EBioMedicine 52 (2019) 102626

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.elsevier.com/locate/ebiom

Research paper

# Foxf1 knockdown promotes BMSC osteogenesis in part by activating the Wnt/β-catenin signalling pathway and prevents ovariectomy-induced bone loss

Gengyang Shen[a,b,c,1], Hui Ren[b,c,1], Qi Shang[a,c,1], Wenhua Zhao[a,c], Zhida Zhang[a,c], Xiang Yu[a,c], Kai Tang[a,c], Jingjing Tang[b,c], Zhidong Yang[b,c], De Liang[b,c], Xiaobing Jiang[b,c,*]

[a] Guangzhou University of Chinese Medicine, Guangzhou 510405, China
[b] Department of Spinal Surgery, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510405, China
[c] Lingnan Medical Research Center of Guangzhou University of Chinese Medicine, Guangzhou 510405, China

ARTICLE INFO

*Article History:*
Received 24 July 2019
Revised 22 November 2019
Accepted 3 December 2019
Available online xxx

*Keywords:*
Foxf1
Osteogenic differentiation
Mesenchymal stem cells
Postmenopausal osteoporosis
Bone metabolism

ABSTRACT

*Background:* Forkhead box protein f1 (Foxf1) is associated with cell differentiation, and may be a key player in bone homoeostasis. However, the effect of Foxf1 on osteogenesis of bone marrow-derived mesenchymal stem cells (BMSCs) and ovariectomy-induced bone loss, as well as its clinical implications, is unknown.
*Methods:* By quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and western blotting, we assayed Foxf1 expression in bone tissue, BMSCs, and bone marrow-derived macrophages (BMMs), derived from ovariectomised (OVX) mice, and during osteogenic differentiation and osteoclast differentiation. Using a loss-of-function approach (small interfering RNA [siRNA]-mediated knockdown) *in vitro*, we examined whether Foxf1 regulates osteoblast differentiation of BMSCs via the Wnt/β-catenin signalling pathway. Furthermore, we assessed the anabolic effect of Foxf1 knockdown (siFoxf1) in OVX mice *in vivo*. We also assayed the expression of Foxf1 in bone tissue derived from postmenopausal osteoporosis (PMOP) patients and its link with bone mineral density (BMD). Finally, we examined the effect of Foxf1 knockdown on the osteoblastic differentiation of human BMSCs.
*Findings:* Foxf1 expression was significantly increased in bone extract and BMSCs from OVX mice and gradually decreased during osteoblastic differentiation of BMSCs but did not differ significantly in OVX mouse-derived BMMs or during osteoclast differentiation. *In vitro*, Foxf1 knockdown markedly increased the expression of osteoblast specific genes, alkaline phosphatase (ALP) activity, and mineralisation. Moreover, siFoxf1 activated the Wnt/β-catenin signalling pathway. The siFoxf1-induced increase in osteogenic differentiation was partly rescued by inhibitor of Wnt signalling (DKK1). In OVX mice, Foxf1 siRNA significantly reduced bone loss by enhancing bone formation. Foxf1 expression levels negatively correlated with reduced bone mass and bone formation in bone tissue from PMOP patients. Finally, Foxf1 knockdown significantly promoted osteogenesis by human BMSCs.
*Interpretation:* Our findings indicate that Foxf1 knockdown promotes BMSC osteogenesis and prevents OVX-induced bone loss. Therefore, Foxf1 has potential as a biomarker of osteogenesis and may be a therapeutic target for PMOP.



## 1. Introduction

Osteoporosis, particularly postmenopausal osteoporosis (PMOP), is a disorder of bone metabolism that affects millions of people worldwide and imposes a major socioeconomic burden [1]. Osteoporotic fractures of the vertebrae and hip are severe age-related morbidities [2]. The bone mass is continuously remodelled and maintained via a process involving a balance between bone formation and resorption. Bone loss occurs if this balance is disrupted and is mediated by osteoblasts (derived from mesenchymal stem cells) and osteoclasts (derived from haematopoietic precursors) [3,4]. Studies focusing on identifying the novel factors in regulating bone homoeostasis are necessary for understanding and treating bone metabolism-related diseases, such as osteoporotic bone loss.

* Corresponding author at: Department of Spinal Surgery, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510405, China.
*E-mail address:* spinedrjxb@sina.com (X. Jiang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ebiom.2020.102626

**Research in context**

*Evidence before this study*

The Fox family members are implicated in controlling cell differentiation, development, metabolism, and ageing. Several Fox family members reportedly regulate bone metabolism. Further, recent studies have demonstrated that Foxf1 cross talks with several bone metabolism-associated factors, such as BMP4, Wnt2, Wnt11, Wnt5A, β-catenin, Erk5, Notch, Hedgehog, and NF-κB. Therefore, we hypothesised that Foxf1 may be a novel potential factor in regulating bone homoeostasis.

*Added value of this study*

We provide the first experimental and clinical evidence that Foxf1 negatively regulates BMSC osteogenesis and oestrogen deficiency-induced osteoporosis. The endogenous expression level of Foxf1 was increased in OVX mice-derived bone tissue and BMSCs and decreased during osteogenic differentiation of BMSCs, whereas no significant difference in Foxf1 expression was observed in OVX mouse-derived BMMs and during osteoclast differentiation. Using a loss-of-function approach *in vitro*, we revealed that Foxf1 knockdown significantly increased BMSC osteogenesis partly by activating the Wnt/β-catenin signalling pathway. *In vivo*, siFoxf1 ameliorated OVX-induced bone loss by promoting osteoblastogenesis and mineralisation. Clinically, Foxf1 expression levels negatively correlated with reduced bone mass and bone formation in bone tissue from PMOP patients. Further, siFoxf1 promoted osteogenic differentiation of human BMSCs. Our data demonstrate that knockdown of Foxf1 promotes BMSCs osteogenesis and prevents OVX-induced bone loss.

*Implications of the available evidence*

Foxf1 may be a biomarker of osteogenesis and a potential therapeutic target for PMOP.

Forkhead box protein f1 (Foxf1), previously designated HFH-8 or Freac-1, is a member of the Fox family of transcription factors, which have a common winged-helix or forkhead domain [5]. Members of the Fox family are implicated in controlling cell differentiation, development, metabolism, and ageing [6–12]. Several Fox family members reportedly regulate bone metabolism. Foxa2 knockdown promoted bone marrow-derived mesenchymal stem cell (BMSC) osteogenesis by modulating the Wnt/β-catenin signalling pathway and improved bone healing in rats [13]. Foxc2 induced osteogenesis by promoting Wnt signalling and BMP4 expression [14,15]. Foxo1 reportedly involved in inhibitory effect of cigarette smoke extract on the osteoblastic differentiation of cultured human periosteum-derived cells [16], whereas Foxo1 negatively regulated osteoclast differentiation and bone resorption [17–19]. Further, Foxf1 cross talks with several bone metabolism-associated factors, such as BMP4, Wnt2, Wnt11, Wnt5A, β-catenin, Erk5, Notch, Hedgehog, and NF-κB [8,12,20–27]. Amongst them, BMP4 and Erk5 regulate osteogenic differentiation [28,29], while Wnt2, Wnt11, Wnt5A, and β-catenin, key components of the Wnt/β-catenin signalling pathway, are required for osteogenesis [30]. The roles of Notch and hedgehog signalling in skeletal development, remodelling, and diseases have been reviewed [31,32]. NF-κB is necessary for osteoclast formation and activity, and suppresses bone formation [33]. Therefore, we hypothesised that Foxf1 may be a novel potential factor in regulating bone homoeostasis.

Here, we examined the Foxf1 expression level in bone extracts and BMSCs from ovariectomised (OVX) mice, its role in osteogenesis by BMSCs and OVX-induced bone loss, and its clinical implications for PMOP. We found that the endogenous expression level of Foxf1 was increased in OVX mice-derived bone tissues and BMSCs and decreased during osteogenic differentiation of BMSCs, but did not differ significantly in OVX mouse-derived BMMs or during osteoclast differentiation. We also explored the effect of the canonical Wnt/β-catenin signalling pathway on Foxf1 knockdown-mediated osteogenic differentiation of BMSCs. And we confirmed that Foxf1 knockdown promoted osteogenesis by BMSCs in part by activating the Wnt/β-catenin signalling pathway *in vitro*. Also, we injected OVX-mice with a Foxf1 small interfering RNA (siRNA) and found that Foxf1 knockdown significantly ameliorated bone loss by enhancing bone formation *in vivo*. Clinically, increased Foxf1 expression was positively related to the reduced bone mineral density (BMD) and bone formation of PMOP patients. Finally, Foxf1 knockdown significantly promoted osteogenesis of human BMSCs.

## 2. Materials and methods

### 2.1. Experimental animals

The animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (No. TCMF1-2019030). The animals were housed in pathogen-free facilities under a 12/12 h light/dark cycle.

### 2.2. Cell culture and reagents

Human BMSCs, from three healthy donors aged from 18 to 45, purchased from Cyagen Biosciences of Guangzhou, were cultured in α-minimum essential medium (α-MEM) containing 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S; all from Gibco, Grand Island, NY, USA) in a 37 °C incubator with a 5% $CO_2$ atmosphere. Adherent human BMSCs were cultured in human BMSC growth medium (Cyagen Biosciences). Mouse BMSCs were isolated and cultured as follows. Briefly, the tibiae and femurs of 8-week-old C57BL/6 mice were aseptically dissected, and bone marrow cells were collected. The adherent cells were digested and cultured until 80% confluence. Cells at passages five to nine were harvested for subsequent experiments. Mouse bone marrow-derived macrophages (BMMs) were grown in culture medium (5 ng/ml macrophage colony-stimulating factor [M-CSF], 1% P/S, and 10% FBS in α-MEM) in 100 mm dishes.

### 2.3. Preparation of PMOP patient-derived bone samples

All protocols were approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (No. ZYYECK [2016]028). We enrolled in this study 20 patients who underwent spine-related surgeries at the Spine surgery Department of the First Affiliated Hospital of Guangzhou University of Chinese Medicine. We obtained written informed consent from all the patients. The inclusion criteria were as follows: PMOP group: (a) postmenopausal females aged 55–80 years at least 5 years since menopause with PMOP (mean lumbar 2–4 BMD T-score, $\leq -2.5$), (b) acute fragile lumbar fractures in the prior 2 weeks and clear indication of vertebroplasty or internal fixation, (c) normal levels of laboratory indicators, particularly serum calcium level ($2.25 \pm 0.13$ *vs* $2.23 \pm 0.07$ in control group), (d) no intake of drugs affecting bone metabolism (*e.g.,* corticosteroids, antacids containing aluminium, and heparin), (e) no systemic disease affecting bone metabolism (*e.g.,* secondary osteoporosis, osteogenesis imperfecta, diabetes), (f) no osteoporotic fracture, and (g) no severe liver or kidney dysfunction. Control group: (a) non-postmenopausal females who had undergone

spinal surgery for a lumbar degenerative disease (*e.g.,* lumbar spondylolisthesis and lumbar spinal stenosis) and (b) no osteoporosis or other metabolism disease. We collected vertebral bone samples as described previously [34].

