Supporting information

Bioactive poly(ether-ether-ketone) nanocomposite scaffold regulates osteoblast/osteoclast activity for regeneration of osteoporotic bone

Mengen Zhao[1], Guo Chen[2], Shixiong Zhang[1], Bin Chen*[,2], Zhaoying Wu*[,1], Chao Zhang*[,1]

1. School of Biomedical Engineering, Shenzhen Campus of Sun Yat-sen University, Shenzhen, Guangdong 518107, China.

2. Division of Orthopaedics and Traumatology, Department of Orthopaedics, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China.

* Correspondence should be addressed to:

Dr. Bin Chen; Tel.: +86-20-87332200-8466; Email: chb@smu.edu.cn

Dr. Zhaoying Wu; Tel: +86-20-39332145; Email: wuzhy37@mail.sysu.edu.cn

Dr. Chao Zhang; Tel.: +86-20-39332145; E-mail: zhchao9@mail.sysu.edu.cn

1.Materials and Methods

1.1 Cells and animals

Dulbecco's modified Eagle Medium/Nutrient mixture F-12 (DMEM/F12, Gibco, USA) supplemented with 10% of fetal bovine serum (FBS, Hyclone, USA), 1% of penicillin and streptomycin (Pen/Strep, Gibco, USA) was used as the growth medium for rat bone marrow stromal cells (rBMSCs); DMEM/F12 supplemented with 10% of FBS, 1% of Pen/Strep, 10 mM of β-glycerolphosphate (Sigma, USA), 100 nM of dexamethasone (Sigma, USA), and 0.05 mM of ascorbic acid (Sigma, USA) was used as the osteogenic medium for rBMSCs; DMEM (Gibco, USA) supplemented with 10% of FBS and 1% of Pen/Strep was used as growth medium for murine preosteoclast cells (RAW 264.7); DMEM supplemented with 10% of FBS, 1% of Pen/Strep and receptor activator of nuclear factor-κB ligand (RANKL, 30 ng/mL, PEPROTECH, USA) was used as osteoclastic medium for RAW 264.7; DMEM/F12 containing 10% of FBS, 1% of Pen/Strep and macrophage colony stimulating factor (M-CSF, 20 ng/ml, PEPROTECH, USA) was used in the culture of rat bone marrow-derived monocytes (rBMMs); DMEM/F12 containing 10% of FBS, 1% of Pen/Strep, M-CSF (20 ng/mL) and RANKL (30 ng/mL) was used as osteoclastic medium for rBMMs.

rBMSCs were isolated from the femoral bone marrow of Sprague Dawley (SD) rat (80 g) (Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China), and cultured in growth medium at 37 °C in a humidified CO_2 incubator. RAW 264.7 were obtained from Procell Biology, China. rBMMs were isolated from 4-week-old SD rat (Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China) and were

cultured in corresponding growth medium. All the cells were routinely sub-cultured upon reaching approximately 80% confluence.

Aged female SD rats (12 weeks, 300−320 g) from the Experimental Animal Centre of Nanfang Hospital (Guangzhou, China) were used in this study. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Southern Medical University, and the animal experiment followed the institutional guidelines.

1.2 Characterization of the bioactive glass and scaffolds

The morphology and composition of A-SrBG nanoparticles were observed using 400F field-emission scanning electron microscopy (FESEM, Quanta, France). The surface microstructure and cross section of the porous scaffolds were observed using FESEM. The scaffolds were also characterized on a 6700 Fourier transform infrared spectroscopy (FTIR, NICOLET, USA) in the wavelength coverage of 4000–400 cm^{-1} and by Empyrean X-ray diffraction (XRD, Kochhar, Netherlands) using Cu Kα radiation (40 kV and 40 mA), at a of the range of 10-80° with a step width of 0.02°.

1.3 Porosity measurement

The porosity of the scaffold was measured in a specific gravity bottle filled with ethanol based on Archimedes' Principle. The porosity of scaffold was calculated as follows:

$$\text{Porosity}\% = \frac{W_{wet} - W_{dry}}{W_1 + W_{wet} - W_3} \times 100$$

where W_1 is the weight of the specific gravity bottle filled with ethanol, W_2 is the weight of the specific gravity bottle with ethanol and scaffold, W_{wet} is the weight of the ethanol-saturated scaffold, and W_{dry} is the weight of the dry scaffold.

