FOXJ3 drives mesenchymal stem cell osteogenic differentiation via the Wnt/β-catenin pathway: A novel regulator implicated in osteoporosis

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Abstract. Osteoporosis involves impaired osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). The present study identified the transcription factor forkhead box (FOXJ3) as a novel regulator of this process. During *in vitro* osteogenic differentiation of BMSCs, FOXJ3 expression progressively increased, and was positively correlated with osteogenic markers Runt-2 transcription factor 2 (RUNX2) and osteocalcin (OCN). Functional studies confirmed the essential role of FOXJ3: Small interfering RNA-mediated knockdown markedly impaired differentiation, as evidenced by reduced alkaline phosphatase (ALP) activity, diminished mineralized nodule formation, and downregulation of RUNX2 and OCN. Conversely, lentivirus-induced FOXJ3 overexpression enhanced these osteogenic markers and outcomes. Mechanistically, FOXJ3 knockdown suppressed active β-catenin expression, indicating Wnt/β-catenin pathway involvement. Crucially, the Wnt/β-catenin agonist SB216763 rescued the inhibitory effects of FOXJ3 knockdown on ALP activity and mineralization. Conversely, the pro-osteogenic effects of FOXJ3 overexpression were abrogated by the Wnt inhibitor XAV939. These findings establish FOXJ3 as a positive regulator of BMSC osteogenic differentiation acting primarily through the Wnt/β-catenin pathway, presenting a novel potential therapeutic target for osteoporosis.

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Introduction

Osteoporosis is a disease characterized by changes in bone microstructure, including thinning of trabeculae and an increased susceptibility to brittle fractures (1). Osteoporosis primarily results from an imbalance in bone metabolism, where bone formation is weakened while bone resorption increases, leading to a loss of bone mass (2). Factors such as aging, inflammation and hormonal changes lead to a reduction in bone formation (3); however, the specific reasons have not yet been fully elucidated.

Bone marrow mesenchymal stem cells (BMSCs) are a type of pluripotent stem cell with the ability to differentiate into three lineages: Osteoblasts, adipocytes and chondrocytes (4). It has previously been shown that the differentiation strength of BMSCs is markedly associated with bone changes *in vivo* (5). Research using tissue engineering functional scaffolds has demonstrated that increasing the osteogenic differentiation ability of BMSCs leads to a notable increase in bone formation *in vivo* (6). Therefore, BMSCs are considered an important target and functional cell for treating diseases characterized by reduced bone formation (7,8). However, the specific regulatory mechanism underlying the osteogenic differentiation of BMSCs has not yet been fully elucidated and requires further investigation.

Forkhead box (FOXJ3) possesses DNA-binding transcriptional activation activity, RNA polymerase II specificity and sequence-specific double-stranded DNA-binding activity. Notably, FOXJ3 participates in the positive regulation of RNA polymerase II transcription. Previous studies have shown that FOXJ3 is related to the progression of various diseases, such as rheumatoid arthritis (9), and spermatogenesis (10). Furthermore, it has been reported to serve an important role in the disease evolution process in cancer (11,12). At the metabolic level, it has been reported that FOXJ3 can promote the thermogenic effect of fat (13). In addition, FOXJ3 can promote the formation of osteoclasts (14). Fat metabolism and osteoclastogenesis in the bone marrow are associated with bone formation and other processes. Given the important role of BMSCs in bone formation, it is crucial to clarify whether FOXJ3 affects the osteogenic differentiation function of BMSCs and its role in bone metabolic diseases. However, to the best of our knowledge, no relevant studies have yet been published.

The Wnt/β-catenin pathway is a crucial pathway that serves important roles in various cell functions, including cell proliferation (15) and differentiation (16). It has previously been shown that this pathway can promote the osteogenic differentiation of BMSCs (17). After activation of the Wnt/β-catenin protein and its entry into the nucleus, it can activate the expression of osteogenic-related molecules, promote the secretion of extracellular matrix proteins, and the synthesis of alkaline phosphatase (ALP) and other substances by BMSCs, thereby promoting mineralization (18). Therefore, the present study aims to investigate whether FOXJ3 is involved in the osteogenic differentiation of BMSCs and whether it exerts its regulatory function through the Wnt/β-catenin pathway, thereby providing a new therapeutic target for bone metabolic diseases.

Materials and methods

BMSC Treatment. Rat BMSCs were obtained from Wuhan Servicebio Technology Co., Ltd. Since BMSCs from passages 3-6 exhibit a homogeneous population, consistent morphology and robust osteogenic differentiation functionality, all experiments were conducted using cells within this passage range. Adherent BMSCs were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd.) in a humidified incubator maintained at 37˚C with 5% CO_2.

Osteoblast differentiation. BMSCs were seeded at a density of $1x10^5$ cells/well in 12-well plates. Following medium renewal on day 2, the BMSCs were induced to differentiate by culturing them in low-glucose DMEM supplemented with 10% FBS, 10^{-8} M dexamethasone (Sigma-Aldrich; Merck KGaA; cat: D4902), 50 μg/ml ascorbic acid 2-phosphate (Sigma-Aldrich, cat: 49752) and 10 mM β-glycerophosphate (Sigma-Aldrich; Merck KGaA; cat. no. G9422). The culture medium was refreshed every 3 days. Osteogenic differentiation medium was supplemented with 5 μM SB216763 (19-21) or 10 μM XAV939 (22,23) (both from Shanghai Aladdin Biochemical Technology Co., Ltd.) to activate or inhibit the Wnt/β-catenin signaling pathway during BMSC culture in 37˚C, respectively. The DMSO group was supplemented with the same volume of DMSO as the groups treated with SB216763 or XAV939.

ALP activity assay. BMSCs were seeded at a density of 1x10 cells/well in 12-well plates. ALP activity was assessed following 10 days of osteogenic differentiation, in accordance with the manufacturer's protocol, using an ALP activity assay kit (Beyotime Biotechnology, cat: C3206). Total protein concentrations in the lysates (Beyotime Biotechnology, cat: P0013) were determined using the bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.). Results were normalized to total protein content and expressed relative to the control condition.