### 2.4. siRNA-mediated knockdown and cell transfection

Foxf1-specific siRNAs (GTGTGACCGAAAGGAGTTT for human and GATCCGGCTAGCGAGTTTA for mouse) and negative control siRNA (siCtrl) (RiboBio, Guangzhou, China) were used to knockdown Foxf1. Transfection of siRNA oligonucleotides was performed using Lipofectamine RNAimax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Foxf1 expression was determined by quantitative reverse transcription PCR (qRT-PCR), western blotting (WB), and immunofluorescence (IF). Transfected cells were passaged and used for downstream analyses.

### 2.5. Cell viability and proliferation assay

The viability of BMSCs harvested on days 1, 3, 5, and 7 was examined by trypan blue staining and using a cell counter. To assay cell proliferation, BMSCs were plated in a 96-well plate and adhered for 24 h. The medium was discarded and BMSCs were treated with 10% CCK-8 (Kumamoto, Japan) in 150 $\mu$l of $\alpha$-MEM without FBS for 3 h in an incubator. The absorbance at 450 nm was determined using a microplate reader.

### 2.6. Osteoblast differentiation assay

To induce the osteogenic differentiation, BMSCs were cultured in osteogenic induction medium ($\alpha$-MEM supplemented with 10% FBS, 1% P/S, 0.2 mM ascorbic acid, 10 mM β-glycerophosphate and $10^{-7}$ M dexamethasone). The osteogenic induction medium was changed every 3 days. After osteogenic induction for 7 days, cells were subjected to alkaline phosphatase (ALP) staining and ALP activity assay. After osteogenic induction for 14 to 28 days, mineral deposition was evaluated by Von Kossa staining.

### 2.7. ALP staining and activity assay

ALP staining was performed as follows. Cells were fixed for 20 min in 4% paraformaldehyde and washed for three times with distilled water. Next, cells were stained with BCIP/NBT Alkaline Phosphatase colour Development Kit (Beyotime, Shanghai, China). To assay ALP activity, cells were lysed with lysis buffer (20 mM pH 7.5 Tris−HCl, 150 mM NaCl, and 1% Triton X-100) in 96-well plates, and the substrates and p-nitrophenol were added. ALP activity was quantified by determining the absorbance at 405/650 nm.

### 2.8. Von Kossa staining

Calcium deposit was assayed by Von Kossa staining. Cells were fixed and washed as for ALP staining. Next, cells were incubated in 5% silver nitrate, exposed to light for 30 min, and washed in 5% sodium thiosulphate for 5 min to remove non-specific staining.

### 2.9. Osteoclast differentiation protocol

For osteoclast differentiation, BMMs were treated with M-CSF (5 ng/ml) and receptor activator of NF-kB ligand (RANKL, 10 ng/ml) (R&D Systems, Minneapolis, MN, USA) for 4 days. Then, cells were fixed in 4% paraformaldehyde for tartrate-resistant acid phosphatase (TRAP) staining. $TRAP^+$ cells with at least three nuclei were considered osteoclasts.

### 2.10. RNA isolation and qRT-PCR

Total RNA was extracted from BMSCs and BMMs with TRIzol reagent (Invitrogen). Bone tissues were precooled in liquid nitrogen and repeatedly ground to a powder in liquid nitrogen. TRIzol was added and the samples were thoroughly homogenised and centrifuged at 4 °C. The supernatant was centrifuged with chloroform to separate RNA from DNA, proteins and other components to obtain total RNA. The absorbance of total RNA was measured at 260 nm (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Perfect Real Time; TaKaRa, Japan) in a reaction volume of 20 $\mu$l with 1 $\mu$l of cDNA as the template. The ABI StepOnePlus™ System (Applied Biosystems, Foster City, CA, USA) with the Power SYBR Green PCR Master Mix (TaKaRa) was applied to quantify transcript levels using the housekeeping gene, β-actin, as an internal reference. The primers (Table S1) were designed by us and synthesised by Sangon Biotech (Shanghai, China). The cycling conditions were 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

### 2.11. Western blotting

Cells were lysed in RIPA lysis buffer (Beyotime) and proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (15%) and were transferred to polyvinylidene fluoride membranes (Millipore, Shanghai, China). The membranes were blocked in non-fat milk (5%) for 2 h at room temperature and incubated for 24 h at 4 °C with primary antibodies against β-actin (1:3000; human/mouse; Cell Signalling Technology, Danvers, MA, USA), Foxf1 (1:500; human/mouse; Biorbyt, San Francisco, CA, USA), and β-catenin (1:500; human/mouse; Cell Signalling Technology). After washing three times for 10 min each with PBST, the membranes were incubated with the corresponding secondary antibodies at room temperature for 2 h and washed three times with PBST. Protein levels were determined by enhanced chemiluminescence (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Band intensities were quantified using Image J software.

### 2.12. Immunofluorescence

Cells were cultured in confocal dishes for evaluation for Foxf1, Runx2, Col1a1, and β-catenin protein expression levels. Briefly, the cells were fixed for 30 min with 4% paraformaldehyde, permeabilized, blocked for 30 min with 0.3% Triton X-100 and 1% bovine serum albumin, and washed. Next, cells were incubated 8 h with primary antibodies against Foxf1 (1:500; Biorbyt), Runx2 (1:1600; Cell Signalling Technology), Col1a1 (1:500; Abcam, Cambridge, UK), and β-catenin (1:1600; Cell Signalling Technology). The cells were incubated with a fluorescence conjugated secondary antibody for 60 min (Beyotime) and with 4′,6-diamidino-2-phenylindole (KeyGen Biotech, Nanjing, China) for 5 min to stain the nuclei. The samples were visualised by confocal laser scanning microscopy (Leica, Wetzlar, Germany).

### 2.13. β-catenin/TCF transcription reporter assay (TOP/FOPflash assay)

To determine the activation status of Wnt signalling, β-catenin/TCF transcriptional activity was assayed by transfecting TOPflash/FOPflash luciferase reporter plasmids (Addgene, Cambridge, MA, USA). Cells ($2 \times 10^4$ per well) were seeded and cultured in 24-well plates for 12 h. Plasmids of TOPflash (with 3 repeats of the TCF-binding site) or FOPflash (with 3 repeats of a mutated TCF-binding site) were transfected into cells according to the manufacturer's instructions. Luciferase activity was measured using the Luciferase Assay System at 48 h after transfection; Renilla luciferase activity was used as the internal control.

### 2.14. OVX-induced bone loss

To evaluate OVX-induced osteoporosis, the bilateral ovaries of anaesthetised 8-week-old C57BL/6 female mice were exposed through a midline incision in the dorsal skin and muscle layer. After ligating the uterine horn, the bilateral ovaries were removed. Then muscle incision was sutured, and the skin was closed. Mice in the sham group underwent the procedures of anaesthesia, incisions, bilateral ovaries exposure and incision closure without bilateral ovaries removed. siRNA delivery began on day 2 after OVX surgery. We injected siFoxf1 (7 mg $kg^{-1}$), siCtrl (7 mg $kg^{-1}$), or PBS (0.2 ml) into the tail vein of OVX or sham-operated mice for 6 weeks ($n$ = 8 per group). The sequence of siFoxf1 *in vivo* is GATCCGGCTAGCGAGTTTA.

### 2.15. Micro-computed tomography

Micro-computed tomography (CT) was performed using a Skyscan 1172 Micro-CT Imaging System (Skyscan, Kontich, Belgium) with a spatial resolution of 12 mm (X-ray source 80 kV/100$\mu$A). NRecon 1.6 and CTAn 1.8 software were used for volumetric reconstructing and analysis, respectively. To evaluate the microarchitecture of L4 vertebrae, the trabecular bone was excised along the border of cortical bone and cancellous bone as the volume of interest (VOI). The trabecular bone volume fraction (BV/TV,%), trabecular number (Tb.N, /mm), trabecular thickness (Tb.Th, mm), and trabecular spacing (Tb.Sp, mm) were calculated within the delimited VOI.

### 2.16. Bone histomorphometry

For histological analysis, 25 mg/kg of calcein was intraperitoneally injected into mice on days 8 and 2 before euthanasia. The histomorphometry of cancellous bone was evaluated using the undecalcified bone samples. Following fixation with 10% paraformaldehyde for 12 h, the vertebral bones were stored in 70% ethanol at 4 °C. The bones were embedded in methylmethacrylate and sectioned at 5 $\mu$m thickness using a microtome. The bone histomorphometry parameters evaluated were the bone formation rate (BFR/BS, $\mu m^3 \cdot \mu m^{-2}$ per day), mineral apposition rate (MAR, $\mu$m per day), mineralizing surface (MS/BS,%), and number of osteoblasts (N.Ob/B.Pm, /mm), number of osteoclasts (N.Oc/B.Pm, /mm) and osteoclast surface (Oc.S/BS,%). We measured these parameters according to the guidelines of ASBMR nomenclature committee [35].

### 2.17. Enzyme-linked immunosorbent assay (ELISA)

The serum concentrations of ALP and TRAP5b were measured using enzyme-linked immunosorbent assay (ELISA) kits from IDS (Fountain Hills, AZ, USA). Mice were fasted for 4 h and the blood was collected by puncturing the cheek pouch.

### 2.18. Statistical analysis

SPSS Statistics version 19.0 (IBM, Chicago, IL, USA) was used to analyse the data. Each experiment *in vitro* was repeated 3 times with similar results. The two-tailed Student's *t*-test was applied for comparisons of two groups and one-way analysis of variance (ANOVA) with the Tukey' *post hoc* test for three or more groups. Comparison between groups was performed using two-way ANOVA by Bonferroni's test for variables with two classifications. A *P*-value of $\leq 0.05$ was considered indicative of statistical significance. Data are expressed as means ± standard deviation (s.d.).