1.4 Mechanical properties

Cylindrical scaffolds with diameter of 12 mm and height of 4 mm, and the mechanical properties of the samples were measured using a LR10KPlus universal testing machine (LLOYD, USA), the load rate was set at 1 mm/min. The compressive strength was obtained, and the compressive modulus was determined as the ratio of compressive stress and strain.

1.5 The loading content and loading efficiency of ALN

10 mg A-SrBG nanoparticles were dissolved in 10 mL NaOH (1 M); the mixture was mixed with OPA/2ME reagent and incubated at room temperature for 10 min. Then, the emission intensity of the solution was measured at 360~600 nm with an excitation wavelength of 360 nm (λmax=470 nm) on a FS5 Fluorescence Spectrometer (Edinburgh, UK) to determine the concentration of ALN. The drug loading content (DLC, %) and drug loading efficiency (DLE) were calculated according to the following formula:

$$\mathrm{DLC} = \frac{\text{amount of loaded of drug}}{\text{amount of drug - loaded nanoparticles}} \times 100\%$$

$$\mathrm{DLE} = \frac{\text{amount of loaded of drug}}{\text{amount of feeding drug}} \times 100\%$$

1.6 ALP Activity and ALP staining

The ALP activity of rBMSCs cultured with extracts of scaffolds was evaluated using an alkaline phosphatase assay kit (Beyotime, China) in accordance with the manufacturer's protocol. Briefly, 2×10^4 rBMSCs per well were seeded in a 24-well plate and cultured in growth medium for 24 h, then the growth medium was replaced

by the osteogenic medium. After 7, 10 and 14 days of culture, the osteogenic medium was removed, and the cells were lysed by addition of 1 mL of 0.2% Triton X-100 solution per well following the manufacturer's protocol. The absorbance of the cell lysate solution at 405 nm was measured on a Gene5 Microplate Reader. rBMSCs cultured in osteogenic medium without scaffold extracts was used as control.

ALP staining was performed using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase color development kit (Beyotime, China). In brief, rBMSCs cultured with the extracts for 10 days were fixed with 4% paraformaldehyde and then incubated with a mixture of BCIP and NBT solution. Finally, cells were rinsed in deionized water and images were acquired under a IX71 microscope (OLYMPUS, Japan).

1.7 Alizarin Red S staining and quantification

The mineralization nodules in rBMSCs in 24-well plate were stained by Alizarin Red S (ARS) after the cells were cultured for 21 days in growth medium/osteogenic medium supplemented with the extracts of scaffolds; breifly, cells were fixed by 4% paraformaldehyde for 30 min, and stained with 1.0 mL of ARS solution for 30 min at room temperature; then excessive ARS solution was aspirated and the cells were washed with distillated water, air-dried and subjected to observation under bright field. To quantify the mineralization, 200 μL of 0.1 M cetylpyridinium chloride was used to dissolve the mineralization nodules; the absorbance of the solution was measured at a wavelength of 570 nm on a microplate reader.

1.8 Immunofluorescence analysis

After 5 days of culture in osteoclastic medium, RAW 264.7 cells were rinsed with PBS for three times and fixed with 4% paraformaldehyde for 10 min at room temperature, and then treated with 0.1% Triton X-100 for 10 min. After blocking with 1% BSA for 30 min, TRITC-conjugated Phalloidin working solution diluted at 1:1000 in labeling buffer were used to stain cytoskeleton for 60 min, and DAPI solution (10 μg/mL) were used to stain nuclei for 10 min. The images were obtained under a IX71 fluorescent microscope (OLYMPUS, Japan). Mature osteoclast occupying rate was determined as the proportion of area occupied by osteoclasts in the well and calculated from Phalloidin stained images using Image J pro.