Alizarin Red S (ARS) staining. The degree of mineralization was determined by ARS staining. BMSCs were seeded at a density of $1x10^5$ cells/well in 12-well plates. After osteogenic differentiation, the cells were fixed with 95% ethanol at 25˚C for 30 min, followed by incubation with 0.1% ARS solution (pH 4.2; Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. To quantify mineralization, the calcium-bound dye was solubilized using 10% cetylpyridinium chloride (Sigma-Aldrich; Merck KGaA) for 1 h in 25˚C, and the eluate was assayed spectrophotometrically at 562 nm. Staining intensity was captured in light microscope and normalized to total protein content and reported relative to the undifferentiated control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from BMSCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), followed by cDNA synthesis via RT with random primers and an M-MLV Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Subsequently, qPCR analyses were performed using a SYBR Green PCR kit (Takara Biotechnology Co., Ltd.). Thermocycling conditions were as follows: 94˚C, 30 sec; 55˚C, 30 sec. Step 3: 72˚C, 1 min). GAPDH expression was used for normalization. The ΔCq values were calculated relative to GAPDH, and relative quantification of gene expression was determined using the $2^{-\Delta\Delta Cq}$ method (24). Each sample was assessed in triplicate. The primers used are shown in Table I.

Western blotting. Cell protein was obtained using lysis buffer (Beyotime Biotechnology, cat: P0013) and quantified by BCA method. Protein lysates (30 μg/lane) underwent electrophoretic separation on 10% SDS-polyacrylamide gels followed by wet transfer to PVDF membranes (Sigma-Aldrich; Merck KGaA). Membranes were then blocked for 1 h in 0.1% TBS-Tween (TBST) containing 5% non-fat dry milk at room temperature. Primary antibody (FOXJ3: Solarbio, Cat: K008825P. active β-catenin: Solarbio, Cat: K009589P. β-catenin: Solarbio, Cat: K008788P. AKT: Solarbio, Cat: K109232P. p-AKT: Solarbio, Cat: Cat:K000186M. ERK: Solarbio, Cat: K200062M. p-ERK: Solarbio, Cat: K009730P. GAPDH: Solarbio, cat. no. K200057M) incubation was performed overnight at 4˚C (1:1,000). After washing, the blots were incubated for 1 h at room temperature with a HRP-linked goat anti-rabbit secondary antibody (1:1,000) (Solarbio, Cat. no. SE132 and SE134). Following three 5-min TBST washes, protein bands were treated by ECL kit (Beyotime Biotechnology, cat: P0018S) and detected by enhanced chemiluminescence after substrate application. GAPDH blotting served as the normalization control. ImageJ software (National Institutes of Health, V1.47) is used for protein quantification.

Lentivirus production and infection. A lentiviral vector encoding FOXJ3 (lentiviral vector backbone: pCDH-EF1a-MCS-IRES-puro; OE-FOXJ3) was generated by Guangzhou iGene Biotechnology Co., Ltd. In short, 293T cells (Guangzhou iGene Biotechnology Co.) were co-transfected using a third-generation lentiviral system, with plasmid ratios of 4 μg (target plasmid): 3 μg (psPAX2): 1 μg (pMD2.G). Virus supernatants were collected in batches at 48 and 72 h after transfection and filtered through a 0.45 μm filter membrane. The virus particles were concentrated by ultracentrifugation (~70,000-100,000 x g, 2 h) in 4˚C, and the precipitate was resuspended in a 500 μl of buffer. Finally, the samples were aliquoted and stored at -80˚C. When cells reached 80-90%

Table I. Primer sequences.

Gene	Primer sequence, 5'-3'
GAPDH	F: AACCCTCAACAGGGATGCTT
	R: GTTCACACCGACCTTCACCA
FOXJ3	F: TTCTCTGGCATTGGGGCAAA
	R: CTGGCATAGCTGTACGGAGG
RUNX2	F: CAACCGAGTCAGTGAGTGCT
	R: CAAACCATACCCAGTCCCTGT
OCN	F: CCGTTTAGGGCATGTGTTGC
	R: CCGTCCATACTTTCGAGGCA

F, forward; FOXJ3, forkhead box J3; OCN, osteocalcin; R, reverse; RUNX2, Runt-related transcription factor 2.

Table II. siRNA sequences.

siRNA	siRNA sequence, 5'-3'
siControl	Sense: UUCUCCGAACGUGUCACGUTT
	Antisense: ACGUGACACGUUCGGAGA ATT
siFOXJ3-1	Sense: CGGGCCUCAACUCCAUAUATT
	Antisense: UAUAUGGAGUUGAGGCCC GTT
siFOXJ3-2	Sense: GGGAAGUGUACAUAGUUA UTT
	Antisense: AUAACUAUGUACACUUCC CTT
siFOXJ3-3	Sense: CUGGAGAGCAGCCUAACAUTT
	Antisense: AUGUUAGGCUGCUCUCCA GTT

FOXJ3, forkhead box J3; si, small interfering.

confluency, lentiviral transduction was performed for 24 h at 37˚C. The viral supernatant was diluted in complete medium to achieve a multiplicity of infection of 10 and was applied to cells supplemented with polybrene (8 μg/ml; Sigma-Aldrich; Merck KGaA) in 24 h. The negative control was prepared by transducing the cells with the lentiviral vector backbone lacking the target gene. Transduction efficiency was assessed by RT-qPCR analysis of FOXJ3 mRNA levels. Subsequent experiments were performed in 24 h later.

Small interfering (si)RNA transfection. Gene silencing was performed using FOXJ3-targeting siRNAs (Shanghai GenePharma Co., Ltd.), with a non-targeting scrambled siRNA (Shanghai GenePharma Co., Ltd.) used as a negative control. Transfection of $1x10^5$ BMSCs was carried out using 50 nM siRNA with Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to standard procedures in 37˚C in 6 h. Subsequent experiments were performed in 6 h later. The siRNA sequences are shown in Table II.

Statistical analysis. All data were obtained from experiments repeated at least three times. Results are presented as the mean ± standard deviation. All statistical analyses were conducted using SPSS 18.0 (IBM Corp.). Paired Student's t-test was used for two-group comparisons, whereas one-way ANOVA with Tukey's HSD post hoc multiple comparisons test applied for multi-group analyses. The Pearson correlation test was performed for correlation analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FOXJ3 expression is positively associated with BMSC osteogenic differentiation. Firstly, osteogenic differentiation of BMSCs was induced and dynamic changes in FOXJ3 expression were detected during this differentiation process. The results of ARS staining indicated that BMSCs were effectively differentiated into osteoblasts after a 14-day culture in osteogenic induction medium (Fig. 1A). RT-qPCR results demonstrated that FOXJ3 expression progressively increased with prolonged osteogenic induction time, reaching peak levels on day 10 of induction with a ~2-fold increase compared with that in the non-induced group (Fig. 1B). Furthermore, RT-qPCR analysis revealed a positive correlation between FOXJ3 expression and the expression of osteogenesis-related genes Runt-related transcription factor 2 (RUNX2) (Fig. 1C) and osteocalcin (OCN) (Fig. 1D). Additionally, western blotting demonstrated a progressive elevation in FOXJ3 protein expression with extended osteogenic induction, reaching a maximum on day 10 of induction (Fig. 1E and F). These findings collectively suggested that FOXJ3 may be positively associated with osteogenic differentiation of BMSCs and could serve a regulatory role in BMSCs osteogenic differentiation processes.

