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USP2-induced upregulation of LEF1 through deubiquitination relieves osteoporosis development by promoting the osteogenic differentiation of bone marrow mesenchymal stem cells

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Abstract

Background Bone marrow mesenchymal stem cells (BMSCs) exhibit therapeutic potential for osteoporosis through their differentiation into osteoblasts. Here, we investigated the role and mechanism of lymphoid enhancer-binding factor 1 (LEF1) in regulating osteogenic differentiation of human BMSCs (hBMSCs).

Methods hBMSCs were exposed to the specific medium to induce their osteogenic differentiation. The ovariectomy (OVX)-induced osteoporotic mouse model was constructed. LEF1 and USP2 mRNA expression was analyzed by quantitative PCR, and protein levels were detected by immunohistochemistry and immunoblotting. Cell proliferation was assessed by CCK-8 assay. Alkaline phosphatase (ALP) expression and activity assay and Alizarin Red staining were used to evaluate osteogenic differentiation. LEF1 protein stability analysis and co-immunoprecipitation (Co-IP) assay were performed to test the USP2/LEF1 interaction.

Results During hBMSC osteogenic differentiation, LEF1 and USP2 levels were increased in hBMSCs. Inhibiting LEF1 or USP2 diminished the proliferation and osteogenic differentiation of hBMSCs in vitro. Mechanistically, USP2 stabilized LEF1 protein by mediating LEF1 deubiquitination. Increased expression of LEF1 reversed USP2 knockdown-imposed suppression on proliferation and osteogenic differentiation of hBMSCs. Moreover, increased USP2 expression reduced bone loss and enhanced osteogenic differentiation in OVX mice. Additionally, LEF1 and USP2 were downregulated in the bone marrow of patients with osteoporosis.

Conclusion Our findings provide the first demonstration of the USP2/LEF1 cascade that enhances the osteogenic differentiation of hBMSCs, broadening the field for the development of BMSCs as effective agents in osteoporosis therapy.

Keywords Osteoporosis, hBMSCs, Osteogenic differentiation, USP2, LEF1

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Introduction

Osteoporosis, characterized by reduced bone mass and microarchitectural deterioration of bone tissue, is a prevalent and growing clinical metabolic bone disorder, resulting in enhanced bone fragility and susceptibility to fractures. This silent killer is often asymptomatic in its early stages but progressively leads to significant morbidity and mortality, including spinal deformation, increased fracture risk, and reduced quality of life [1–3]. This clinical disease poses a critical health problem among the middle-aged and elderly populations, particularly in China, where the prevalence among individuals over 50 years old reaches 19.2%, with postmenopausal women being more severely affected [4, 5]. The pathogenesis of osteoporosis involves multiple interconnected factors, including genetic predisposition (e.g. GLP-1R polymorphisms) and epigenetic dysregulation (e.g. Nrf2 inactivation) [6, 7]. Current therapeutic strategies primarily focus on calcium and vitamin D supplementation, bone-enhancing medications, and bone repair treatments to alleviate symptoms [8–11]. Various biochemical markers of bone turnover have been used to manage therapy monitoring in osteoporotic patients [12, 13]. However, these treatments are limited by the need for long-term administration and potential side effects [14–16]. Of particular interest, bone marrow mesenchymal stem cells (BMSCs) have the potential to differentiate into osteoblasts, promoting bone formation and repair [17]. Studies have highlighted the efficacy of BMSCs in reducing trabecular bone loss and improving bone structure and fracture healing in osteoporotic models [18, 19]. The exploration of BMSCs as a therapeutic option holds promise in this debilitating disease but requires a clearer understanding of the mechanisms of their osteogenic differentiation.