1.9 Quantitative polymerase chain reaction (qPCR)

rBMSCs/RAW 264.7 cells were seeded in a 6-well plate at a density of 2.0×10^5 cells/well and cultured in growth medium for 24 h, then the growth medium was replaced by the osteogenic/osteoclastic medium. After predetermined time of culture, the medium was removed, and total RNA was extracted using the RNase Eraser (world Foregene, China). Concentration and purity of the extracted RNA were detected using a NanoDrop spectrophotometer (Thermo Scientific, USA). Then, the total RNA was reverse transcribed into cDNA using a PrimeScript RT Master Mix (Takara, Japan), and real-time PCR was performed using the SYBR Green system (Takara, Japan) in an Applied Biosystems 7500 Fast Real-Time PCR System. The sequences of the forward and reverse primers for target genes were listed in **Table S1**. The relative expression levels for each gene were calculated and normalized to GAPDH.

1.10 Cranial defect model in osteoporotic rat

To induce the osteoporosis model, animals were anaesthetised through intraperitoneal injection of 2% (w/v) pentobarbital (40 mg/kg). Then, rats received bilateral 10-mm linear incisions through the lumbar skin followed by gentle removal of both ovaries. The tissue layers were then repositioned and sutured. Three months after surgery, a critical-sized cranial defect with a size of 8 mm in diameter and 2 mm in depth was created using a miniature hand-held cranial drill (RWD life science, China). The scaffolds (Ø8×2 mm) were implanted into the cavity defects. After 6 and 12 weeks of surgery, the animals were sacrificed and all harvested craniums were fixed in 4% paraformaldehyde before further analysis.

Table S1 Primer sequences used for qRT-PCR gene expression analysis.

Genes	5′-3′	Primer sequences
GAPDH	Sense	5′- AAATGGTGAAGGTCGGTGTGAAC-3′
	Anti-sense	5′- CAACAATCTCCACTTTGCCACTG-3′
ALP	Sense	5′-AGCGACACGGACAAGAAGC-3′
	Anti-sense	5′-GGCAAAGACCGCCACATC-3′
RUNX2	Sense	5′-GCACCCAGCCCATAATAGA-3′
	Anti-sense	5′-TTGGAGCAAGGAGAACCC-3′
COL I	Sense	5′-CCTGAGCCAGCAGATTGAGA-3′
	Anti-sense	5′-TCCGCTCTTCCAGTCAGA-3′
OCN	Sense	5′-AGCAGCTTGGCCCAGACCTA-3′
	Anti-sense	5′-TAGCGCCGGAGTCTGTTCACTAC-3′
TRAP	Sense	5′-TACCTGTGTGGACATGACC-3′
	Anti-sense	5′-CAGATCCATAGTGAAACCGC-3′
MMP9	Sense	5′-TCCAGTACCAAGACAAAGCCTA-3′
	Anti-sense	5′-TTGCACTGCACGGTTGAA-3′
Cath K	Sense	5′-CAGCAGAACGGAGGCATTGA-3′
	Anti-sense	5′-CCTTTGCCGTGGCGTTATAC-3′
NFATc1	Sense	5′-GGTAACTCTGTCTTTCTAACCTTAAGCTC-3′
	Anti-sense	5′-GTGATGACCCCAGCATGCACCAGTCACAG-3′

Table S2 Porosity and mechanical properties of the scaffolds.

Scaffolds	Compressive strength (MPa)	Elastic modulus (MPa)	Porosity (%)
PEEK	6.6±0.37	188.6±10.6	63.50 ±4.1
ASP20	5.9±0.42	136.7±9.8	73.12 ±3.3
ASP40	5.3±0.35	112.6±7.5	80.04 ±4.8