Loss of FOXJ3 in vitro inhibits the osteogenic differentiation of BMSCs. To investigate the regulatory role of FOXJ3 in BMSCs osteogenic differentiation, BMSCs were transfected with siRNA to knock down FOXJ3 expression. The results of RT-qPCR demonstrated that siFOXJ3-1 and siFOXJ3-2 exhibited significant knockdown efficiencies, whereas siFOXJ3-3 showed no substantial effect compared to the siControl group (Fig. 2A). Western blotting further confirmed that siFOXJ3-1 and siFOXJ3-2 effectively reduced FOXJ3 protein expression in BMSCs (Fig. 2B and C). Therefore, siFOXJ3-1 and siFOXJ3-2 for the subsequent experiments. Following FOXJ3 knockdown, osteogenic differentiation was induced in BMSCs. Quantitative ALP analysis revealed decreased ALP activity in both siFOXJ3-1 and siFOXJ3-2 groups compared with that in the control, indicating that FOXJ3 knockdown suppressed ALP activity in BMSCs (Fig. 2D). ARS staining showed that the numbers of osteogenic nodules in the siFOXJ3-1 and siFOXJ3-2 groups were markedly reduced compared with those in the control group (Fig. 2E). Quantitative analysis of ARS staining further confirmed that knockdown of FOXJ3 expression inhibited osteogenic differentiation of BMSCs (Fig. 2F). RT-qPCR demonstrated that the expression of osteogenic differentiation-related genes RUNX2 and OCN was suppressed following FOXJ3 knockdown (Fig. 2G). These

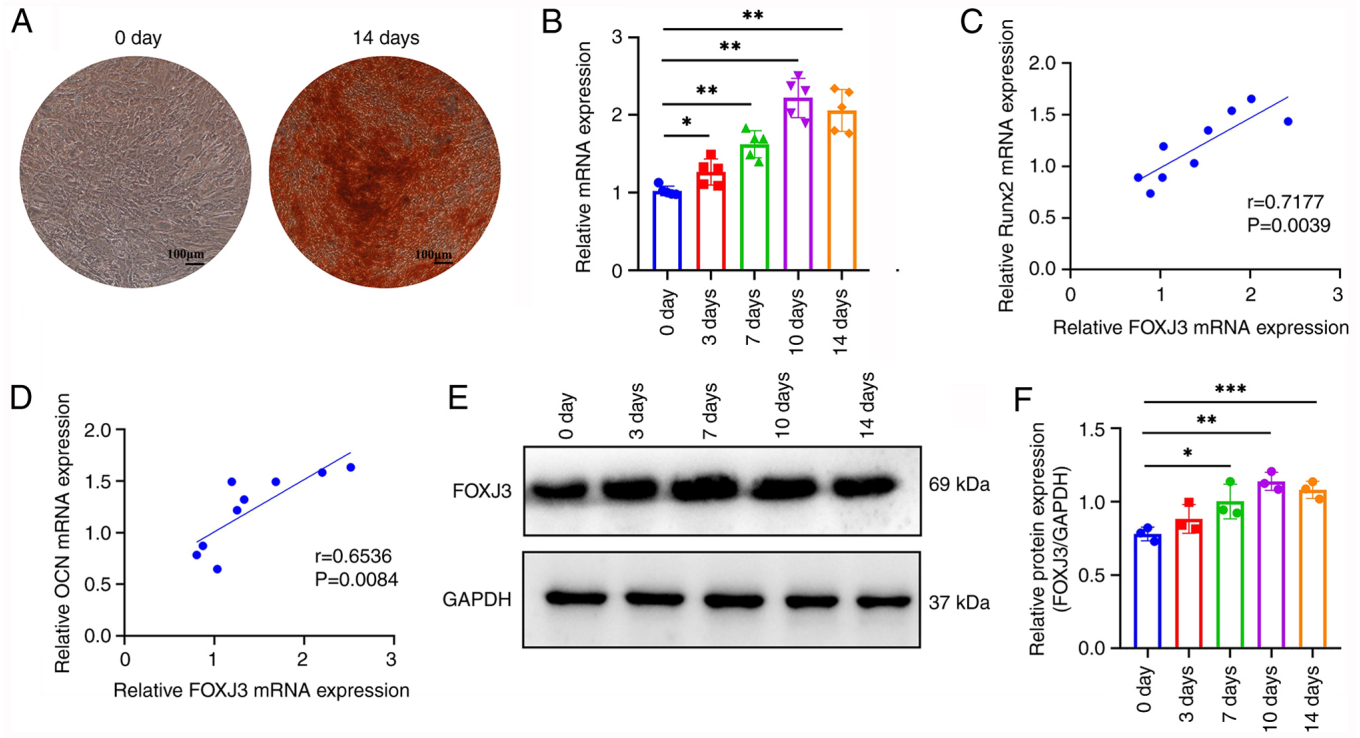

Figure 1. FOXJ3 expression is positively associated with the osteogenic differentiation of BMSCs. (A) Alizarin Red S staining demonstrated that BMSCs were effectively differentiated into osteoblasts. (B) RT-qPCR detection of FOXJ3 expression changes during the osteogenic differentiation of BMSCs (n=5 biologically independent samples). (C) Correlation between the expression levels of FOXJ3 and the osteogenesis-related gene RUNX2, detected by RT-qPCR (n=9 biologically independent samples). (D) Correlation between FOXJ3 and the osteogenesis-related gene OCN, detected by RT-qPCR (n=9). (E) Western blotting results demonstrated a progressive elevation in FOXJ3 protein expression with extended osteogenic induction. (F) Semi-quantitative analysis of western blotting (n=3). *P<0.05, **P<0.01, ***P<0.001. BMSC, bone marrow mesenchymal stem cell; FOXJ3, forkhead box J3; OCN, osteocalcin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RUNX2, Runt-related transcription factor 2.

findings collectively demonstrated that FOXJ3 knockdown may impair the osteogenic differentiation potential in BMSCs.