## 3. Results

### 3.1. Foxf1 expression was upregulated in OVX mice-derived and BMSCs, but not in OVX-derived BMMs

We assayed endogenous expression level of Foxf1 in vertebrae, BMSCs, and BMMs from OVX mice and evaluated bone loss in OVX mice by micro-CT (Fig. S1a–e). Compared with the control, vertebrae from OVX mice showed a marked increase in Foxf1 expression (Fig. 1a–c). Foxf1 expression was markedly upregulated in OVX-derived BMSCs relative to the control (Fig. 1d–f), but did not differ significantly between OVX-derived BMMs and the control (Fig. 1g–i). Next, we investigated endogenous Foxf1 expression on days 0, 3, and 7 of BMSCs osteogenesis (Fig. S2a) and on days 0, 3, and 5 of osteoclast differentiation (Fig. S2b). Compared with the undifferentiated BMSCs, differentiated BMSCs showed a significant decrease in Foxf1 expression (Fig. 2a–c). However, there was no significant difference in Foxf1 expression in OVX-derived BMMs after osteoclast differentiation (Fig. 2d–f).

### 3.2. Foxf1 knockdown did not affect BMSC viability or proliferation but enhanced osteoblast differentiation

To explore the role of Foxf1 in osteogenesis by BMSCs, Foxf1 expression was downregulated in BMSCs by siRNA-mediated knockdown and assayed by qRT-PCR, IF, and WB 5 days after transfection. The mRNA and protein levels of Foxf1 were markedly reduced compared with the negative control (Fig. S3a–e).

To determine whether Foxf1 knockdown affected BMSC viability and proliferation, we cultured BMSC for 1, 3, 5, and 7 days and performed trypan blue staining and CCK-8 assay, respectively. There was no significant difference in BMSC viability and proliferation between the siFoxf1 and siCtrl groups (Fig. S4a–d).

We investigated the osteogenic and mineralisation potential of BMSCs by performing ALP staining, ALP activity assay, and Von Kossa staining. Foxf1 knockdown increased ALP staining and activity compared to the control (Fig. 3a and c). Similarly, a greater number of mineralised nodules was observed in the siFoxf1 group than in the siCtrl group (Fig. 3b).

To determine the effect of siFoxf1 on osteogenesis by BMSCs, the expression of *Runx2*, alkaline phosphatase (*Alp*), osterix (*Osx*), osteocalcin (*Ocn*), and *Col1a1* was tested by qRT-PCR; Runx2 and Col1a1 protein levels were determined by IF. *Runx2, Alp, Osx, Ocn*, and *Col1a1* mRNA levels were markedly upregulated in the siFoxf1 group at days 3 and 7 relative to the siCtrl group (Fig. 3d). Also, Runx2 and Col1a1 protein levels were increased at day 3 in the Foxf1-knockdown group (Fig. 3e–h).

### 3.3. Foxf1 knockdown activated the Wnt/β-catenin signalling pathway

We explored the role of canonical Wnt/β-catenin signalling in regulating Foxf1 knockdown-mediated osteogenesis by BMSCs. Topflash activity was determined by luciferase reporter assay and the β-catenin expression by qRT-PCR, WB, and IF after 3 days of BMSCs osteogenesis. The mRNA expression levels of *Oct4, Cyclin D1, C*-myc, and *CD44* were determined by qRT-PCR. Compared with the siCtrl groups, Topflash activity in the Foxf1 knockdown group was significantly upregulated (Fig. 4a). Also, the Foxf1 knockdown BMSCs exhibited higher levels of β-catenin mRNA and protein (Fig. 4b and c). IF showed that siFoxf1 increased nuclear accumulation of β-catenin protein at day 3 (Fig. 4d and e). Finally, the siFoxf1 group showed markedly increased expression of *Oct4, Cyclin D1, C*-myc, and *CD44* mRNA (Fig. 4f).

### 3.4. siFoxf1-mediated upregulation of BMSCs osteogenesis was in part reversed by DKK1

Several factors antagonise Wnt signalling, such as recombinant Dickkopf related protein 1 (DKK1) [36]. To verify the role of Wnt/β-catenin

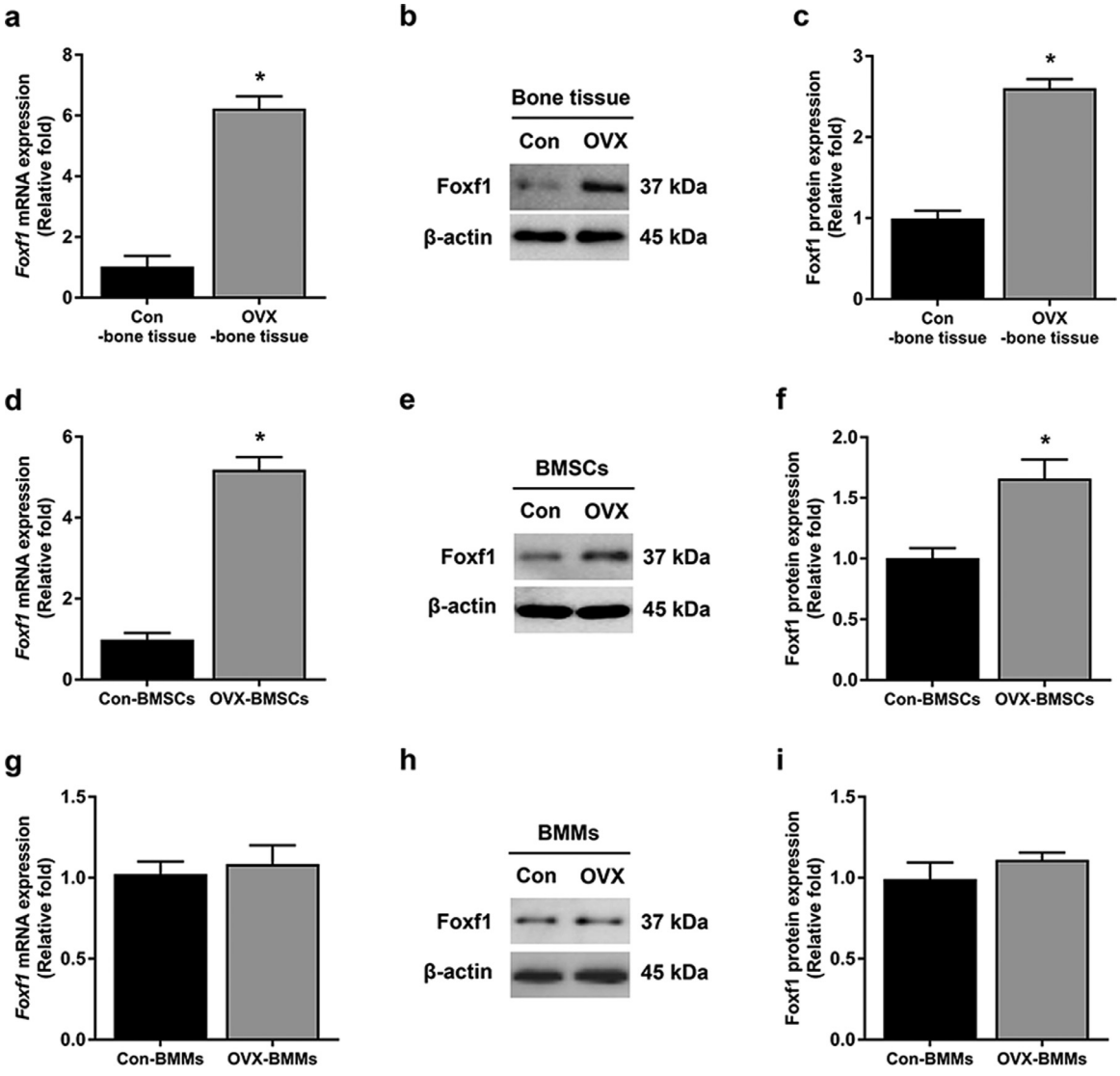


**Fig. 1.** Foxf1 expression level in bone tissues, BMSCs, and BMMs derived from OVX mice. (a) qRT-PCR analysis revealed that OVX mice-derived vertebrae tissues showed a marked increase in *Foxf1* mRNA expression level compared with the controls. Mean ± SD, $n = 5$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (b and c) WB analysis showed that OVX mice-derived vertebrae tissues showed a significant increase in Foxf1 protein expression level, Mean ± SD, $n = 5$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (D) qRT-PCR analysis revealed that OVX mice-derived BMSCs showed a marked increase in *Foxf1* mRNA expression level. Mean ± SD, $n = 5$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (e and f) WB analysis showed that OVX mice-derived BMSCs showed a significant increase in Foxf1 protein expression level. Mean ± SD, $n = 5$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (g–i) No significant difference in Foxf1 mRNA and protein expression levels was observed in OVX-derived BMMs compared with that in the control. Mean ± SD, $n = 5$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test.

pathway in siFoxf1-induced osteogenesis by BMSCs, we analysed the effect of DKK1 (PeproTech, Rocky Hill, NJ, USA). Following treatment with DKK1 for 3 days, Topflash activity and β-catenin expression were markedly reduced (Fig. 5a–c). Importantly, DKK1 treatment partly rescued the promotion of BMSC osteogenesis induced by Foxf1 knockdown, as evidenced by the decreased expression of Runx2 and Col1a1 (Fig. 5d). Additionally, enhanced ALP activity (Fig. 5e) and a greater number of mineralised nodules (Fig. 5f) were observed in the siFoxf1 group compared to the siFoxf1+DKK1 group.

