides were chosen randomly for microscopic observation with a fluorescence microscope (Olympus Inc., Tokyo, Japan).

Molecular docking

The molecular structure of Cal was drawn with ChemBioDraw and ChemBio3D was used to minimize its energy. The crystal structure of human CaSR protein with the agonist of tryptophan derivative (PDB code 5FBH) was obtained from the Protein Data Bank (https://www.rcsb.org/). After being minimized using PyMoL (version 1.7.6), the lowest energy conformations for docking were determined via default parameters. The protein-ligand docking analysis was conducted using the AutoDock Tools (version 1.5.6) which can provide the ligand binding flexibility with the binding pocket residues. The images of 3D views were finally generated using the UCSF PyMoL and the image of 2D view was generated by Ligplot+ software.

Scanning electron microscope (SEM) observation

For the SEM observation, the PEM-coated scaffolds and glass plates were used after dried them in a vacuum oven. For observations of the cell morphology on scaffold, OVX-rBMSCs were seeded onto each scaffold for 14 days. Cells were then washed in PBS and fixed with 2.5% glutaraldehyde for 1 hour at room temperature,

followed by washing in the same buffer. The samples were dehydrated using a graded ethanol series from 50 to 100% and dried in vacuum oven for 4 hours. Finally, all the samples were sputtered with a thin layer of gold and observed with a Hitachi S 4000 SEM (Hitachi).

Cell viability in scaffolds

The cytotoxicity of scaffolds on OVX-rBMSCs were detected with the cell counting kit-8 (CCK-8; Dojindo Co, Kumamoto, Japan) according to the manufacturer's protocol. At the indicated time (3, 7, 14 days), the scaffolds with cells mounted were transferred to a new plate. Then, the cells were washed with phosphate-buffered saline (PBS), and 500 μl of DMEM containing 50 μl of CCK-8 solution was added to each well of the plate and incubated for another 2 h at 37 °C. The absorbance at 450 nm of the wells was then measured using a micro-plate reader. Additionally, the blank group (cells are directly seeded in the 24-wells plate) in 3 days, was used as a contrast to calculate the seeding rates of each scaffold.

qRT-PCR

The total RNA of OVX-rBMSCs cultured in different scaffold were extracted using TRIzol reagent (Invitrogen). 1000ng of total RNA was reverse transcribed to synthesize cDNA (MBI Fermantas, Germany). For the quantitative realtime PCR (qPCR), a total 10 μl of reaction volume was used, including 5 μl of 2 × SYBR Master Mix, 0.25 μl of each primer and 4.5 μl of diluted cDNA. Parameters of RT-PCR were: 10 min 95 °C, followed by 40 cycles of 15 s 95 °C and 1 min 60 °C. The reaction was performed using CFX96Real-Time PCR System (Bio-Rad Laboratories, California, USA). The cycle threshold (Ct) values were collected and normalized to the level of GAPDH. The level of relative mRNA of each target gene was calculated by using the $2^{-\triangle\triangle Ct}$ method. The primers of Cola1, OCN, Runx-2 and CaSR were designed with the aid of NCBI Primer-Blast Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), which were listed as follows: Cola1 (F) 5′-GACCTCCGGCTCCTGCTCCT-3′ (R)

5′-TCGCACACAGCCGTGCCATT-3′; OCN (F) 5′- CGGCCCTGAGTCTGACAAA -3′, (R) 5′- ACCTTATTGCCCTCCTGCCTT -3′; Runx-2, (F) 5′-TGTCCATCTCCAGCCGTGTC -3′, (R) 5′- TCTGTCTGTGCCTTCTTGGTTC -3′; CaSR (F) 5′-TAGCAGCCCGCGCCACTCTA-3′, (R) 5′-TGCCTCTCTGGCTGCGGGAA-3′; ALP (F) 5′-AACGTGGCCAAGAACATCATCA-3′ (R) 5′-TGTCCATCTCCAGCCGTGTC-3′; GADPH (F) 5′-AGAAGGTGGTGAAGCAGGCGG-3′, (R) 5′-ATCCTTGCTGGGCTGGGTGG-3′.

Osteoclastogenesis assay

In *vitro*, BMMs were plated at a density of 1×10^4 cell/well in 24-well plates and treated in complete medium containing M-CSF and RANKL (50 ng/mL) with or without different scaffolds. The medium were replaced every 2 days. After 5 days, cultured cells were fixed with 0.25% glutaraldehyde for 20 minutes at room temperature and stained for tartrate-resistant acid phosphatase (TRAP) using the Acid Phosphatase, Leukocyte TRAP Kit (Sigma), following the manufacturer’s protocol. The images were captured under a light microscope, and the cells with more than 3 nuclei were determined as TRAP-positive multinucleated cells.