In vitro overexpression of FOXJ3 promotes BMSCs osteogenic differentiation. To further elucidate the regulatory role of FOXJ3 in osteogenic differentiation, lentiviral infection was used to overexpress FOXJ3 in BMSCs. The results of RT-qPCR showed that the expression levels of FOXJ3 in BMSCs were significantly increased after lentiviral infection (Fig. 3A). Western blotting also revealed that FOXJ3 protein expression was elevated in the OE-FOXJ3 group compared with that in the control group (Fig. 3B). Protein semi-quantification demonstrated a ~2-fold increase in protein expression in the OE-FOXJ3 group relative to the control group (Fig. 2C). Following FOXJ3 OE, osteogenic differentiation was further induced in BMSCs. Quantitative detection of ALP revealed that ALP activity in the OE-FOXJ3 group was significantly increased compared with that in the control group (Fig. 3D). ARS staining results demonstrated a marked increase in osteogenic nodules within the OE-FOXJ3 group relative to the control group (Fig. 3E), and quantitative analysis of ARS staining further confirmed that FOXJ3 overexpression enhanced osteogenic differentiation of BMSCs (Fig. 3F). Additionally, RT-qPCR revealed that the expression levels of the osteogenic differentiation-related genes RUNX2 and OCN were upregulated following FOXJ3 overexpression (Fig. 3G). These findings collectively demonstrated that FOXJ3 gain-of-function may promote osteogenic differentiation in BMSCs.

FOXJ3 regulates the Wnt/β-catenin pathway. To investigate the mechanism by which FOXJ3 regulates BMSC osteogenic differentiation, osteogenic differentiation was induced after knocking down FOXJ3 expression, and the expression levels of proteins in common osteogenic differentiation pathways, including the Wnt/β-catenin, PI3K/AKT and MAPK/ERK pathways, were examined via western blotting. The results revealed that the expression levels of active β-catenin were decreased in the siFOXJ3-1 and siFOXJ3-2 groups compared with those in the control group, whereas p-AKT and p-ERK expression showed no significant differences (Fig. 4A and B). These results indicated that FOXJ3 may primarily promote BMSCs osteogenic differentiation by regulating the Wnt/β-catenin pathway.

FOXJ3 modulates the osteogenic differentiation of BMSCs through the Wnt/β-catenin pathway. To further elucidate the role of the Wnt/β-catenin pathway in FOXJ3-mediated regulation of BMSCs osteogenic differentiation, rescue experiments were performed using pathway-specific inhibitors or agonists. Western blotting initially confirmed alterations in the expression levels of proteins associated with the Wnt/β-catenin pathway following combined FOXJ3 knockdown and treatment with SB216763, a Wnt/β-catenin pathway agonist. The results showed that the expression of active β-catenin in the siFOXJ3 group was decreased compared with that in the siControl group; however, after the addition of the pathway agonist SB216763, levels of active β-catenin were increased to levels