### 3.5. siFoxf1 prevents OVX-induced bone loss

To explore the anabolic effect of siFoxf1 *in vivo*, we injected siFoxf1 (7 mg $kg^{-1}$), siCtrl (7 mg $kg^{-1}$), or PBS (0.2 ml) into OVX- or sham-operated mice for 6 weeks (Fig. 6a). As expected in the Sham mice, OVX significantly reduced bone mass indicated by 38.31% decrease of BV/TV, 38.27% decrease of Tb.N, and 33.24% decrease of Tb.Th., and 36.46% increase of Tb.Sp compared with the Sham group (Fig. 6b, c–f, columns 1 vs. 4) revealed by the micro-CT analysis. Further, siFoxf1 treatment remarkedly increased bone mass, as evidenced by the increased BV/TV, Tb.N, and Tb.Th, and reduced Tb.Sp in both the Sham group and the OVX group (Fig. 6b, c–f, columns 1 vs. 3, columns 4 vs. 6). Interestingly, compared with the siFoxf1+Sham group, the siFoxf1+OVX group exhibited the reduced bone mass indicated by 26.3% decrease of BV/TV, 22.8% decrease of Tb.N, and 27.8% decrease of Tb.Th. (Fig. 6b, c–f, columns 3 vs. 6). Although siFoxf1 treatment can not completely protect siFoxf1-treated mice against OVX-induced bone loss, the bone mass of siFoxf1-treated OVX mice was almost restored to the pre-surgery values. The anabolic effect of siFoxf1 was confirmed by the marked increases in the number of osteoblasts, mineral apposition rate, and bone formation rate (Fig. 6g–j). Nevertheless, siFoxf1 did not influence the expression of bone resorption parameters, including Oc.S/BS and N.Oc/B.Pm value (Fig. 6k and l). siFoxf1 also increased *Runx2* and *Col1a1* mRNA levels (markers of osteoblast activity) in bone extracts but did not alter levels of *TRAP* (marker of osteoclast activity) (Fig. 7a–c). Similarly, the serum bone-specific ALP level was increased in siFoxf1-treated mice, while the

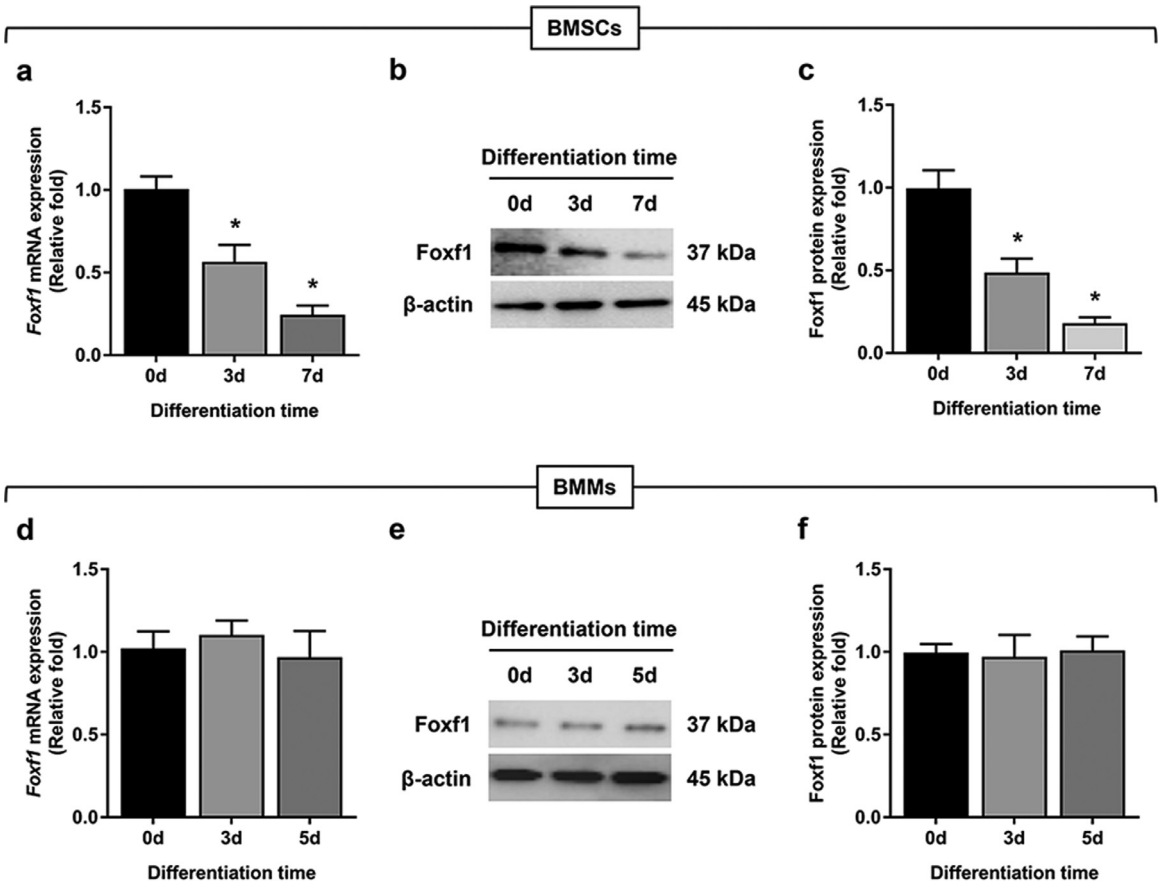


**Fig. 2.** Foxf1 expression level during osteogenic differentiation and osteoclast differentiation. (a) qRT-PCR analysis demonstrated that the mRNA expression of *Foxf1* decreased significantly after osteogenic differentiation. Mean ± SD, $n = 3$ biologically independent samples, $^{*}P < 0.05$ vs. 0 d by ANOVA with Tukey's *post hoc* test. (b and c) WB analysis showed that the protein expression of Foxf1 decreased markedly after osteogenic differentiation. Mean ± SD, $n = 3$ biologically independent samples, $^{*}P < 0.05$ vs. 0 d by ANOVA with Tukey's *post hoc* test. (d–f) No significant difference in Foxf1 mRNA and protein expression levels was observed in BMMs after osteoclast differentiation. Mean ± SD, $n = 3$ biologically independent samples. All *in vitro* experiments were repeated 3 times with similar results.

serum TRAP5b level was unaffected (Fig. 7d and e). We also assayed the Foxf1 mRNA and protein levels in bone tissue to verify *in vivo* tissue delivery and uptake (Fig. 7f and h). Foxf1 and β-catenin protein levels were reduced and elevated, respectively, in bone tissue from siFoxf1-injected mice (Fig. 7g and h). Therefore, Foxf1 knockdown could prevent OVX-induced bone loss by promoting bone formation.

### 3.6. *Foxf1 expression increased with decreasing BMD and bone formation in PMOP patients and suppressed osteogenesis by human BMSCs*

To explore the relationship between Foxf1 expression and human PMOP, bone tissue from PMOP patients (Fig. 8a) were subjected to qRT-PCR and WB. Compared with the control group, the PMOP group showed markedly decreased bone formation markers levels (*Runx2* and *Col1a1*, Fig. 8b and c) and increased Foxf1 expression (Fig. 8d–e). Also, *Runx2* and *Col1a1* levels decreased with the decreasing BMD in PMOP patients (Fig. 8f and g), while the expression of Foxf1 increased with decreasing BMD and bone formation markers levels in PMOP patients (Fig. 8h–j).

To investigate the effect of Foxf1 overexpression on the osteogenic potential of human BMSCs, we co-transfected siFoxf1 into human BMSCs at day 0 of osteogenic induction; ALP activity and calcium deposition were significantly increased in the siFoxf1 group (Fig. 8k-l). Furthermore, *Runx2* and *Col1a1* mRNA levels were increased in siFoxf1-treated human BMSCs compared with the controls (Fig. 8m). Therefore, Foxf1 negatively regulates osteogenesis by human BMSCs.

## 4. Discussion

We identified an important role of Foxf1 in the control of osteogenesis by BMSCs. Foxf1 is a marker of the nucleus pulposus and participates in transcriptional control of lung morphogenesis [6–12]. Minogue et al. reported that BMSCs and adipose tissue-derived mesenchymal stem cells (ADMSCs), following differentiation in type I collagen gels, showed significantly increased expression of Foxf1 [37]. Bohnenpoll et al. found that the sonic hedgehog (SHH)/Foxf1/BMP4 axis regulated growth and differentiation of mesenchymal tissue during ureter development [20]. Crosstalk between osteogenesis and angiogenesis in now accepted as a new mechanism of bone metabolism [38,39]. Foxf1 reportedly mediates vascular sprouting in endothelial progenitor [40] and hedgehog signalling-induced murine vasculogenesis [41]. In addition, Foxf1 enhanced angiogenesis and increased bevacizumab resistance in colorectal cancer cells by increasing the transcription level of vascular endothelial growth factor A1 (VEGFA) [42]. Foxf1 also played an important role in embryonic vasculature formation via VEGF signalling in endothelial cells [43]. Further, conditional Foxf1 overexpression induced lethal lung hypoplasia and vascular defects in mice [44]. BMP4, Wnt2, Wnt11, Wnt5A, β-catenin, Erk5, Notch2, Hedgehog, and NF-κB, important regulators of bone metabolism, reportedly interact with Foxf1 [8,12,20–27]. The above support our hypothesis that Foxf1 is a key player in osteogenesis.

Thus, we assayed Foxf1 expression in bone tissue, BMSCs, and BMMs from OVX mice, and during osteogenic differentiation and osteoclast differentiation, by qRT-PCR and WB. Foxf1 expression was