In *vivo*, the tissue sections of different groups were fixed and stained also with the Acid Phosphatase, Leukocyte TRAP Kit (Sigma) following the manufacturer's protocol. The stained sections were photographed digitally under a microscope.

SUPPLEMENTAL FIGURES AND LEGENDS

Figure S1.

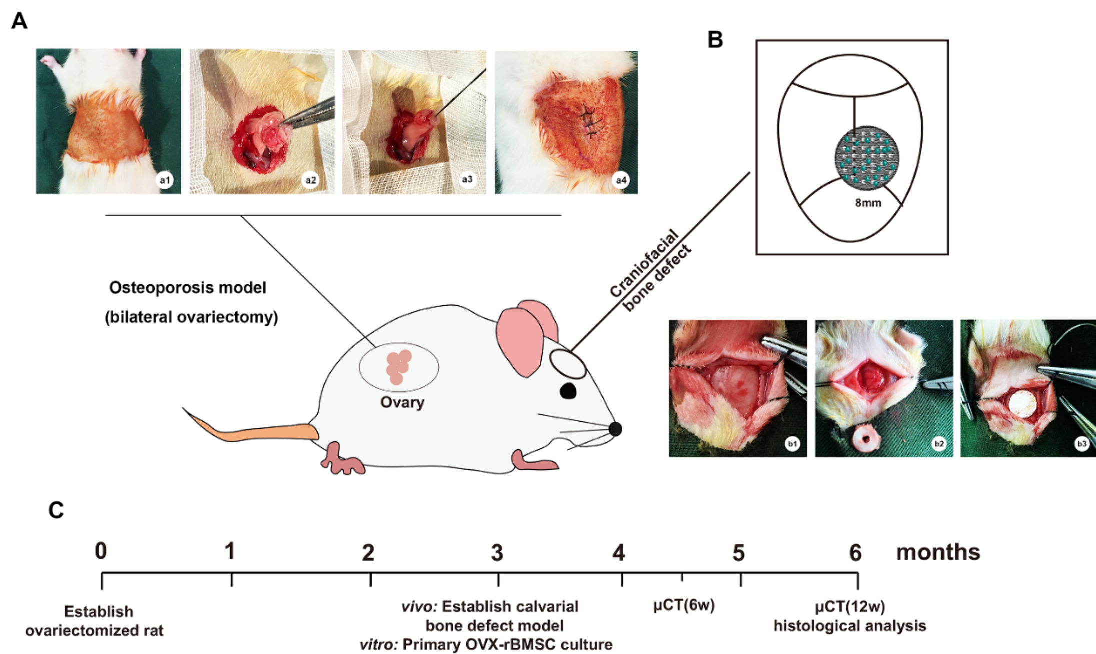


Figure S1. Surgical procedure (A) Rat osteoporosis model was created by bilateral ovariecotomy (B) 8-mm cranial defect was created with intersection of sagittal suture and coronal suture as center of round defect. (C) Time point of surgical procedures.

Figure S2.