Lymphoid enhancer-binding factor 1 (LEF1), a key transcription factor, functions as a critical modulator in diverse biological processes by affecting cellular proliferation, differentiation, senescence and survival [20]. LEF1 is a key component of the Wnt/β-catenin signaling pathway, modulating cell fate decisions and playing an essential role in the development of several tissues, including bone [21]. Research on LEF1 has unveiled its involvement in multiple diseases. For example, LEF1 serves as a novel marker for chronic lymphocytic leukemia/small lymphocytic lymphoma and has been implicated in abnormal expression patterns in leukemia, lymphoma, and lung adenocarcinoma [22–24]. Subsequent reports also demonstrate its association with allergic disease, osteoarthritis, and Hutchinson-Gilford progeria syndrome [25–27]. Furthermore, direct associations between LEF1 and osteogenesis or fracture healing are demonstrated during osteoporosis [28, 29]. Depletion of LEF1 also has relevance to suppressed osteogenic differentiation of BMSCs [30], while LEF1 upregulation can enhance BMSC proliferation and osteogenic differentiation [31, 32]. However, the mechanisms underlying LEF1's action on affecting osteogenic differentiation of human BMSCs (hBMSCs) are not yet fully understood.

Ubiquitination and deubiquitination are ubiquitous post-translational modifications that regulate protein stability, degradation, cellular signaling, and other biological processes [33]. USP2, a multifunctional deubiquitinating enzyme, possesses significant activity in reversing ubiquitination marks on target proteins, thereby influencing their stability and activity [34, 35]. Recent work has illumined the vital role of USP2 in bone formation and osteoporosis pathogenesis [36]. Moreover, USP2 is capable of regulating osteogenic differentiation of hBMSCs depending on the Wnt/β-catenin signaling [37].

In this report, we have utilized a specific medium to induce osteogenic differentiation of hBMSCs, unveiling the promoting role of LEF1 in hBMSC osteogenic differentiation. Moreover, we identify the deubiquitinase USP2 as an important regulator of LEF1 in the context of affecting hBMSC osteogenic differentiation. These findings reveal mechanisms driving hBMSC osteogenic differentiation and broaden the field for the development of BMSCs as effective agents in osteoporosis therapy.

Table 1 Clinical features of the patients with osteoporosis and normal

	Normal($n=23$)	osteoporosis ($n=29$)	*P* value
Age (years)	61.21 ± 3.03	62.58 ± 3.94	0.18
BMI (kg/m^2)	25.86 ± 3.24	24.63 ± 3.57	0.20
Weight (kg)	70.95 ± 10.96	66.16 ± 9.33	0.10
Height (cm)	161.62 ± 10.45	156.18 ± 9.71	0.06
DBP (mmHg)	74.59 ± 12.37	73.41 ± 12.04	0.73
SBP (mmHg)	137.32 ± 21.93	138.47 ± 22.02	0.85
PTH (pg/ml)	38.30 ± 7.35	34.27 ± 7.49	0.06
25(OH)D (ng/ml)	24.75 ± 4.82	22.82 ± 5.77	0.20
Phosphorus (mmlo/l)	1.38 ± 0.22	1.49 ± 0.25	0.09

Data are mean ± SD. SD: standard deviation; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PTH: parathyroid hormone; 25(OH)D: 25-hydroxyvitamin D

Materials and methods

Human bone marrow specimens

With approval from the Ethics Committee of Kailuan General Hospital, we collected 52 bone marrow samples from the anterior superior iliac spine from two subject groups: patients diagnosed with osteoporosis ($n=29$) and age- and sex-comparable healthy participants ($n=23$), both sourced from Kailuan General Hospital. Clinical features of these participants were shown in Table 1. Individuals were excluded from the study if they had a history of cancer, cardiovascular diseases, metabolic disorders, or any other bone-related disorders. The epidemiological information of the study subjects were shown in

Table S1. Informed consent was given by all subjects for the use of their bone marrow specimens in the current research.