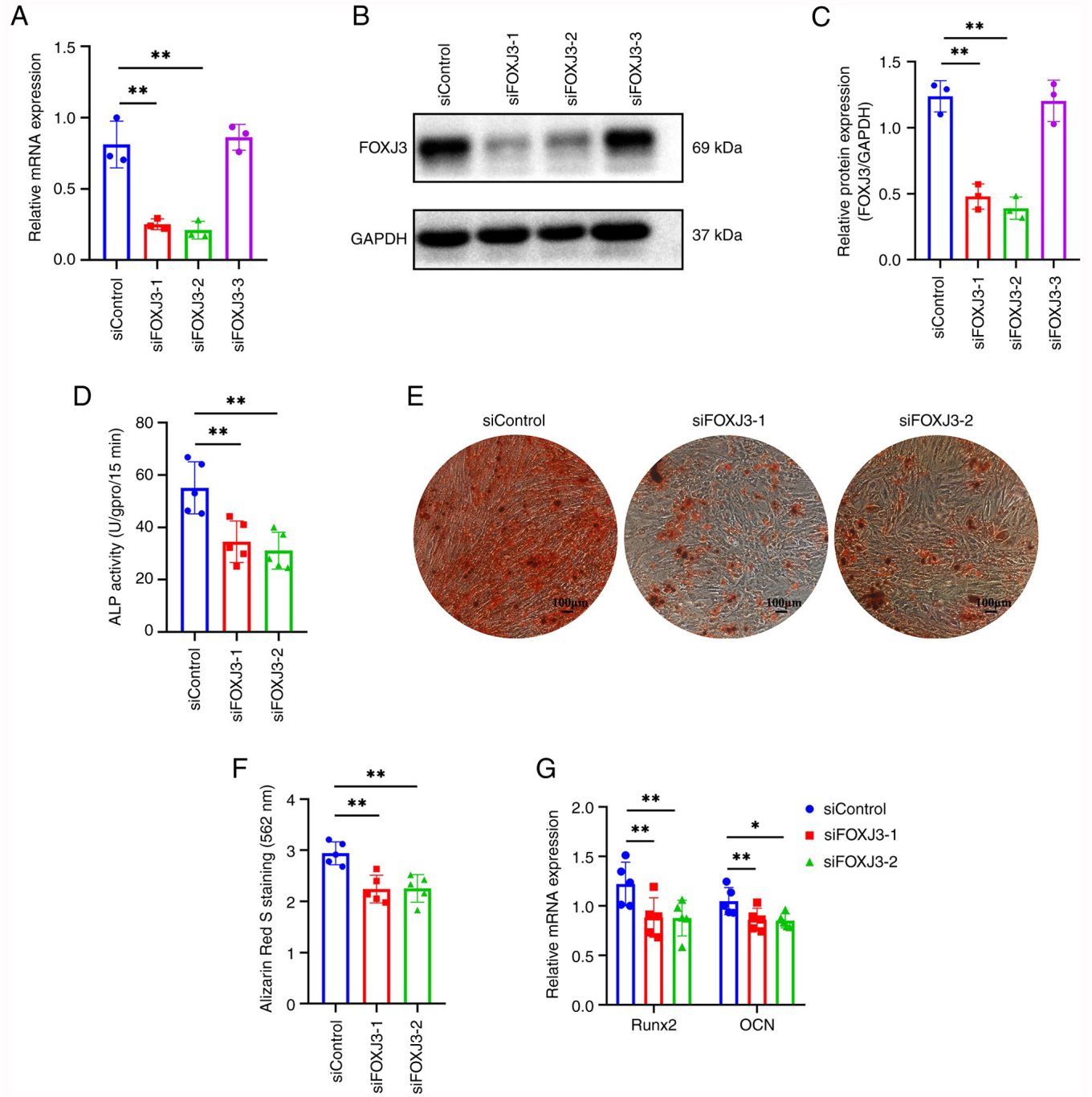

Figure 2. *In vitro* knockdown of FOXJ3 inhibits osteogenic differentiation of BMSCs. (A) Knockdown efficiency of siFOXJ3 was detected at the transcriptome level using RT-qPCR (n=3 biologically independent samples). (B) Protein knockdown efficiency of siFOXJ3 was evaluated through western blotting (n=3 biologically independent samples). (C) Semi-quantitative analysis of western blotting (n=3 biologically independent samples). (D) ALP activity detection revealed decreased ALP activity in BMSCs after FOXJ3 knockdown (n=5 biologically independent samples). (E) Alizarin Red S staining demonstrated reduced osteogenic nodule formation in BMSCs following FOXJ3 knockdown. (F) Quantitative analysis of Alizarin Red S staining indicated impaired osteogenic differentiation capacity in BMSCs with FOXJ3 knockdown. (G) RT-qPCR analysis showed osteogenic differentiation-associated genes RUNX2 and OCN were downregulated in BMSCs with FOXJ3 knockdown (n=5). *P<0.05, **P<0.01. ALP, alkaline phosphatase; BMSC, bone marrow mesenchymal stem cell; FOXJ3, forkhead box J3; OCN, osteocalcin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RUNX2, Runt-related transcription factor 2; si, small interfering.

comparable with the control (Fig. S1A and B). Quantitative ALP analysis revealed that ALP activity was reduced in the siFOXJ3 group compared with that in the siControl group, whereas the addition of the pathway agonist SB216763 significantly enhanced ALP activity (Fig. 5A). Furthermore, ARS staining and quantification demonstrated fewer osteogenic nodules in the siFOXJ3 group compared with that in the siControl group, whereas the addition of the pathway agonist SB216763 restored the osteogenic differentiation capacity of BMSCs (Fig. 5B and C).

Furthermore, after overexpressing FOXJ3, the findings were further validated using the Wnt/β-catenin pathway inhibitor XAV939. Western blotting demonstrated that active β-catenin expression was elevated in the OE-FOXJ3 group