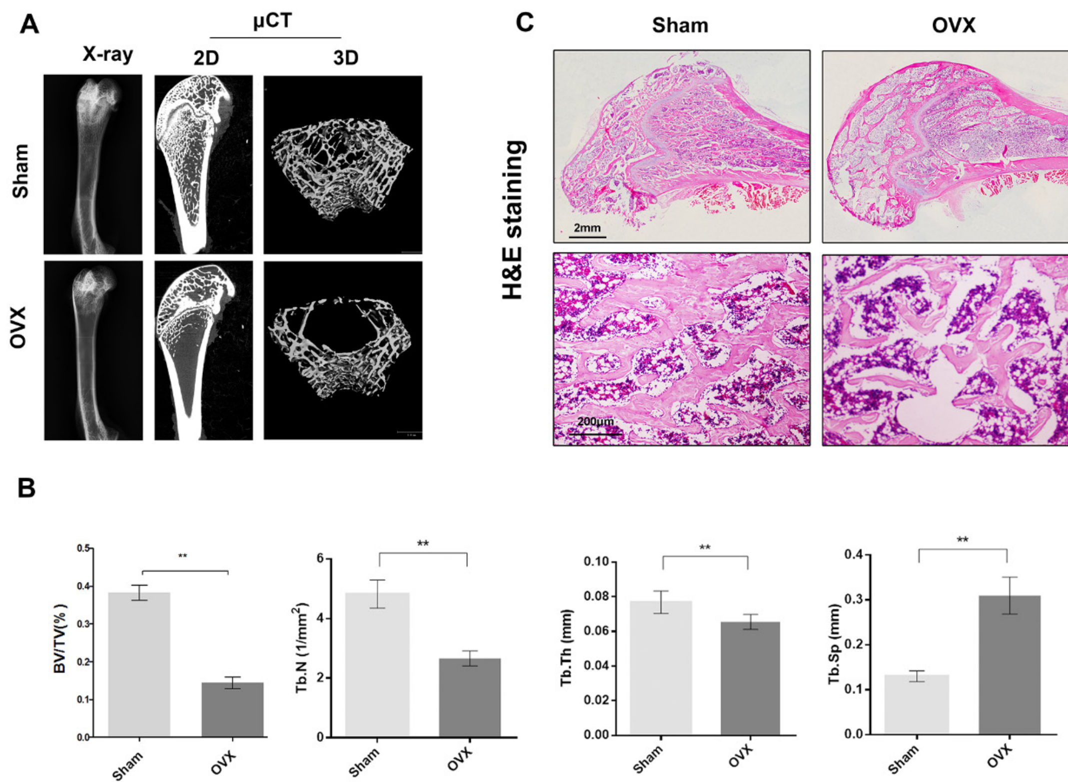
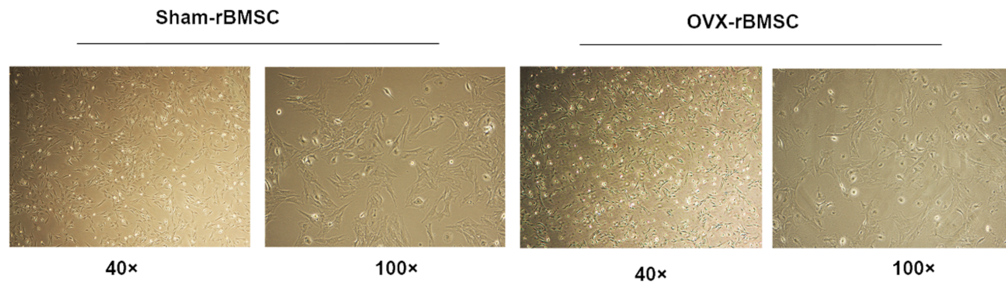