Cell culture and osteoblastic differentiation induction

We employed hBMSCs (Cat No. IMP-H054, Immocell, Xiamen, China) in this study and cultivated them at 37 °C in a humidified atmosphere with 5% CO_2 (Thermo Fisher Scientific, Geel, Belgium) using standard grown medium provided by Immocell. The culture medium was replaced every three days, and hBMSCs at passages 2–4 were used for all in vitro experiments.

Osteoblastic differentiation was induced using a previously described method [38], hBMSCs were maintained in a specific medium consisting of 10% FBS DMEM with low glucose (Life Technologies, Cergy Pontoise, France), 10 mM β-glycerolphosphate (Sigma-Aldrich, Shanghai, China), 50 ng/mL L-ascorbic acid (Abcam), and 0.1 μM dexamethasone (Beyotime, Shanghai, China). Induction was sustained for a period of 7–14 days, with the induced medium being replaced every three days.

Plasmid constructs, lentivirus particles and cell transfection

For in vivo research, we obtained lentivirus particles expressing Mouse USP2 and vector lentivirus controls from Genomeditech (Shanghai, China). From Miaoling Biology (Wuhan, China), we procured pLV3-U6-USP2(human)-shRNA-Puro (sh-USP2), pLV3-U6-LEF1(human)-shRNA-Puro (sh-LEF1), nontarget shRNA (sh-NC), pML-EnCMV-LEF1(human)-1-3×FLAG-WPRE-SV40-Neo (oe-LEF1), and corresponding control vector for in vitro studies.

Transient transfection of hBMSCs with shRNA or shRNA + plasmid was conducted using RFect DNA Transfection Reagent as suggested by the supplier (Baidai, Changzhou, China). Then, 48 h post transfection, hBMSCs were induced for osteoblastic differentiation for 7 days in the induced medium or subjected to transfection efficiency detection.

Analysis of LEF1 and USP2 mRNA expression

Frozen bone marrow specimens or cultured hBMSCs (induction for 0, 1, 3, 7, and 14 days or after 48 h transfection) underwent RNA preparation using the Direc-zol RNA Miniprep Kit as described by the vendor (Zymo Research, Irvine, CA, USA). Using 200 ng of RNA, we synthesized cDNA utilizing the QuantiTect Reverse Transcription Kit and following the provided protocols (Qiagen, Crawley, UK). For the quantitative PCR analysis, we employed the Rotorgene 6000 (Qiagen) with QuantiTect SYBR Green (Qiagen) and primers designed for LEF1 (5'-CCCGTGAAGAGCAGGCTAAA-3'-sense and 5'-AGGCAGCTGTCATTCTTGGA-3'-antisense) or USP2 (5'-GCCGATTTCCCGGGGAGG-3'-sense and 5'-ATCTGTGTAGCGGGCCGATT-3'-antisense). Under the application of the $2^{-\Delta\Delta Ct}$ method, LEF1 and USP2 mRNA levels were determined, with GAPDH serving as a reference gene for normalization (5'-TTCTTTTGCGTC GCCAGCC-3'-sense and 5'-TCCCGTTCTCAGCCTTG AC-3'-antisense).

Generation of the osteoporotic mouse model and administration of lentivirus

The Animal Care and Use Ethics Committee of Kailuan General Hospital approved all animal procedures. To establish an osteoporotic mouse model induced by ovariectomy (OVX), we acquired 24 female C57BL/6J mice aged 10 weeks from Vital River Laboratory (Beijing, China) and divided them into four groups: sham ($n = 6$), OVX ($n = 6$), OVX + Vector ($n = 6$), and OVX + oe-USP2 ($n = 6$). The osteoporotic model was established using a standard OVX method. In brief, under anesthesia, mice in the OVX group underwent the OVX procedure for bilateral oophorectomy, while the sham group only had the adipose tissue around the ovaries excised. Four weeks post-surgery, the lateral knee was dislocated, exposing the intermalleolar groove. Then, 15 μL of oe-USP2 lentivirus (5×10^7 TU) or vector virus was injected into the bone marrow cavity through fossa trochanteric femur. Throughout the experiment, all mice were maintained on a regular diet. At the conclusion of the experiment (8 weeks later), all mice were euthanized, and their femurs were collected for further analysis.