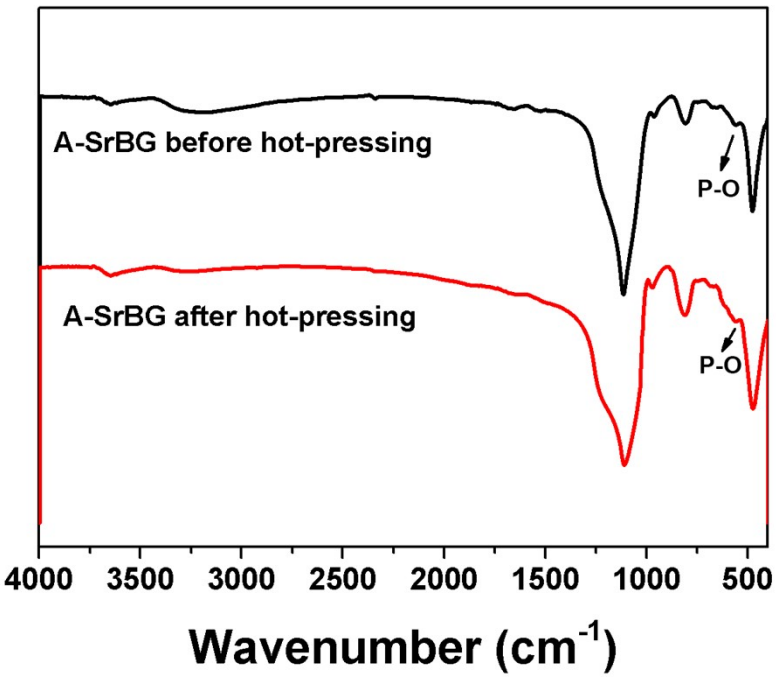

Figure S1 FTIR spectra of A-SrBG nanoparticles before and after hot-pressing.

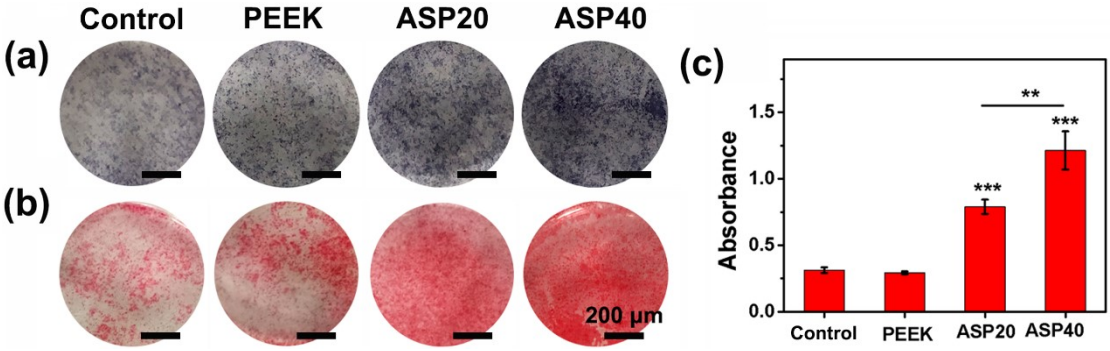

Figure S2 ALP staining (a) ARS staining (b) and ARS quantification (c) of rBMSCs cultured with the extracts of different scaffolds. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