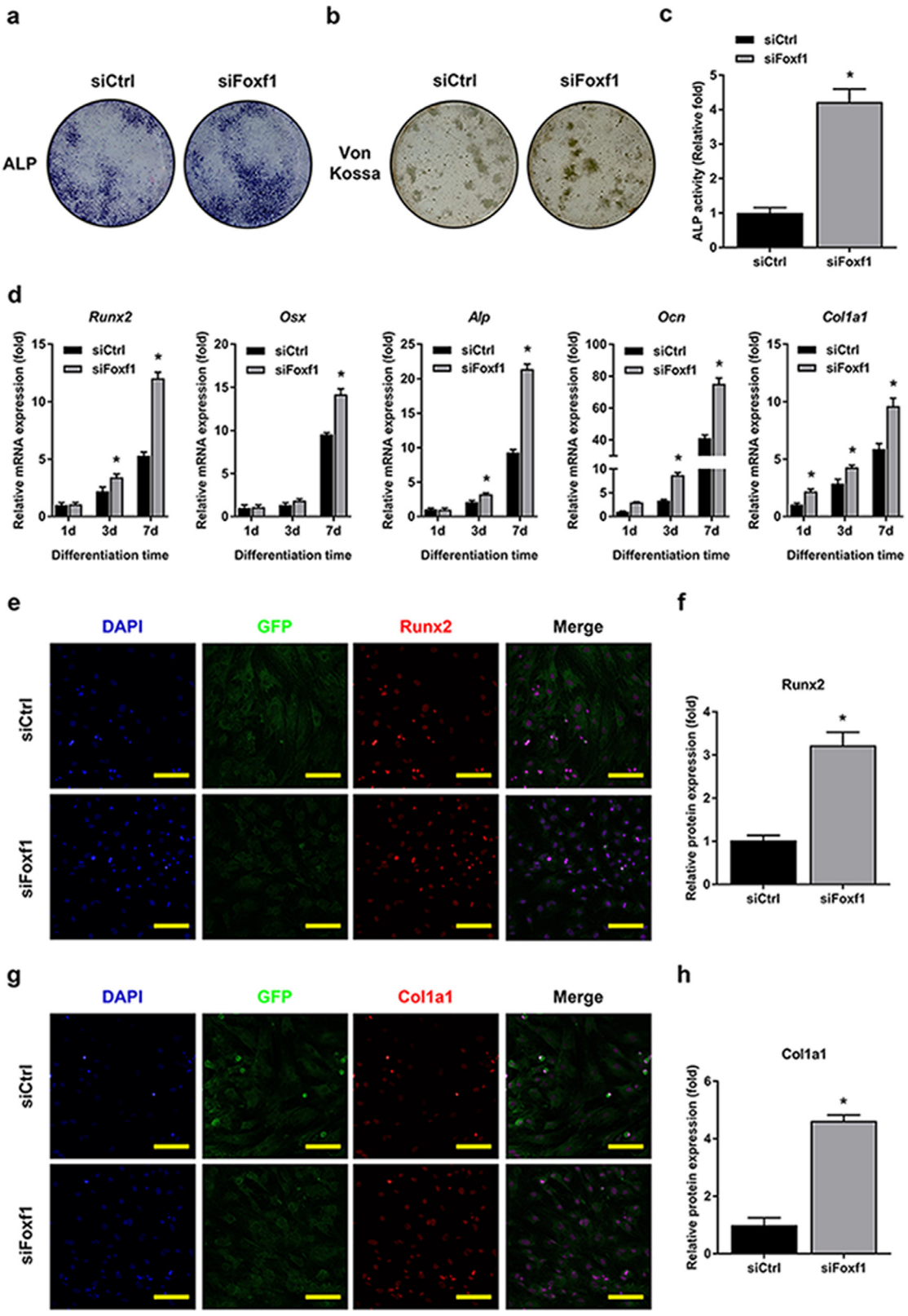


**Fig. 3.** Foxf1 knockdown enhanced the osteogenesis of BMSCs. (a and b) Representative images of ALP staining and Von Kossa staining of BMSCs transfected with siFoxf1. $n = 3$ biologically independent samples. (c) Quantitative analysis of the ALP activity of BMSCs transfected with siFoxf1. Mean ± SD, $n = 3$ biologically independent samples, $^*P < 0.05$ vs siCtrl by Student's *t*-test. (d) qRT-PCR analysis showed that siFoxf1 treatment significantly increased the mRNA expression of osteogenic markers *Runx2, Alp, Osx, Ocn*, and *Col1a1*. Mean ± SD, $n = 3$ biologically independent samples, $^*P < 0.05$ vs. siCtrl by Student's *t*-test. (e-h) IF analysis revealed that siFoxf1 treatment promoted the protein expression level of Runx2 and Col1a1. Scale bars, 50 $\mu$m. Mean ± SD, $n = 3$ biologically independent samples, $^*P < 0.05$ vs. siCtrl by Student's *t*-test. All *in vitro* experiments were repeated 3 times with similar results.

significantly increased in bone extracts and BMSCs from OVX mice and gradually decreased during osteoblast differentiation of BMSCs but it did not differ significantly in OVX mouse-derived BMMs and during osteoclast differentiation. Foxf1 knockdown promoted ALP activity and mineralisation. Expression levels of osteogenic markers (Runx2 and Col1a1) and osteogenesis-related factors (*Alp, Osx*, and

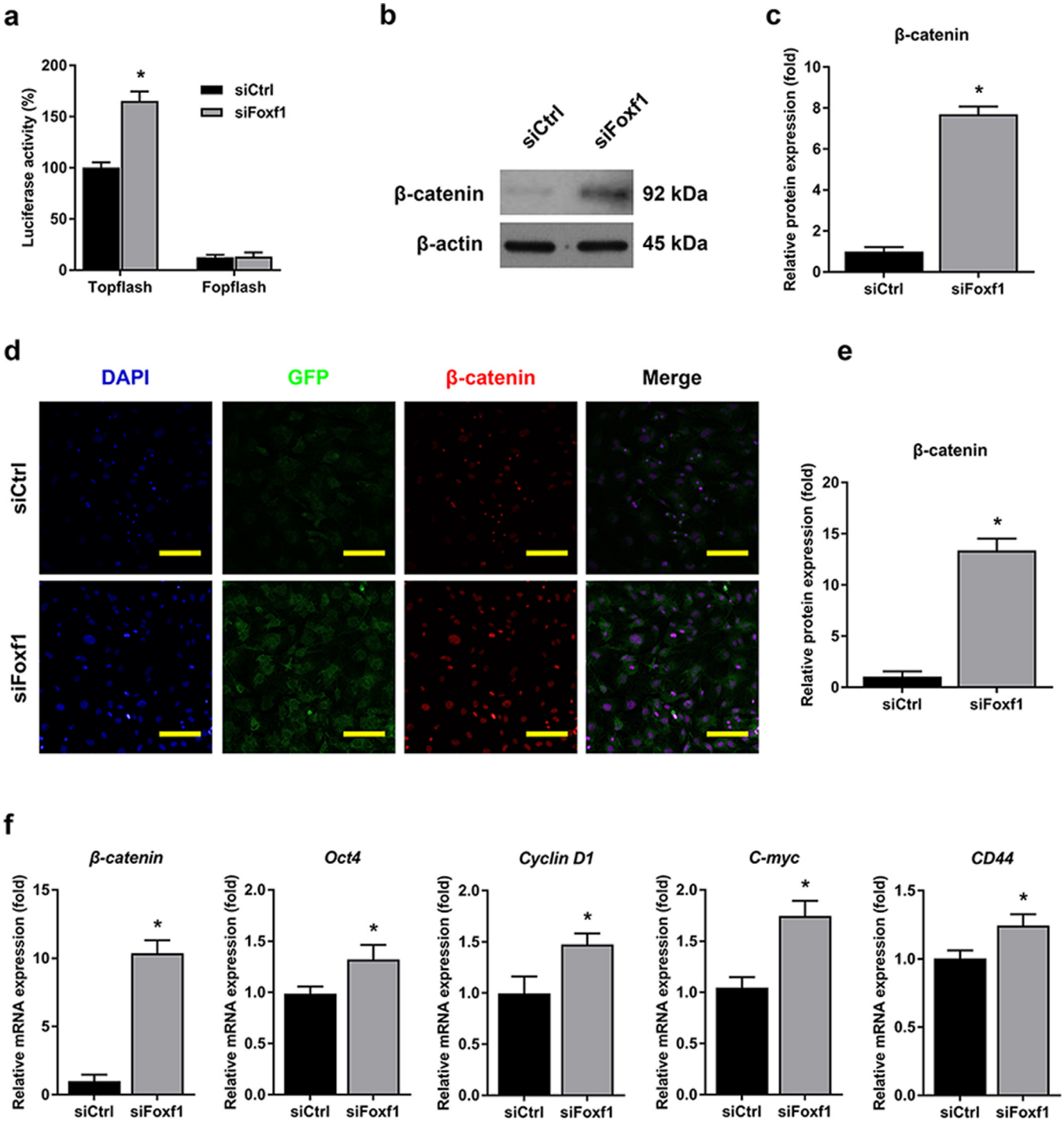


**Fig. 4.** Foxf1 knockdown activated the Wnt/β-catenin signalling pathway. (a) Luciferase reporter assay showed siFoxf1 treatment significantly increased the Topflash activity. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ vs. siCtrl by Student's $t$-test. (b and c) WB analysis revealed that siFoxf1 treatment significantly promoted β-catenin protein expression level. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ vs. siCtrl by Student's $t$-test. (d and e) IF analysis demonstrated that siFoxf1 treatment induced more accumulation of β-catenin protein in the nucleus. Scale bars, 50 $\mu$m. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ vs. siCtrl by Student's $t$-test. (f) qRT-PCR analysis showed that siFoxf1 treatment significantly increased the mRNA expression level of β-*catenin, Oct4, Cyclin D1, C-myc*, and *CD44*. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ vs. siCtrl by Student's $t$-test. All *in vitro* experiments were repeated 3 times with similar results.

*Ocn*) were significantly increased by Foxf1 knockdown, as determined by qRT-PCR and IF. Additionally, siFoxf1 did not modulate the proliferation of BMSCs. Therefore, Foxf1 suppressed osteogenesis by BMSCs *in vitro*.

The Wnt/β-catenin signalling pathway regulates osteogenic differentiation of BMSCs, bone formation, and bone metabolism disorders [45,46]. Activation of this canonical pathway depends on cell membrane binding of Wnt and frizzled receptors and the LRP co-receptor, which inhibits glycogen synthase kinase-3β (GSK3β) expression and stabilises β-catenin in the cytosol, promoting its nucleus translocation. In the nucleus, β-catenin enhances transcription regulated by T-cell factor 4 (TCF4)/lymphoid enhancing factor-1 (LEF1), and the β-catenin-TCF4 complex activates transcription of downstream target genes [47]. In addition, Fox family members modulate bone formation by regulating the Wnt signalling pathway. For instance, Foxo1, 3, and 4 reportedly inhibit bone formation by suppressing Wnt/β-catenin/TCF