Histological analysis and immunohistochemistry

Harvested mouse femurs underwent fixation in 4% paraformaldehyde and the subsequent paraffin-embedding, following by the preparation of femur Sect. (4 μm). For histological analysis by H&E staining, we utilized an H&E staining Kit as suggested by the producer (Beyotime). For RUNX2 probing, we conducted an immunohistochemical assay as reported [39] with rabbit monoclonal to RUNX2 (Cat No. PA5-86506, 1 to 100, Invitrogen, Saint-Aubin, France). After capturing images with the Axiovert 200 M microscope (Zeiss, Oberkochen, Germany), we analyzed histological changes and examined RUNX2 expression alterations.

Immunoblot analysis

hBMSCs were induced for osteoblastic differentiation for 0, 7, and 14 days, subjected to the relevant transfection for 48 h, or subjected to osteogenic induction for 7 days following 48 h of transfection. Collected bone marrow specimens, cultured hBMSCs, or harvested mouse femurs underwent protein extraction under the use of the Protein Extraction Kit and accompanying instructions (Abcam). As described elsewhere [39],

we performed western blot using rabbit monoclonal to LEF1 (Cat No. ab137872, 1 to 1,000, Abcam, Cambridge, UK), rabbit polyclonal to USP2 (Cat No. 10392-1-AP, 1 to 1,500, Proteintech, Wuhan, China), rabbit monoclonal to RUNX2 (Cat No. PA5-86506, 1 to 800, Invitrogen), rabbit polyclonal to osteopontin (OPN, Cat No. 22952-1-AP, 1 to 3,000, Proteintech), rabbit polyclonal to ubiquitin (Ub, Cat No. 10201-2-AP, 1 to 5,000, Proteintech), or rabbit polyclonal to GAPDH (Cat No. ab9485, 1 to 2,500, Abcam). After the ECL procedure (Merck Millipore, Darmstadt, Germany) for signal development, we obtained images by iBright 1500 System (Invitrogen).

Cell proliferation assay

hBMSCs were either induced for osteoblastic differentiation over a 14-day time course (0, 1, 3, 7, 14 days), or subjected to osteogenic induction at shorter intervals (0, 24, 48, 72 h) following 48 h of transfection. At the indicated time points, we evaluated cell growth by measuring the number of viable cells by CCK-8 assay (MedChemExpress, Monmouth Junction, NJ, USA) following the suggestions provided by the supplier. Utilizing the Viktor X3 reader (Perkin Elmer, Turku, Finland), the absorbance at 450 nm was gauged, which was proportional to the viable cell count.

Alkaline phosphatase (ALP) staining and activity assay

Following a 7- or 14-day induction period, the hBMSCs underwent ALP staining using an ALP Color Development Kit as recommended by the vendor (Beyotime). Prior to staining, the hBMSCs were subjected to fixation with 4% paraformaldehyde. Microscopic images were subsequently captured using the Axiovert 200 M microscope. Meantime, we determined ALP activity under the use of an ALP Activity Assay Kit as per the supplier's instructions (Beyotime). The Viktor X3 reader was used for the measurement of the absorbance at 405 nm.

Alizarin red staining

As reported previously [40], we evaluated calcium deposition during osteoblastic differentiation by Alizarin Red staining following a 7- or 14-day induction period. In brief, after fixation with 4% paraformaldehyde, the hBMSCs were subjected to staining with Alizarin Red (2%) solution as described by the supplier (Solarbio, Beijing, China). We used the Axiovert 200 M to obtain microscopic images. Additionally, for quantitative assessment, cetylpyridinium chloride from Solarbio was employed for the measurement of the absorbance at 570 nm.