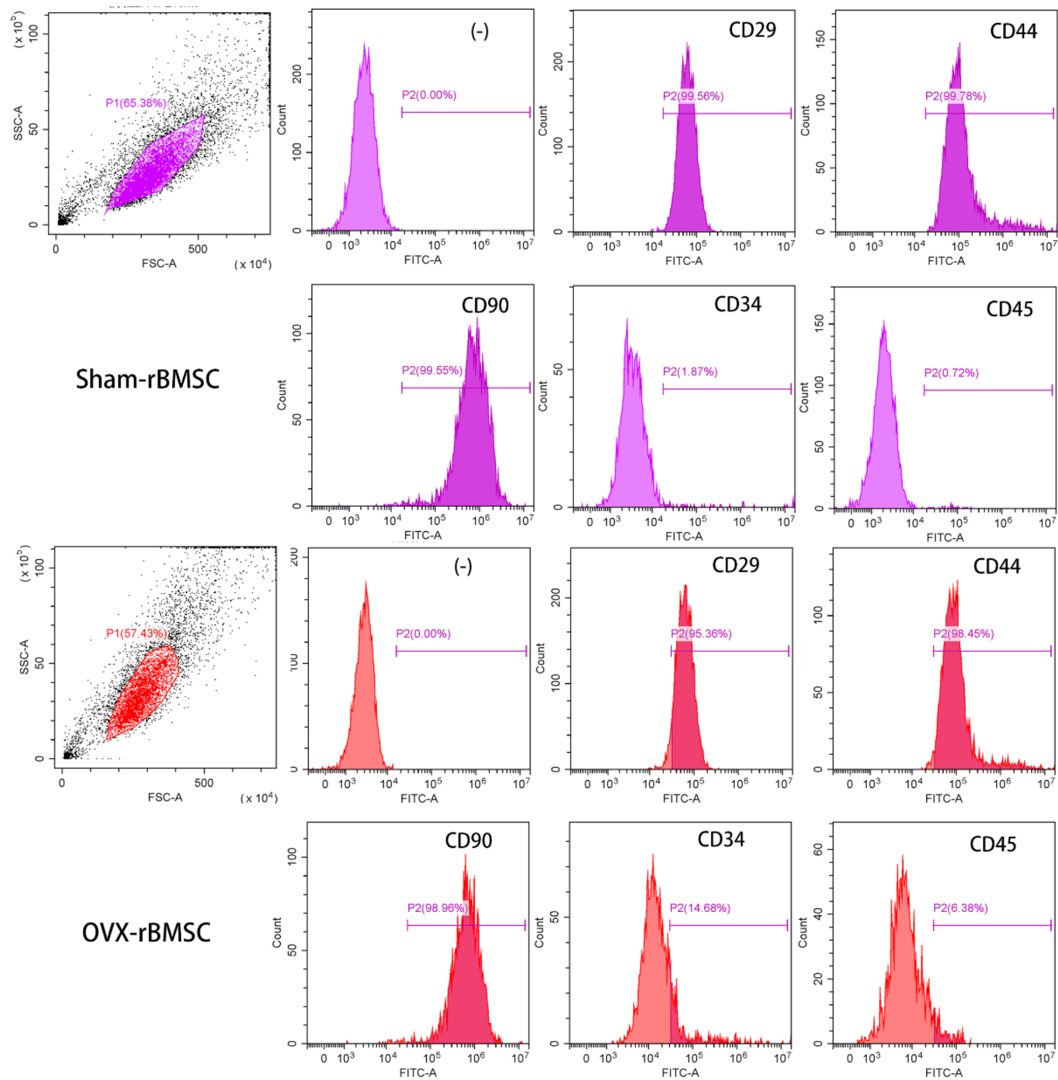
Figure S2. Determination of osteoporosis model (A) Representative μCT images of microarchitecture in distal femur from sham and OVX rats, Bone radiographs of excised femur were taken with Digital X-ray machine (Kubtec, USA) and the X-ray source voltage set at 55 kV and beam current set at 145 μA. For microarchitecture, trabecular bone architecture of the left femora and new bone formation in calvarium were determined by using microcomputed tomography (μCT) (MicroCT m100, SCANCO Medical, Switzerland) (B) Quantitative analysis of the trabecular bone volume/total volume (BV/TV), Trabecular Number (Tb.N), Trabecular Thickness, (Tb.Th), and Trabecular Separation/Spacing , (Tb.Sp). (C) H & E staining of the tibia of sham and OVX rats. The data in the figures were represented as the means ± S.D. Significant differences between the different groups are indicated as **P<0.01, *p<0.05, n=5.