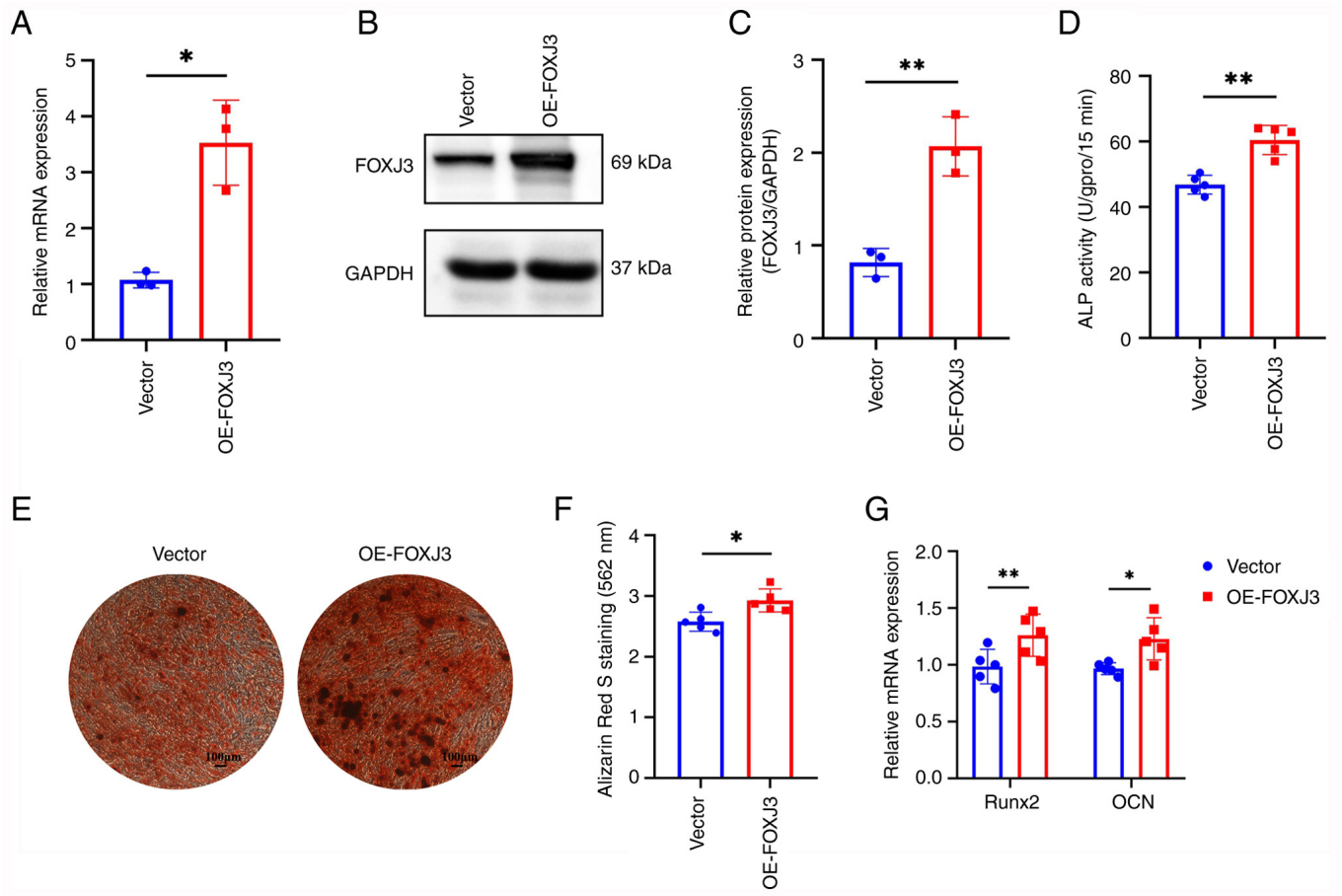

Figure 3. FOXJ3 promotes osteogenic differentiation of BMSCs *in vitro*. (A) OE efficiency of OE-FOXJ3 was detected at the transcriptome level by RT-qPCR. (B) Protein overexpression efficiency of OE-FOXJ3 was verified by western blotting. (C) Semi-quantitative analysis of western blotting (n=3). (D) ALP activity detection was performed in BMSCs after FOXJ3 OE (n=5). (E) Alizarin Red S staining was conducted to assess osteogenic nodules in BMSCs following FOXJ3 OE. (F) Quantitative analysis of Alizarin Red S staining demonstrated enhanced osteogenic differentiation capacity in BMSCs after FOXJ3 OE (n=5 biologically independent samples). (G) RT-qPCR assay detected upregulated expression of the osteogenic differentiation-related genes RUNX2 and OCN in BMSCs post-FOXJ3 OE (n=5 biologically independent samples). *P<0.05, **P<0.01. ALP, alkaline phosphatase; BMSC, bone marrow mesenchymal stem cell; FOXJ3, forkhead box J3; OCN, osteocalcin; OE, overexpression; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RUNX2, Runt-related transcription factor 2.

compared with that in the vector group; however, this enhancement was reversed following treatment with the pathway inhibitor XAV939, restoring active β-catenin expression to control levels (Fig. S1C and D). ALP activity was significantly enhanced in the OE-FOXJ3 group relative to the vector group, whereas this effect was attenuated upon pathway inhibitor treatment (Fig. 5D). ARS staining and quantification showed increased osteogenic nodule formation in the OE-FOXJ3 group compared with that in the vector group, whereas this pro-osteogenic effect was abolished in the OE-FOXJ3 + XAV939 group, with nodule formation returning to baseline control levels (Fig. 5E and F). Therefore, these results indicated that FOXJ3 could regulate the osteogenic differentiation of BMSCs in a Wnt/β-catenin pathway-dependent manner.

Discussion

The present study provided compelling evidence establishing the transcription factor FOXJ3 as a novel and important positive regulator of osteogenic differentiation in BMSCs and identified its crucial dependence on the Wnt/β-catenin signaling pathway. The findings suggested the potential role of FOXJ3 in in the development of osteoporosis, offering a promising novel target for therapeutic intervention.

The pivotal role of BMSCs in maintaining bone homeostasis and their dysfunction in osteoporosis is well-established (25). As multipotent progenitors residing in the bone marrow, BMSCs possess the capacity to differentiate into osteoblasts, the bone-forming cells essential for skeletal integrity and repair (26,27). In osteoporosis, an age-related imbalance occurs where the commitment of BMSCs shifts from osteogenesis towards adipogenesis, coupled with a general decline in their osteogenic potential and proliferative capacity (28). Previous studies have indicated that numerous transcription factors (including RUNX2 and Osterix/SP7) are master regulators of osteogenesis (29,30). FOXJ3, a member of the FOX family of transcription factors, which are characterized by a conserved winged-helix DNA-binding domain, represents a hitherto unrecognized regulator of cell differentiation (13). Although FOXJ3 has been implicated in other biological processes, such as spermatogenesis and cellular stress responses (12), its specific functions in bone metabolism and BMSC biology have remain unexplored. Notably, some other FOX members, such as FOXO1, have been implicated in the oxidative stress