Prediction of deubiquitinating enzymes related to LEF1

The related deubiquitinases that have the potential to modulate LEF1 deubiquitination were predicted by the computational algorithm Ubibrowser2.0 at http://ubibrowser.bio-it.cn/ubibrowser_v3/.

Assessment of LEF1 protein stability

To analyze the impact of USP2 on LEF1 protein stability, sh-NC- or sh-USP2-transfected hBMSCs were exposed to 20 mg/mL cycloheximide (CHX, Selleck, Shanghai, China), which was used to suppress protein synthesis. At 0, 4, 8, and 12 h post-treatment, we extracted total protein and analyzed them using immunoblot analysis.

Co-immunoprecipitation (Co-IP) assay

To analyze the relationship between USP2 and LEF1, we performed Co-IP experiments. Under the application of the commercially available Co-IP Kit as described by the vendor (Beyotime), sh-NC-introduced, sh-USP2-transfected, or untransfected hBMSCs were subjected to IP assays with rabbit polyclonal to USP2 (Cat No. 10392-1-AP, Proteintech), rabbit monoclonal to LEF1 (Cat No. ab137872, Abcam), or Isotype IgG antibody (Cat No. ab172730, Abcam). Briefly, the Protein A + G magnetic beads pre-treated with the relevant antibody were added into cellular lysates. Upon addition, incubation was done for 6 h at 4 °C. We then harvested the beads for protein isolation to detect the enrichment abundance of USP2, LEF1, or ubiquitinated LEF1.

Statistical analysis

Unless specified otherwise, all results presented in Figures were shown as mean ± SD. Values of statistical $P < 0.05$, by a one-way ANOVA with Tukey's *post hoc* test or a two-tailed Student's *t*-test, were defined as statistically significant.

Results

LEF1 expression is increased during hBMSC osteogenic differentiation

We first examined LEF1 expression levels in bone marrow samples from patients with osteoporosis and healthy participants. Quantitative PCR and immunoblot analyses revealed that LEF1 expression was markedly reduced at both mRNA and protein levels in bone marrow of osteoporosis patients compared to healthy controls (Fig. 1A and B). Then, hBMSCs were exposed to the specific medium to induce their osteogenic differentiation for 14 days. Notably, during osteogenic differentiation of hBMSCs, LEF1 mRNA levels increased progressively (Fig. 1C). Similarly, the expression of LEF1 protein in hBMSCs was higher on day 7 of induction compared with that on day 0 and further increased on day 14 compared to the seventh day (Fig. 1D). Furthermore, osteogenesis-associated proteins RUNX2 and OPN were upregulated in induced hBMSCs on day 7 compared to those on day 0 and further elevated on day 14 (Fig. 1E), confirming the successful