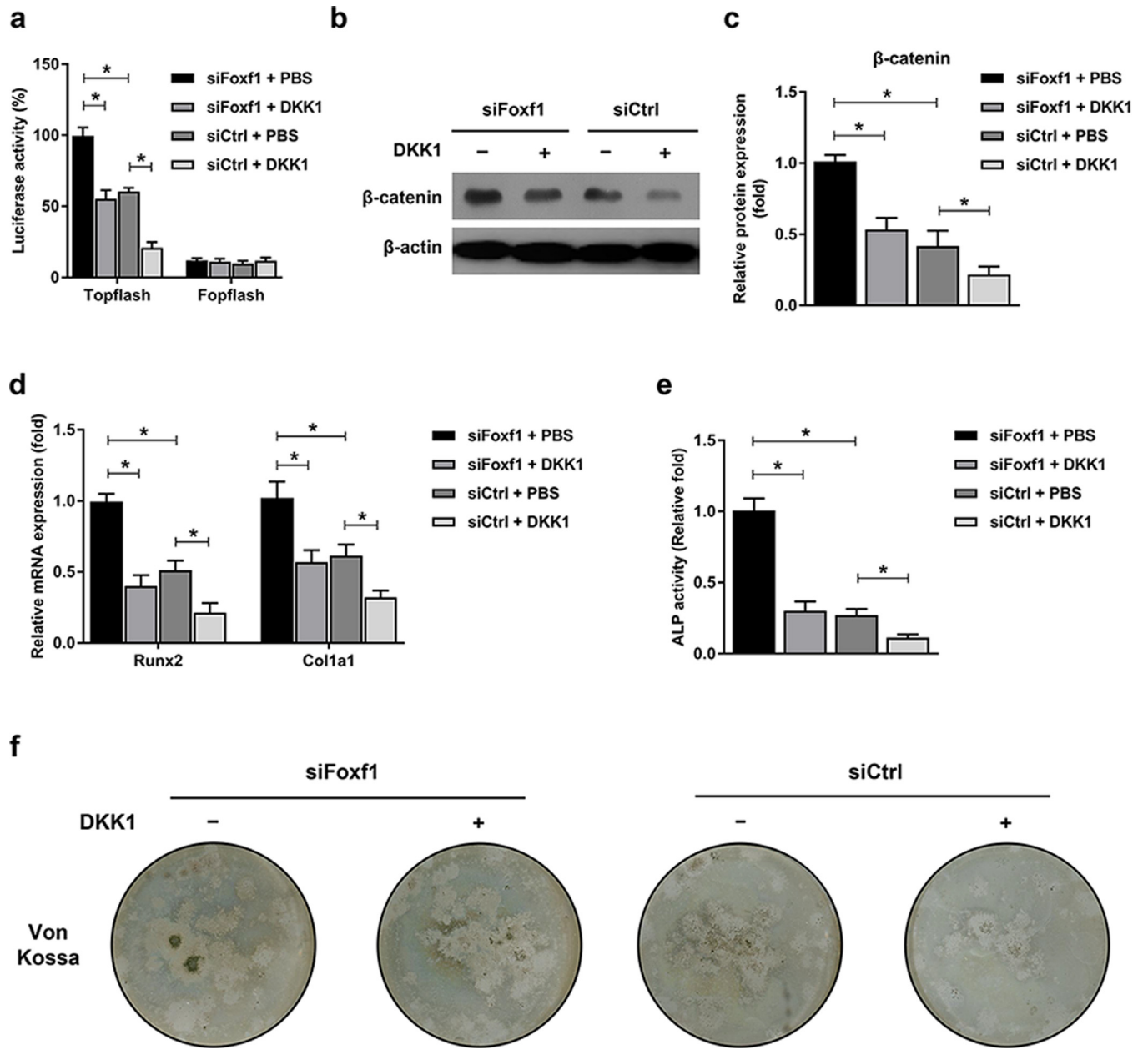


**Fig. 5.** siFoxf1-mediated BMSCs osteogenesis upregulation was partly reversed by DKK1. (a) Luciferase reporter assay showed DKK1 treatment (0.5 $\mu$g/ml) significantly rescued the increased Topflash activity induced by siFoxf1 treatment. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ by ANOVA with Tukey's *post hoc* test. (b and c) WB analysis revealed that DKK1 treatment markedly reversed the increased β-catenin protein expression level induced by siFoxf1 treatment. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ by ANOVA with Tukey's *post hoc* test. (d) qRT-PCR analysis showed that DKK1 treatment markedly rescued the increased *Runx2* and *Col1a1* mRNA expression levels induced by siFoxf1 treatment. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ by ANOVA with Tukey's *post hoc* test. (e) Quantitative analysis of the ALP activity of BMSCs treated with DKK1 and transfected with siFoxf1. Mean ± SD, $n$ = 3 biologically independent samples, $^*P < 0.05$ by ANOVA with Tukey's *post hoc* test. (f) Representative images of Von Kossa staining of BMSCs treated with DKK1 and transfected with siFoxf1. $n$ = 3 biologically independent samples. All *in vitro* experiments were repeated 3 times with similar results.

signalling [48]. Further, Foxf1 was reported to control murine gut development by suppressing mesenchymal Wnt signalling and enhancing extracellular matrix production [12]. Considering the connection between Foxf1 and Wnt signalling, we examined the role of Wnt signalling in Foxf1-mediated osteogenesis by BMSCs. The Wnt/β-catenin signalling pathway was associated with the Foxf1 knockdown-enhanced osteogenic differentiation of BMSCs. Moreover, DKK1 partially rescued the increased osteogenesis of BMSCs induced by Foxf1 knockdown and significantly decreased the expression levels of *Runx2* and *Col1a1*, as well as calcified nodules formation. Therefore, the Wnt/β-catenin signalling pathway is crucial for Foxf1 knockdown-induced osteogenesis by BMSCs.

The skeleton is affected by ageing-associated changes, and promotion of osteogenesis ameliorates PMOP. Teriparatide, a parathyroid hormone, is the only osteoanabolic agent widely used clinically [49]. However, teriparatide therapy is associated with low compliance due to the daily injections and high cost. Also, the anabolic effect of this drug may promote osteosarcoma [50]. We investigated the anabolic effect of Foxf1 knockdown *in vivo* in OVX mice by injecting siFoxf1 or siCtrl intermittently for > 6 weeks. siFoxf1 markedly alleviated trabecular bone loss in OVX mice. Also, the change in bone formation indices and markers indicated that effect of siFoxf1 may be due to the promotion of bone formation. Additionally, Foxf1 expression levels negatively correlated with reduced bone mass and bone formation in

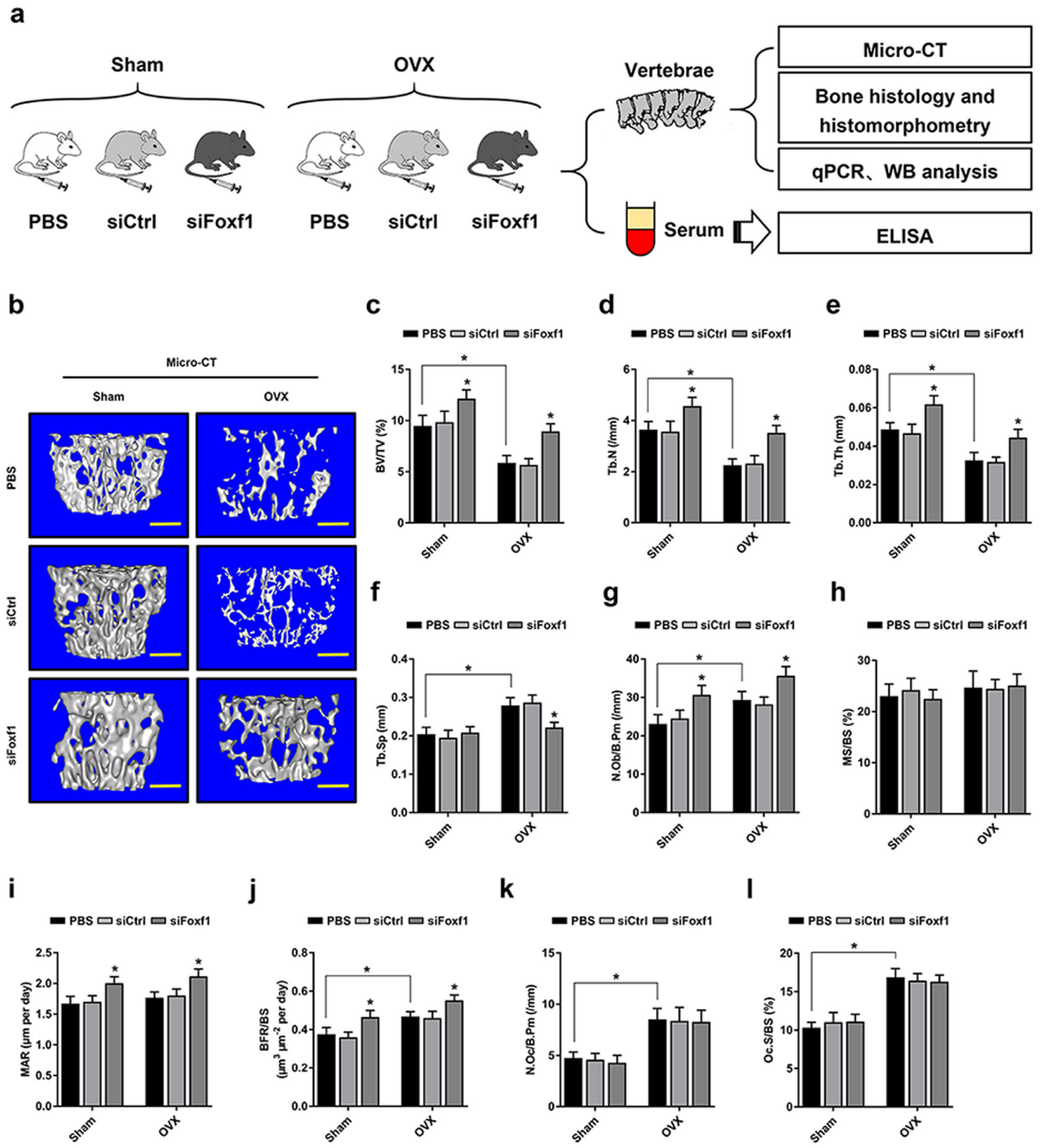


**Fig. 6.** siFoxf1 delivery prevented OVX-induced bone loss. (a) The *in vivo* study design. (b) Representative images of micro-CT scanning of lumbar 4 vertebrae. Scale bars, 500 $\mu$m. $n$ = 8 biologically independent samples. (c–i) Histomorphometric analysis of the trabecular bone in vertebrae, including BV/TV, Tb.Th, Tb.N, Tb.Sp, bone formation parameters (BFR/BS, MAR, MS/BS, and N.Ob/B.Pm), and bone resorption parameters (Oc.S/BS and N.Oc/B.Pm). Mean ± SD, $n$ = 8 biologically independent samples, $^{*}P < 0.05$ by two-way ANOVA.

bone tissue from PMOP patients. Furthermore, siFoxf1 significantly increased osteogenic differentiation of human BMSCs *in vitro*, suggesting it may be a therapeutic target for PMOP.

This is to our knowledge the first report of a role for Foxf1 in osteogenesis by BMSCs, OVX-induced bone loss, and PMOP. However, this study had two limitations. First, we did not evaluate the effect of Foxf1 on other important bone metabolism processes, such as adipogenic and angioblastic differentiation. Second, the molecular mechanism of bone formation is complex and so further work, involving for instance genetically engineered mice, is needed.