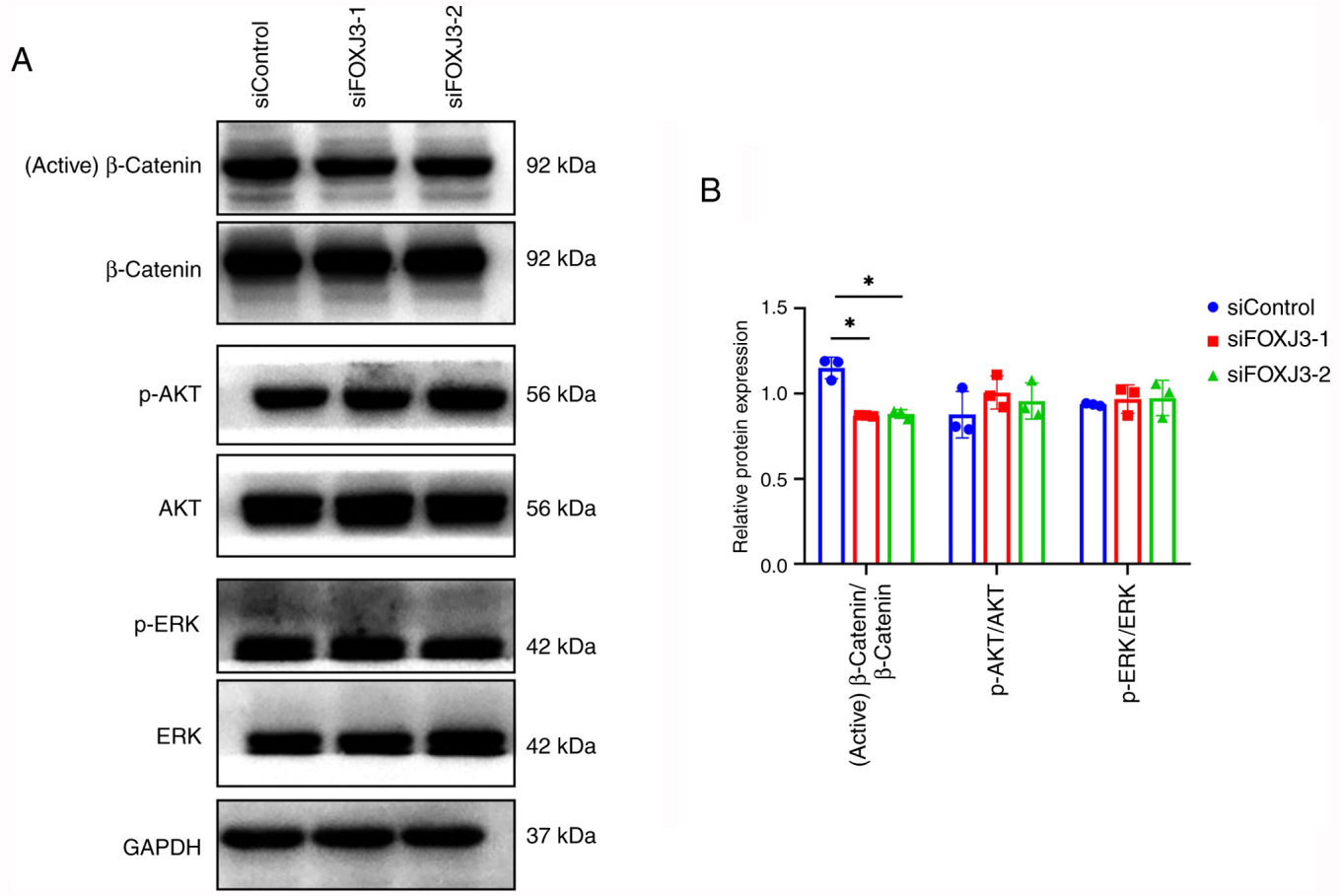

Figure 4. FOXJ3 regulates the Wnt/β-catenin pathway. (A) Changes in the expression levels of proteins in the pathways associated with osteogenic differentiation after FOXJ3 knockdown were detected using western blotting. (B) Semi-quantitative analysis of western blotting demonstrated that FOXJ3 knockdown inhibited the Wnt/β-catenin pathway (n=3). *P<0.05. FOXJ3, forkhead box J3; p-, phosphorylated; si, small interfering.

response in bone, and FOXC2 has been shown to be involved in BMP2 signaling (31), thus indicating that FOXJ3 may also be involved in osteogenesis.

The present study first revealed that FOXJ3 was upregulated during *in vitro* osteogenic differentiation, and further results indicated that a positive association existed between FOXJ3 and osteogenic differentiation of BMSCs. Furthermore, the siRNA-mediated knockdown of FOXJ3 resulted in a marked suppression of the osteogenic potential of BMSCs. Conversely, lentiviral overexpression of FOXJ3 robustly enhanced osteogenesis. These findings are important in identifying FOXJ3 as a novel modulator of BMSCs osteogenesis. While previous studies have explored factors such as microRNAs (32), long non-coding RNAs (33) and epigenetic regulators (34) in BMSC osteogenesis, the identification of the role of a transcription factor such as FOXJ3 may provide a novel mechanism and potential target. The present results demonstrated that manipulating FOXJ3 levels alone was sufficient to markedly alter the osteogenic differentiation trajectory of BMSCs, highlighting its potency as a regulator. FOXJ3, alongside other identified positive regulators of BMSCs osteogenesis, such as specific isoforms of Dlk1, may expand the known factors that potentially manipulate bone formation (35). Moreover, investigating FOXJ3 expression in well-characterized human osteoporosis cohorts, particularly its association with bone mineral density, fracture history or response to existing therapies, represents a critical next step to validate its clinical relevance. Such studies could further establish FOXJ3 as a potential diagnostic biomarker or therapeutic target in osteoporosis.

The canonical Wnt pathway is a well-established and powerful promoter of osteoblast differentiation and bone formation (36). Here, FOXJ3 knockdown specifically reduced the levels of active (non-phosphorylated) β-catenin, while leaving the PI3K/AKT and MAPK/ERK pathways unaffected. The selective impact of FOXJ3 on Wnt/β-catenin signaling suggests a focused regulatory mechanism. This finding is consistent with the results of previous studies emphasizing the critical role of precise Wnt pathway modulation in bone anabolism and its therapeutic exploitation (37,38). For example, romosozumab, an anti-sclerostin antibody that enhances Wnt signaling, has been shown to exert notable efficacy in treating patients with osteoporosis (39). The finding that FOXJ3 acts upstream of β-catenin activation adds a novel layer to this complex regulatory network. Previous studies have suggested that FOXJ3 can act as a recruited transcription factor to promote osteoclast formation (40,41). If both osteoclasts and osteoblasts exist *in vivo*, FOXJ3 may have regulatory effects on both types of cells. Whether it promotes or inhibits osteoporosis depends on whether its effect on bone formation is greater than that on bone resorption. This not only involves the quantity of