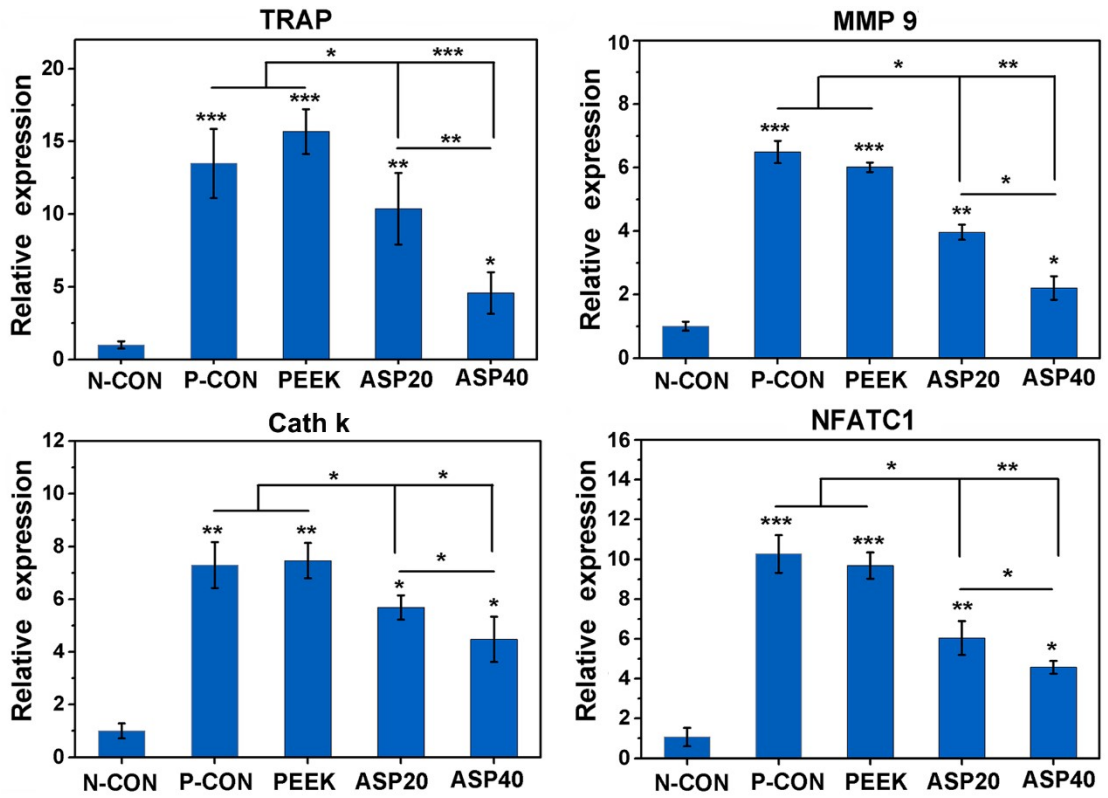

Figure S3 Osteoclastogenesis-related genes (g) of RAW 264.7 cells cultured in all groups. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