In summary, we provide the first experimental and clinical evidence that Foxf1 negatively regulates BMSC osteogenesis and oestrogen deficiency-induced osteoporosis. The endogenous expression level of Foxf1 was increased in OVX mice-derived bone tissue and BMSCs and decreased during osteogenic differentiation of BMSCs, whereas no significant difference in Foxf1 expression was observed

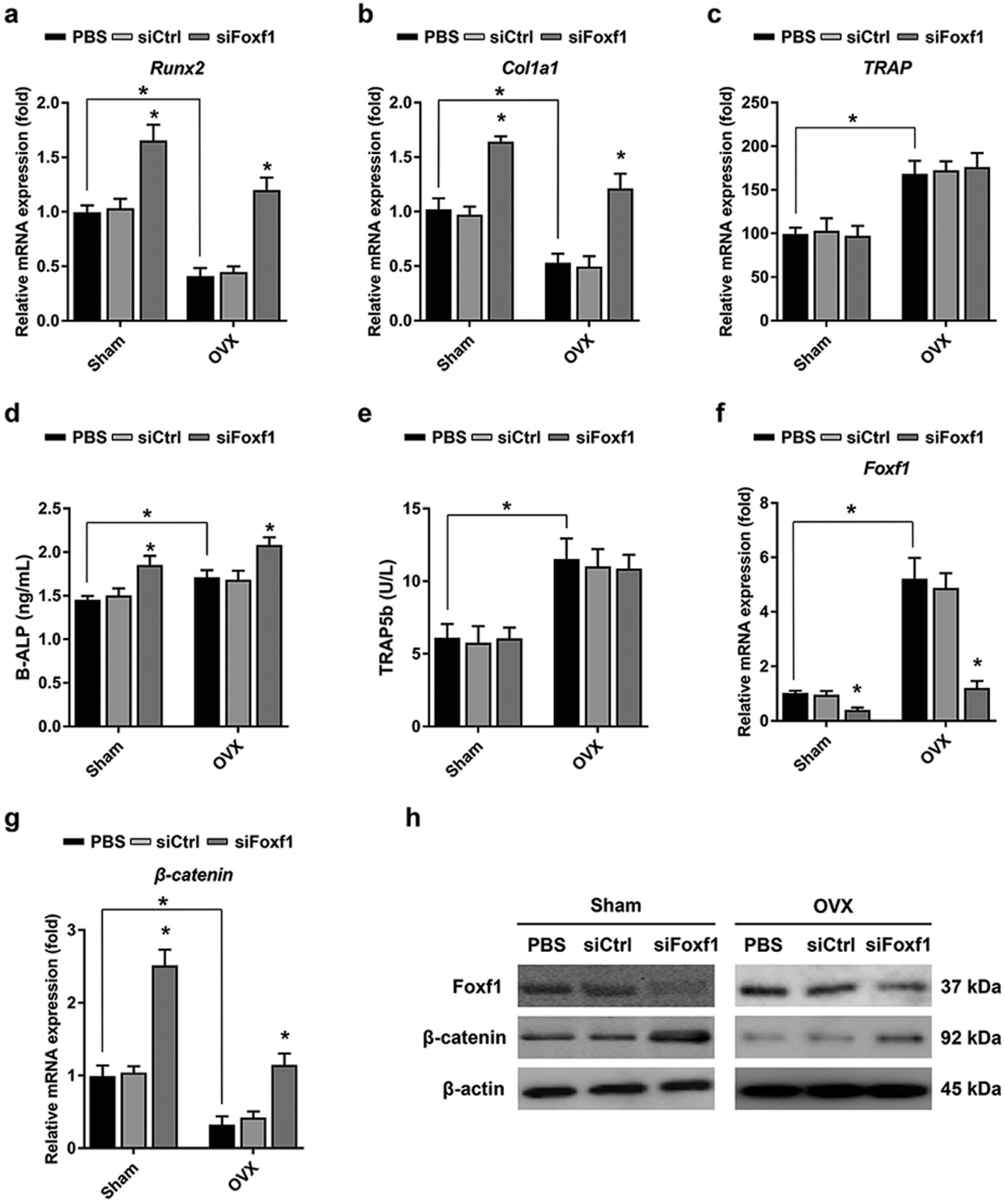


**Fig. 7.** siFoxf1 delivery increased bone formation *in vivo*. (a–c) qRT-PCR analysis showed that siFoxf1 treatment increased the *Runx2* and *Col1a1* mRNA expression levels in bone tissue had no effect on *TRAP* mRNA expression level. Mean ± SD, $n = 8$ biologically independent samples, $^*P < 0.05$ by two-way ANOVA. (d and e) ELISA assay revealed that siFoxf1 treatment increased the expression levels of serum ALP, whereas no significant difference in serum TRAP5b expression level was observed. Mean ± SD, $n = 8$ biologically independent samples, $^*P < 0.05$ by two-way ANOVA. (f) qRT-PCR analysis confirmed the significant decrease in *Foxf1* expression level in Sham- and OVX-derived bone tissues with siFoxf1 delivery. Mean ± SD, $n = 8$ biologically independent samples, $^*P < 0.05$ by two-way ANOVA. (g) qRT-PCR analysis revealed that siFoxf1 delivery markedly increased the β-*catenin* mRNA expression level *in vivo*. Mean ± SD, $n = 8$ biologically independent samples, $^*P < 0.05$ by two-way ANOVA. (h) WB analysis showed that siFoxf1 delivery significantly decreased Foxf1 protein expression level and increased β-catenin protein expression level in Sham- and OVX-derived bone tissues. $n = 8$ biologically independent samples.

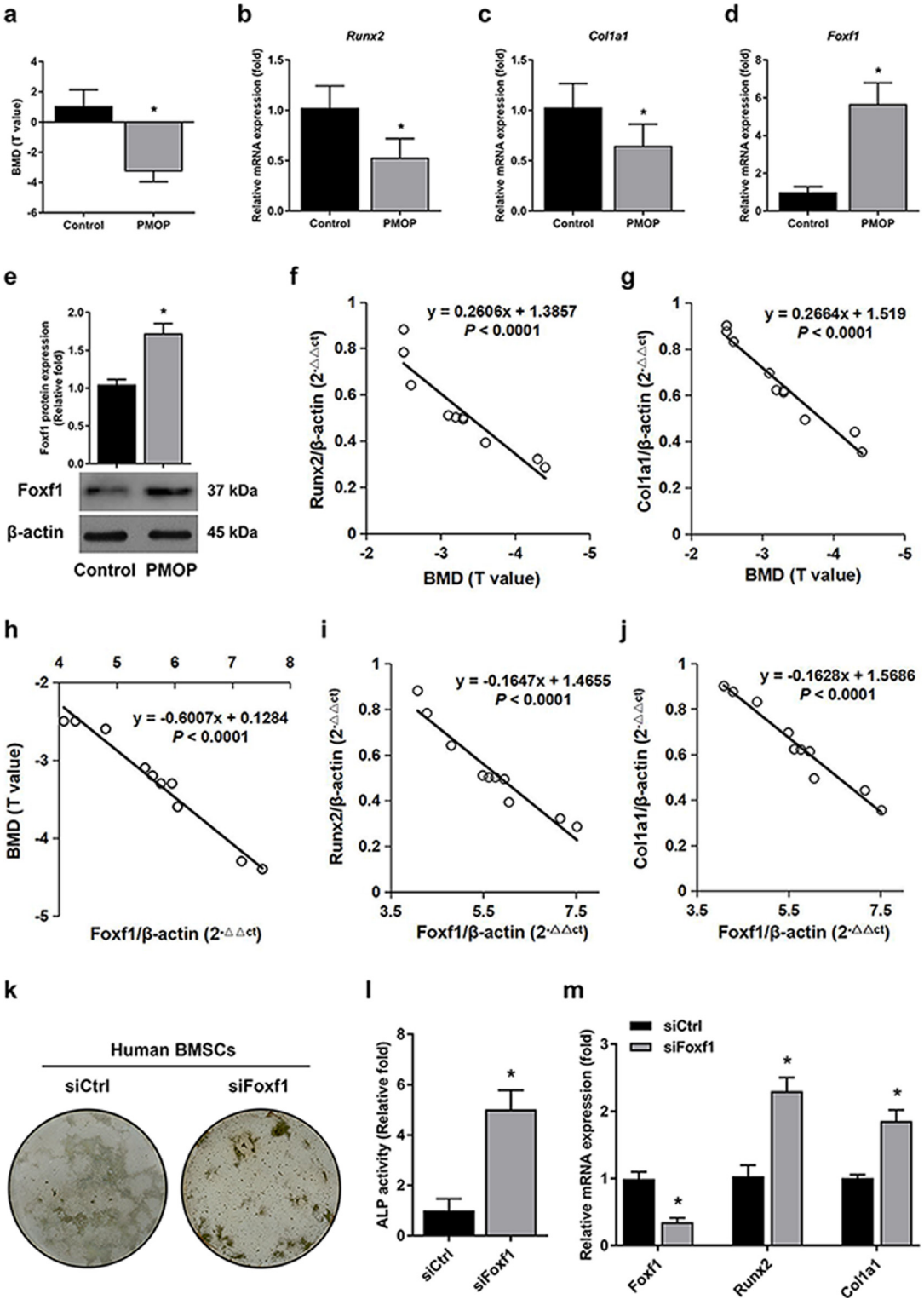


**Fig. 8.** Foxf1 expression in bone extracts derived from PMOP patients and siFoxf1 promotes human BMSCs osteogenesis. (a) BMD of the control group and the PMOP group. Mean ± SD, $n = 10$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (b and c) qRT-PCR analysis showed that *Runx2* and *Col1a1* mRNA expression level were significantly decreased in PMOP patients-derived bone tissues. Mean ± SD, $n = 10$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (d and e) qRT-PCR and WB analysis revealed that Foxf1 mRNA and protein expression level were markedly enhanced in PMOP patients-derived bone tissues. Mean ± SD, $n = 10$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (f and g) Correlation analysis demonstrated that the expression level of *Runx2* and *Col1a1* mRNA were decreasing along with the decreasing BMD levels in PMOP patients. $n = 10$ biologically independent samples. (h) Correlation analysis demonstrated that the expression level of *Foxf1* mRNA was increasing along with the decreasing BMD levels in PMOP patients. $n = 10$ biologically independent samples. (i and j) Correlation analysis demonstrated that Foxf1 expression levels negatively correlated with reduced *Runx2* and *Col1a1* mRNA levels in bone tissue from PMOP patients. $n = 10$ biologically independent samples. (k) Representative images of Von Kossa staining of human BMSCs treated with siFoxf1. $n = 3$ biologically independent samples. (l) Quantitative analysis of the ALP activity of human BMSCs transfected with siFoxf1. Mean ± SD, $n = 3$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. (m) qRT-PCR analysis showed that siFoxf1 treatment significantly decreased *Foxf1* mRNA expression level and increased *Runx2* and *Col1a1* mRNA expression levels. Mean ± SD, $n = 3$ biologically independent samples, $^*P < 0.05$ by Student's *t*-test. All *in vitro* experiments were repeated 3 times with similar results.

in OVX mouse-derived BMMs and during osteoclast differentiation. Using a loss-of-function approach *in vitro*, we revealed that Foxf1 knockdown significantly increased BMSC osteogenesis partly by activating the Wnt/β-catenin signalling pathway. *In vivo*, siFoxf1 ameliorated OVX-induced bone loss by promoting osteoblastogenesis and mineralisation. Clinically, Foxf1 expression levels negatively correlated with reduced bone mass and bone formation in bone tissue from PMOP patients. Further, siFoxf1 promoted osteogenic differentiation of human BMSCs. Our data demonstrate that knockdown of Foxf1 promotes BMSCs osteogenesis and prevents OVX-induced bone loss, suggesting it may be a biomarker of osteogenesis and a potential therapeutic target for PMOP.