Figure S3

A



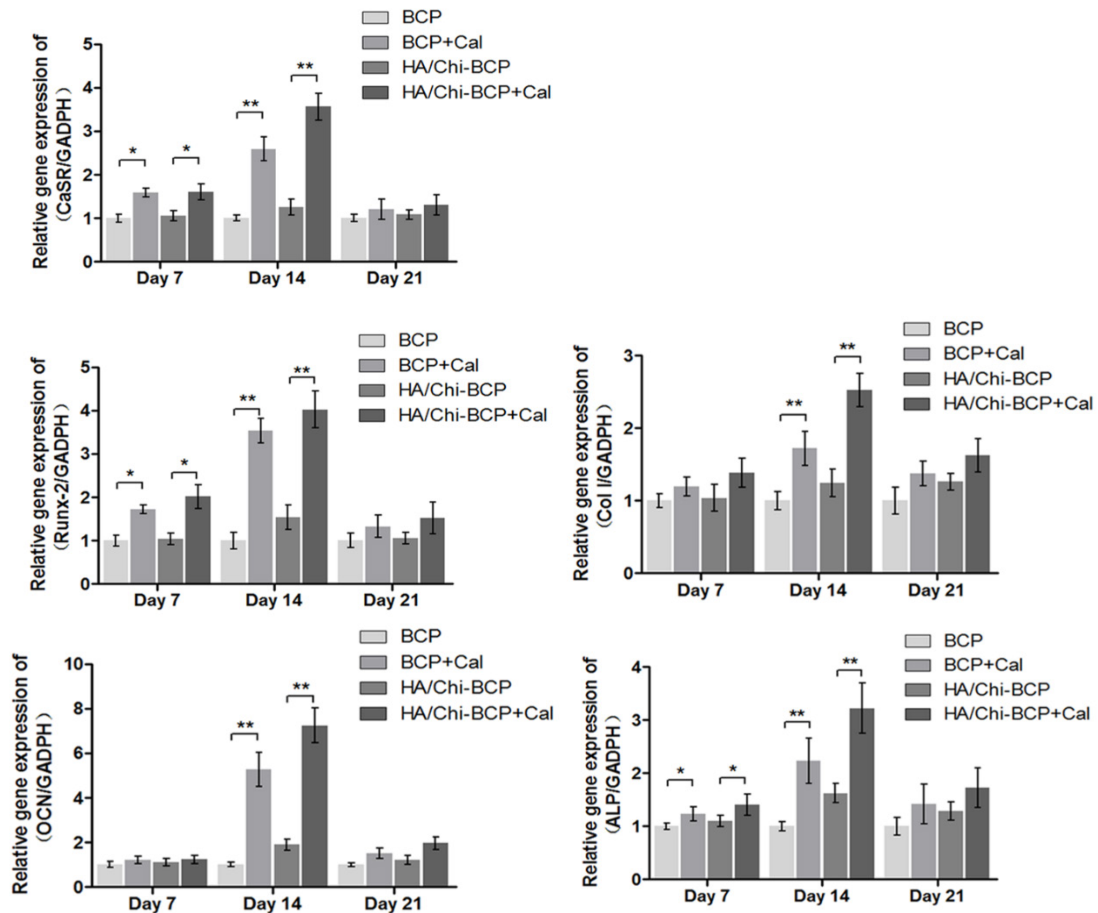
B



Supplementary Figure S 3. Determination of primary rBMSCs derived from sham and OVX rats.

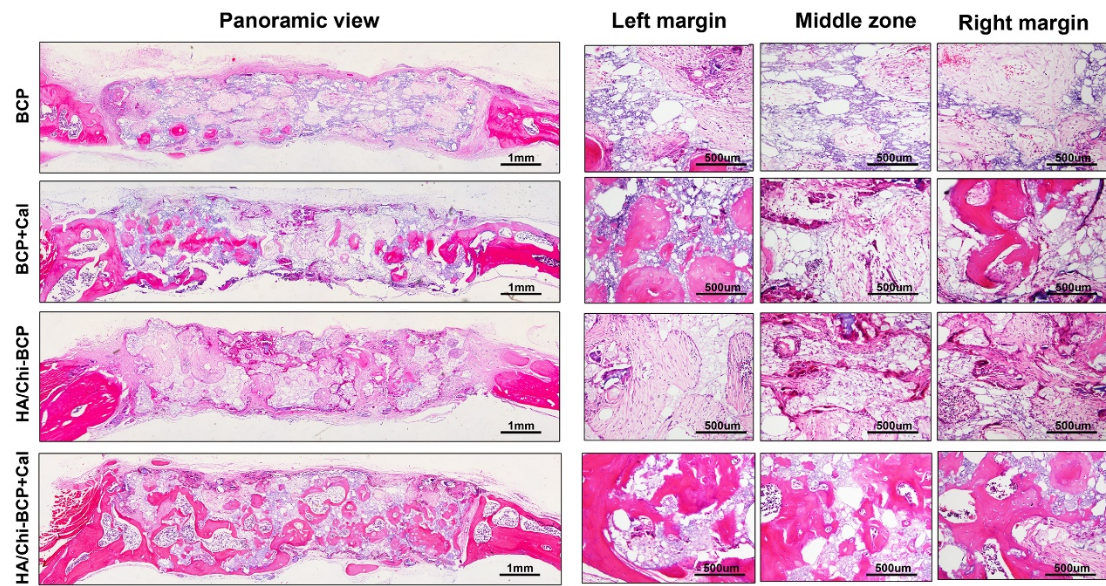
(A) Morphologic difference between sham-rBMSCs and OVX-rBMSCs. (B) Flow cytometric analysis of CD29, CD34, CD44, CD45, CD90 in rBMSCs d derived from Sham and OVX rats.