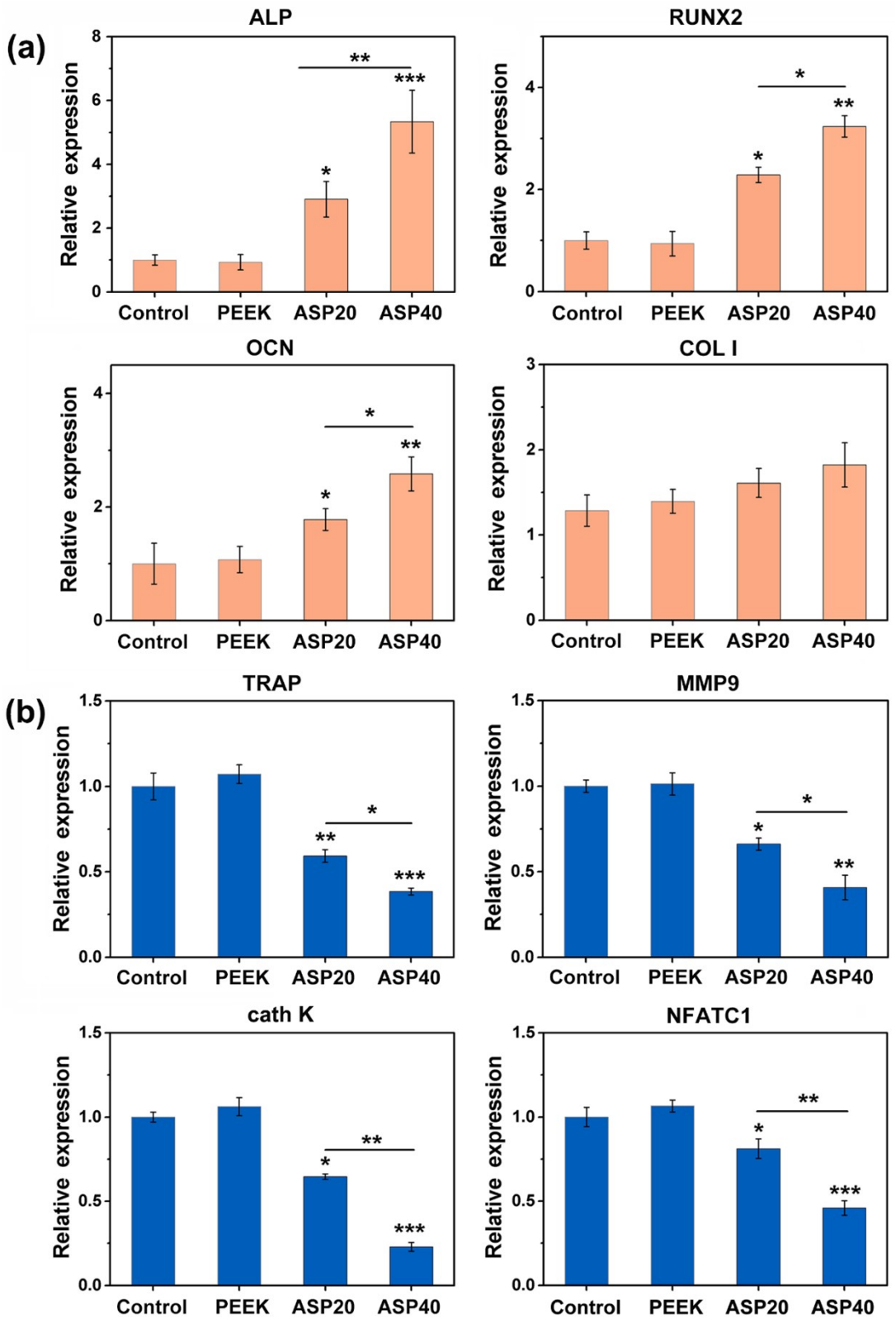

Figure S4 The relative mRNA expression of osteogenesis-related genes (a) and osteoclastogenesis-related genes (b) of cells cultured in co-culture system. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

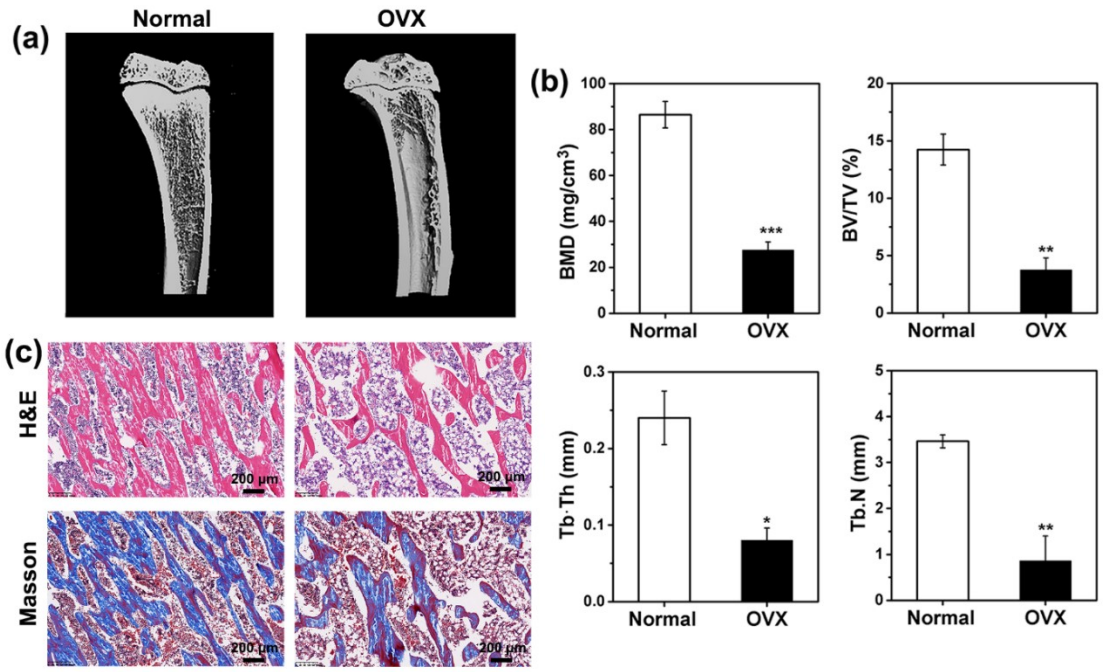

Figure S5 The μ-CT images (a), quantitative analysis of BV/TV, BMD, Tb.Th and Tb.N(b), H&E and Masson's trichrome staining (c) of normal and OVX femur. (*p < 0.05, **p < 0.01, ***p < 0.001)

The μ-CT images of ovariectomized (OVX) rats showed a loss of trabecular bone volume and density, as compared with sham-operated rats **(Figure S5a)**. Quantitative analysis of distal femur were further conducted; BMD, BV/TV, Tb.Th and Tb.N were significantly lower in OVX rats than in Sham rats **(Figure S5b)**. Furthermore, H&E and Masson-trichrome staining **(Figure S5c)** also supported noticeable changes between the two groups. A trabecular rod-like deterioration was represented in OVX rats, which was different from the healthy trabecular bone with plate-like structure in Sham rats. These results suggested that the rat model of osteoporosis was successfully established in this study.