## Authors contributions

G.S., H.R., and X.J. designed the study. G.S., H.R., Q.S., W.Z., Z.Z., X.Y. and K.T. performed the experiments. J.T., Z.Y., D.L. and X.J. collected surgically human samples. G.S. wrote the original draft of the manuscript. G.S., H.R., Q.S. and X.J. reviewed and edited the manuscript. G.S., H.R., J.T. Z.Y., D.L. and X.J. supervised the research.

## Declarations of Competing Interest

The authors declare no competing interests.

## Acknowledgements

This work was supported in part by the following grants: Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (2018), National Natural Science Foundation of China (81904225, 81503591, 81774338, 81674000), Key Project of Basic Research and Applied Basic Research of Department of Education of Guangdong Province (2018KZDXM021), The youth scientific research training project of GZUCM (2019QNPY04), Excellent Young Scholars Project of China Association of Traditional Chinese Medicine (CACM-2017-QNRC1-01), Special Research Project for the Construction of the National TCM Clinical Research Base of the State Administration of Traditional Chinese Medicine (JDZX2015078), Guangdong Natural Science Foundation (2018A030310615), Scientific Research Project of Guangdong Traditional Chinese Medicine Bureau (20191107). The funding institutions had not any role in study design, data collection, data analysis, interpretation or writing of the report in this study.

## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102626.

## References

[1] Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. Lancet 2011;377:1276–87.
[2] Cummings SR, Melton LJ. Epidemiology and outcomes of osteoporotic fractures. Lancet 2002;359:1761–7.
[3] Marie PJ. Targeting integrins to promote bone formation and repair. Nat Rev Endocrinol 2013;9:288–95.
[4] Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504–8.
[5] Katoh M, Katoh M. Human FOX gene family. Int J Oncol 2004;25:1495–500.
[6] Mahlapuu M, Enerbäck S, Carlsson P. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signalling, causes lung and foregut malformations. Development 2001;128:2397–406.
[7] Kalinichenko VV, Zhou Y, Shin B, et al. Wild-type levels of the mouse Forkhead Box f1 gene are essential for lung repair. Am J Physiol Lung Cell Mol Physiol 2002;282:L1253–65.
[8] Kalinichenko VV, Gusarova GA, Kim IM, et al. Foxf1 haploinsufficiency reduces Notch-2 signalling during mouse lung development. Am J Physiol Lung Cell Mol Physiol 2004;286:L521–30.
[9] Ormestad M, Astorga J, Carlsson P. Differences in the embryonic expression patterns of mouse Foxf1 and -2 match their distinct mutant phenotypes. Dev Dyn 2004;229:328–33.
[10] Kim IM, Zhou Y, Ramakrishna S, et al. Functional characterization of evolutionarily conserved DNA regions in forkhead box f1 gene locus. J Biol Chem 2005;280:37908–16.
[11] Lomenick JP, Hubert MA, Handwerger S. Transcription factor FOXF1 regulates growth hormone variant gene expression. Am J Physiol Endocrinol Metab 2006;291:E947–51.
[12] Ormestad M, Astorga J, Landgren H, et al. Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signalling and promoting extracellular matrix production. Development 2006;133:833–43.
[13] Ye C, Chen M, Chen E, et al. Knockdown of FOXA2 enhances the osteogenic differentiation of bone marrow-derived mesenchymal stem cells partly via activation of the ERK signalling pathway. Cell Death Dis 2018;9:836.
[14] Gozo MC, Aspuria PJ, Cheon DJ, et al. Foxc2 induces Wnt4 and Bmp4 expression during muscle regeneration and osteogenesis. Cell Death Differ 2013;20:1031–42.
[15] Zhou P, Li Y, Di R, et al. H19 and Foxc2 synergistically promotes osteogenic differentiation of BMSCs via Wnt-β-catenin pathway. J Cell Physiol 2019;234:13799–806.
[16] Sung IY, Park BC, Hah YS, et al. FOXO1 Is Involved in the Effects of Cigarette Smoke Extract on Osteoblastic Differentiation of Cultured Human Periosteum-derived Cells. Int J Med Sci 2015;12:881–90.
[17] Bartell SM, Kim HN, Ambrogini E, et al. FoxO proteins restrain osteoclastogenesis and bone resorption by attenuating H2O2 accumulation. Nat Commun 2014;5:3773.
[18] Tan P, Guan H, Xie L, et al. FOXO1 inhibits osteoclastogenesis partially by antagnozing MYC. Sci Rep 2015;5:16835.
[19] Dou C, Li N, Ding N, et al. HDAC2 regulates FoxO1 during RANKL-induced osteoclastogenesis. Am J Physiol Cell Physiol 2016;310:C780–7.
[20] Bohnenpoll T, Wittern AB, Mamo TM, et al. A SHH-FOXF1-BMP4 signalling axis regulating growth and differentiation of epithelial and mesenchymal tissues in ureter development. PLoS Genet 2017;13:e1006951.
[21] Astorga J, Carlsson P. Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4. Development 2007;134:3753–61.
[22] Ustiyan V, Bolte C, Zhang Y, et al. FOXF1 transcription factor promotes lung morphogenesis by inducing cellular proliferation in fetal lung mesenchyme. Dev Biol 2018;443:50–63.
[23] Zhao L, Liu Y, Tong D, et al. MeCP2 Promotes Gastric Cancer Progression Through Regulating FOXF1/Wnt5a/β-Catenin and MYOD1/Caspase-3 Signalling Pathways. EBioMedicine 2017;16:87–100.
[24] Katoh M, Katoh M. Transcriptional mechanisms of WNT5A based on NF-kappaB, Hedgehog, TGFbeta, and Notch signalling cascades. Int J Mol Med 2009;23:763–9.
[25] Katoh M, Katoh M. Transcriptional regulation of WNT2B based on the balance of Hedgehog, Notch, BMP and WNT signals. Int J Oncol 2009;34:1411–5.
[26] Fulford L, Milewski D, Ustiyan V, et al. The transcription factor FOXF1 promotes prostate cancer by stimulating the mitogen-activated protein kinase ERK5. Sci Signal 2016;9:ra48.
[27] Kalinichenko VV, Bhattacharyya D, Zhou Y, et al. Foxf1 +/- mice exhibit defective stellate cell activation and abnormal liver regeneration following CCl4 injury. Hepatology 2003;37:107–17.
[28] Tsialogiannis E, Polyzois I, Oak Tang Q, et al. Targeting bone morphogenetic protein antagonists: in vitro and in vivo evidence of their role in bone metabolism. Expert Opin Ther Targets 2009;13:123–37.
[29] Maria S, Samsonraj RM, Munmun F, et al. Biological effects of melatonin on osteoblast/osteoclast cocultures, bone, and quality of life: Implications of a role for MT2 melatonin receptors, MEK1/2, and MEK5 in melatonin-mediated osteoblastogenesis. J Pineal Res 2018:64.
[30] Appelman-Dijkstra NM, Papapoulos SE. Clinical advantages and disadvantages of anabolic bone therapies targeting the WNT pathway. Nat Rev Endocrinol 2018;14:605–23.
[31] Zanotti S, Canalis E. Notch Signalling and the Skeleton. Endocr Rev 2016;37:223–53.
[32] Alman BA. The role of hedgehog signalling in skeletal health and disease. Nat Rev Rheumatol 2015;11:552–60.
[33] Novack DV. Role of NF-κB in the skeleton. Cell Res 2011;21:169–82.
[34] Ren H, Yu X, Shen G, et al. miRNA-seq analysis of human vertebrae provides insight into the mechanism underlying GIOP. Bone 2019;120:371–86.
[35] Dempster DW, Compston JE, Drezner MK, et al. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 2013;28:2–17.
[36] Pinzone JJ, Hall BM, Thudi NK, et al. The role of Dickkopf-1 in bone development, homeostasis, and disease. Blood 2009;113:517–25.
[37] Minogue BM, Richardson SM, Zeef LA, Freemont AJ, Hoyland JA. Characterization of the human nucleus pulposus cell phenotype and evaluation of novel marker gene expression to define adult stem cell differentiation. Arthritis Rheum 2010;62:3695–705.
[38] Xu R, Yallowitz A, Qin A, et al. Targeting skeletal endothelium to ameliorate bone loss. Nat Med 2018;24:823–33.
[39] Xie H, Cui Z, Wang L, et al. PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis. Nat Med 2014;20:1270–8.
[40] Sturtzel C, Lipnik K, Hofer-Warbinek R, et al. FOXF1 Mediates Endothelial Progenitor Functions and Regulates Vascular Sprouting. Front Bioeng Biotechnol 2018;6:76.
[41] Astorga J, Carlsson P. Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4. Development 2007;134:3753–61.
[42] Wang S, Xiao Z, Hong Z, et al. FOXF1 promotes angiogenesis and accelerates bevacizumab resistance in colorectal cancer by transcriptionally activating VEGFA. Cancer Lett 2018;439:78–90.

[43] Ren X, Ustiyan V, Pradhan A, et al. FOXF1 transcription factor is required for formation of embryonic vasculature by regulating VEGF signalling in endothelial cells. Circ Res 2014;115:709–20.
[44] Dharmadhikari AV, Sun JJ, Gogolewski K, et al. Lethal lung hypoplasia and vascular defects in mice with conditional Foxf1 overexpression. Biol Open 2016;5:1595–606.
[45] Baron R, Kneissel M. WNT signalling in bone homeostasis and disease: from human mutations to treatments. Nat Med 2013;19:179–92.
[46] Hartmann C. A Wnt canon orchestrating osteoblastogenesis. Trends Cell Biol 2006;16:151–8.
[47] Canalis E. Wnt signalling in osteoporosis: mechanisms and novel therapeutic approaches. Nat Rev Endocrinol 2013;9:575–83.
[48] Iyer S, Ambrogini E, Bartell SM, et al. FOXOs attenuate bone formation by suppressing Wnt signalling. J Clin Invest 2013;123:3409–19.
[49] Hodsman AB, Bauer DC, Dempster DW, et al. Parathyroid hormone and teriparatide for the treatment of osteoporosis: a review of the evidence and suggested guidelines for its use. Endocr Rev 2005;26:688–703.
[50] Canalis E, Giustina A, Bilezikian JP. Mechanisms of anabolic therapies for osteoporosis. N Engl J Med 2007;357:905–16.