FigureS4



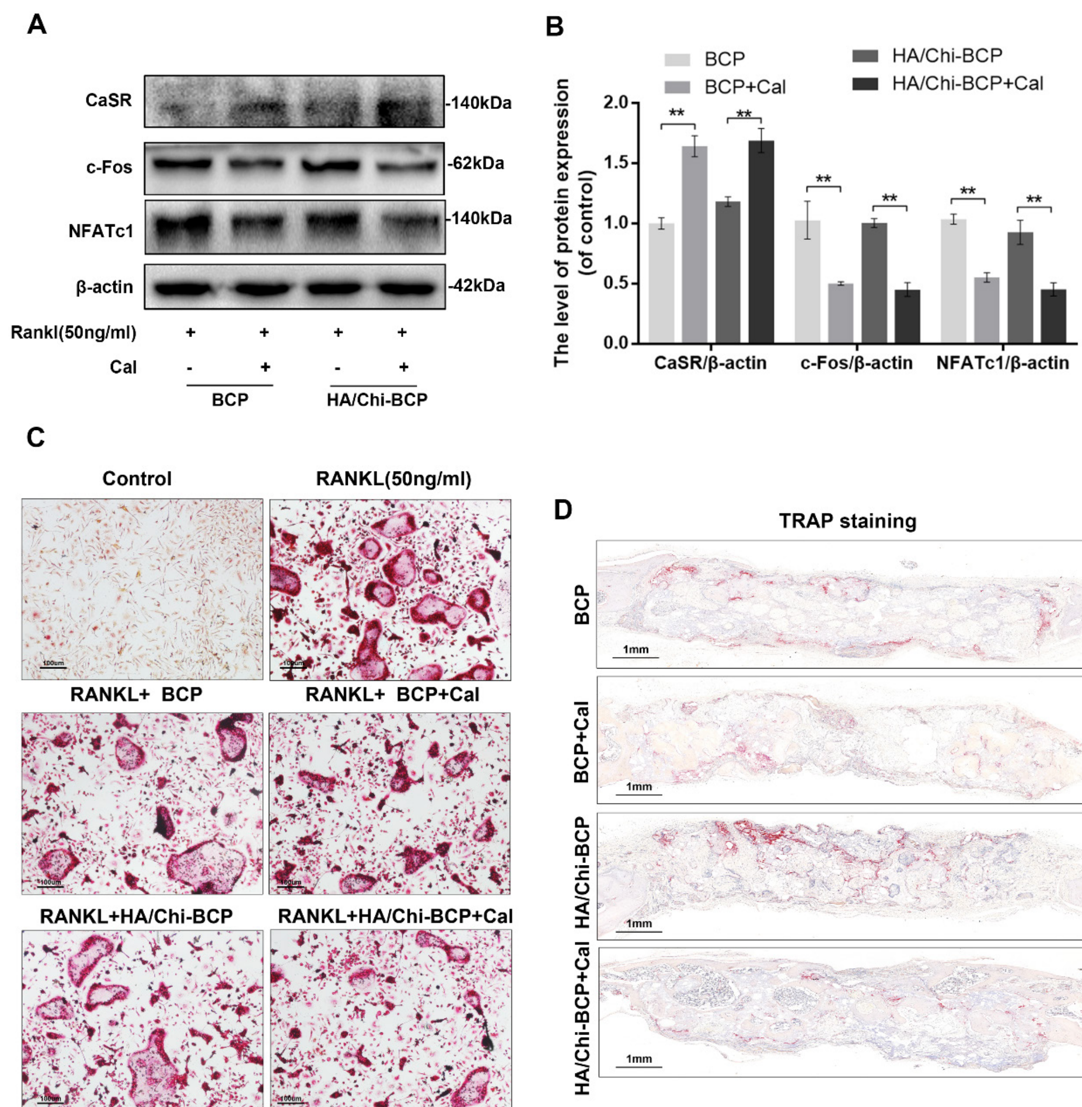
Supplementary FigureS4. qRT-PCR results of different scaffolds on the mRNA expression of CaSR, Runx-2, Col I, OCN and ALP at day7, day14 and day21. The data in the figures were represented as the means ± S.D. Significant differences between the different scaffold groups are indicated as **P<0.01, *p<0.05, n=5.

FigureS5



Supplementary FigureS5. H&E staining of decalcified bone consist of full image and magnified images from different site of defect area (Scar Bar = 1mm/500μm).

FigureS6



Supplementary FigureS6. (A-B). Representative western blot results and quantification data for CaSR, c-Fos and NFATc1 in the BMMs. (C). Representative TRAP staining images of RANKL-induced osteoclast formation treated with or without different scaffolds. (D). Representative

images of decalcified bone stained with TRAP from different scaffolds treated rats. Data are presented as the mean ± S.D. Significant differences among scaffold groups are indicated as ** $P < 0.01$, $n = 5$.