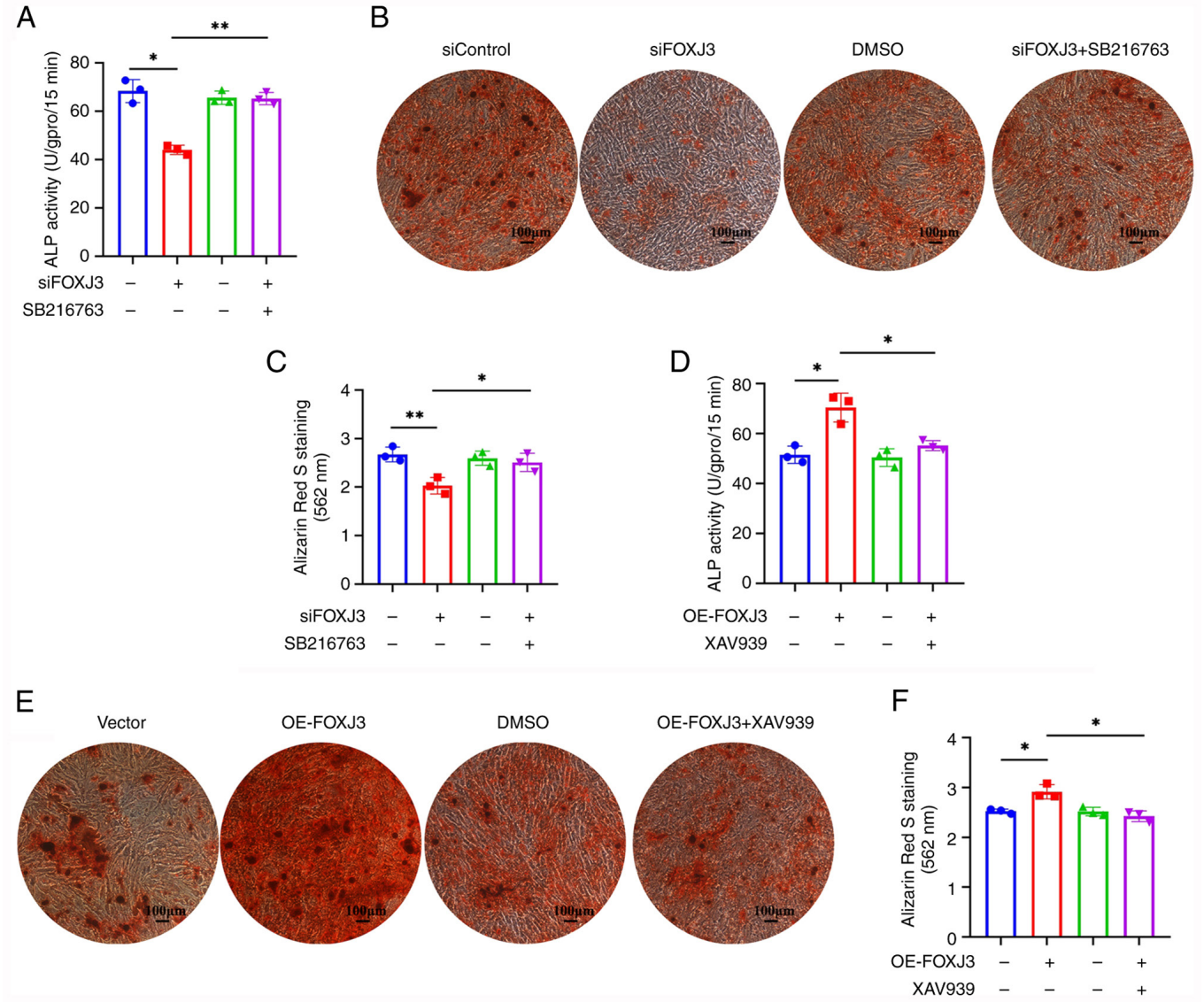

Figure 5. FOXJ3 regulates BMSC osteogenic differentiation through the Wnt/β-catenin pathway. (A) Detection of ALP activity in BMSCs after FOXJ3 knockdown and treatment with the Wnt/β-catenin pathway agonist SB216763 (n=3 biologically independent samples). (B) Alizarin Red S staining and (C) quantification of osteogenic nodules in BMSCs after FOXJ3 knockdown and treatment with the Wnt/β-catenin pathway agonist SB216763 (n=3 biologically independent samples). (D) Detection of ALP activity in BMSCs after FOXJ3 OE and treatment with the Wnt/β-catenin pathway inhibitor XAV939 (n=3 biologically independent samples). (E) Alizarin Red S staining and (F) quantification of osteogenic nodules in BMSCs after FOXJ3 OE and treatment with the Wnt/β-catenin pathway inhibitor XAV939 (n=3 biologically independent samples). *P<0.05, **P<0.01. ALP, alkaline phosphatase; BMSC, bone marrow mesenchymal stem cell; FOXJ3, forkhead box J3; OE, overexpression; si, small interfering.

osteoblasts and osteoclasts, but also the activity of the cells and their proportion of their roles in bone formation. The present study lacks animal experiments; therefore, whether FOXJ3 will aggravate osteoporosis remains unknown. To assess this, research using high-quality tools, such as gene knockout mice, is needed.

The rescue experiments in the present study demonstrated the pathway dependence and enhance the impact of the study. The use of the specific Wnt/β-catenin agonist SB216763 effectively reversed the inhibitory effects of FOXJ3 knockdown on β-catenin activation, ALP activity and mineralization. Conversely, the pro-osteogenic effects of FOXJ3 overexpression were negated by the Wnt pathway inhibitor XAV939. These experiments indicated that the ability of FOXJ3 to promote BMSC osteogenic differentiation requires a functional Wnt/β-catenin pathway, thus integrating FOXJ3 into a well-characterized and therapeutically relevant signaling axis. However, the exact molecular mechanism by which FOXJ3 regulates β-catenin activation remains to be fully determined, which is a promising direction for future research.

Placing the current findings within the broader context of osteoporosis research underscores their potential importance. Osteoporosis therapies have traditionally focused on anti-resorptive agents (such as bisphosphonates and denosumab) (42), however, while they are effective, these treatments primarily prevent bone loss rather than robustly rebuild bone. The development of true bone-forming (anabolic) agents, such as teriparatide [a parathyroid hormone (PTH) analogue], abaloparatide (a PTH-associated protein analogue) and the aforementioned romosozumab, represents a major advance (43). However, limitations

remain, including cost, administration routes and potential side effects (44). Identifying novel upstream regulators such as FOXJ3, which positively drives osteogenesis through a fundamental anabolic pathway (Wnt/β-catenin), provides novel options for therapeutic development. Strategies may involve small molecules or biologics designed to enhance FOXJ3 expression or activity directly within BMSCs or osteoprogenitors, or gene therapy approaches. This approach aligns with the growing interest in stem cell-based therapies and targeting stem cell dysfunction in age-associated diseases such as osteoporosis (45-47). Enhancing the intrinsic osteogenic potential of endogenous BMSCs via FOXJ3 modulation could offer a powerful strategy for bone regeneration. Moreover, future studies should include *in vivo* models, such as FOXJ3-knockout mice or local injection of FOXJ3-modulating vectors in osteoporotic animal models, to further validate its role.

In conclusion, the present study advances the understanding of the molecular control of BMSC osteogenic differentiation and the pathogenesis of osteoporosis. Robust mechanistic evidence was provided demonstrating that FOXJ3 exerts its pro-osteogenic effects primarily, if not exclusively, through the potent Wnt/β-catenin signaling pathway. This dependency was conclusively proven through targeted pathway rescue experiments. The integration of functional cellular assays and mechanistic pathway analysis provided a strong foundation for considering FOXJ3 as a promising new molecular target for the development of novel anabolic therapies aimed at restoring bone formation in osteoporosis and other bone-deficit conditions. While the present study provided strong evidence for the role of FOXJ3 *in vitro* and its clinical association, certain limitations warrant mention and guide future research. First, the findings were based on *in vitro* models, which may not fully recapitulate the complex bone microenvironment. Second, the precise molecular mechanism by which FOXJ3 regulates β-catenin remains unclear. Third, clinical patient-derived data, to assess the association between FOXJ3 expression and osteoporosis severity or treatment outcomes, were not included. Thus, future research focusing on *in vivo* validation and detailed mechanistic assessment will be crucial to fully realize the therapeutic potential of targeting the FOXJ3-Wnt/β-catenin axis, and to confirm the role of FOXJ3 in osteoporosis and its translational potential.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HX performed the cell experiments and wrote the initial manuscript and submitted the paper for publication. JL contributed to some cell experiments. WH conducted the statistical analysis of the data. YQ conceived the study, supervised the research and revised the manuscript. HX and JL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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