Research paper

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

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\textbf{ABSTRACT}

\textbf{Background:} Forkhead box protein f1 (Foxf1) is associated with cell differentiation, and may be a key player in bone homeostasis. 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.

\textbf{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) \textit{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) \textit{in vivo}.

\textbf{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. \textit{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.

\textbf{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.

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\textbf{Keywords:}

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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 morbidity [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 homeostasis are necessary for understanding and treating bone metabolism-related diseases, such as osteoporotic bone loss.

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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 in 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.

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 homeostasis.

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.

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% CO2 atmosphere. Adherent human BMSCs were cultured in human BMSC growth medium (Cyagen Biosciences). Mouse BMSCs were isolated and cultured as follows. Briefly, theibia 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 BMD T-score, ≤ −2.5), (b) acute fragile lumbar fractures in the prior 2 weeks and clear indication of vertebralplasty or internal fixation, (c) normal levels of laboratory indicators, particularly serum calcium level (2.25 ± 0.13 vs 2.23 ± 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 spondyloolisthesis and lumbar spinal stenosis) and (b) no osteoporosis or other metabolic disease. We collected vertebral bone samples as described previously [34].

2.4. siRNA-mediated knockdown and cell transfection

Foxf1-specific siRNAs (CGTGTGGGAAGACGACTT for human and GATCGGCTTACGGGGTTTTA for mouse) and negative control siRNA (sciCtrl) (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 μl of α-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 (α-MEM supplemented with 10% FBS, 1% ITS, 0.2 mM ascorbic acid, 10 mM β-glycerophosphate and 10 mM 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 μl with 1 μl 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⁻ΔΔCt 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 × 10⁴ 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 GATCCGGCTAGC-GAGTTTA.

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/100μA). 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 euthanisation. 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 μm thickness using a microtome. The bone histomorphometry parameters were evaluated using the bone formation rate (BFR/BS, μm²/μm² per day), mineral apposition rate (MAR, μm 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’s 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 ≤ 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-mediating 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, 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
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, Top\textsuperscript{fl}ash activity and \(\beta\)-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\textsuperscript{-1}), siCtrl (7 mg kg\textsuperscript{-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 remarkably 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–i). Nevertheless, siFoxf1 did not influence the expression of bone resorption parameters, including Ocs/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
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 murine 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
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...
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. 4](image-url)
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 nodule 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. 5. siFoxf1-mediated BMSCs osteogenesis upregulation was partly reversed by DKK1. (a) Luciferase reporter assay showed DKK1 treatment (0.5 μg/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.
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. 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 μm. n = 8 biologically independent samples. (c–l) 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.
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.

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Supplementary materials

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