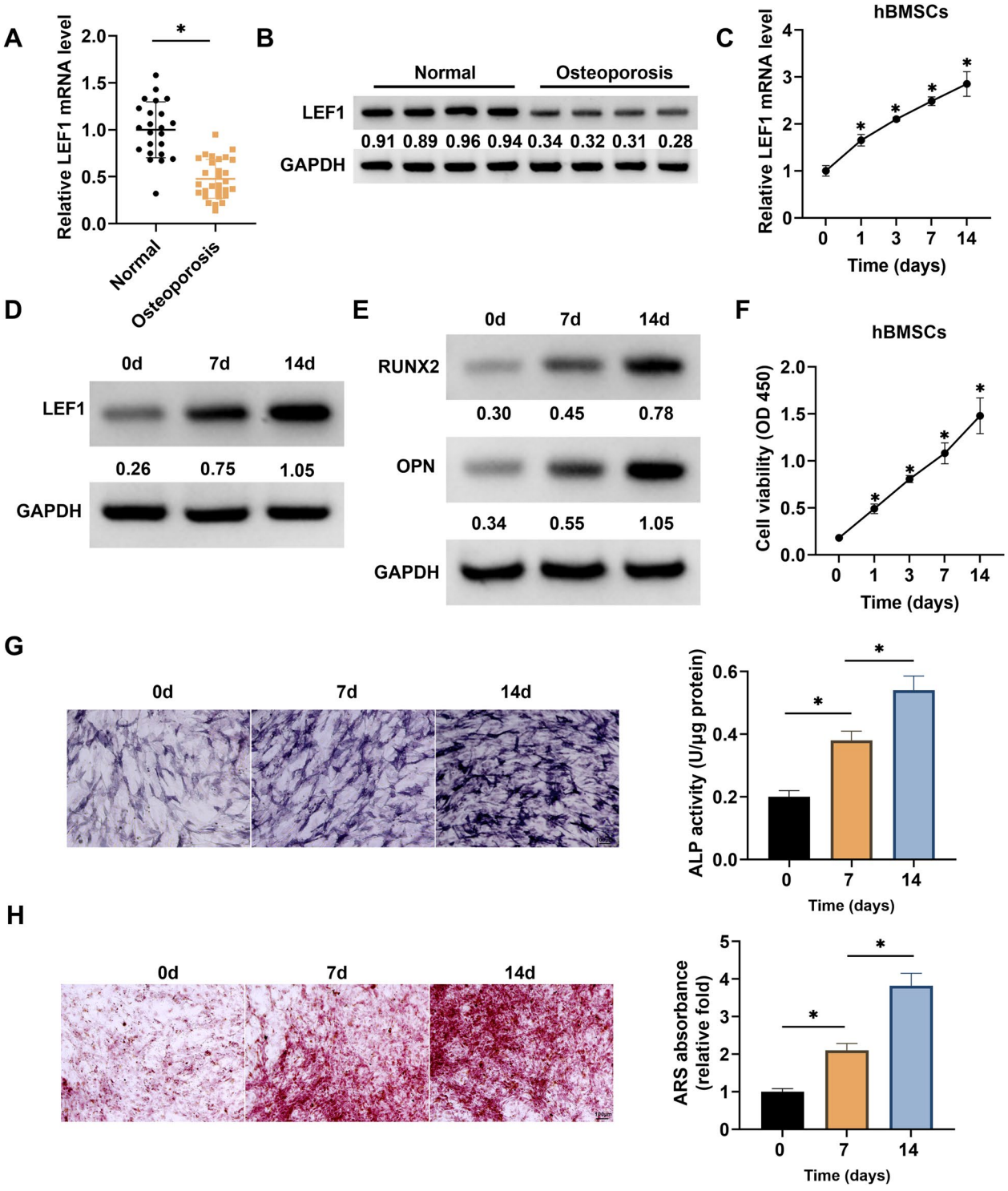

Fig. 1 LEF1 is downregulated in bone marrow of osteoporosis patients and increased in hBMSCs during osteogenic differentiation. (**A**) Quantitative PCR of LEF1 mRNA expression in bone marrow samples from patients with osteoporosis ($n=29$) and healthy participants ($n=23$). (**B**) Immunoblotting of LEF1 protein abundance in bone marrow samples from patients with osteoporosis ($n=4$) and healthy participants ($n=4$). (C-H) hBMSCs were exposed to the specific medium to induce osteogenic differentiation for 14 days. (**C**) LEF1 mRNA expression in induced hBMSCs in the process of osteogenic differentiation ($n=3$). (**D**) LEF1 protein abundance by immunoblotting in hBMSCs on days 0, 7 and 14 after induction. (**E**) The expression of RUNX2 and OPN proteins in hBMSCs on days 0, 7 and 14 after induction. (**F**) Proliferation analysis by CCK-8 assay with hBMSCs in the process of osteogenic differentiation ($n=3$). (**G**) Microscopic images revealing ALP expression in hBMSCs on days 0, 7 and 14 after induction and quantification of ALP activity using ALP activity assay ($n=3$). Scale bar: 100 μm. (**H**) Representative Alizarin Red Staining (ARS) showing calcium deposits in hBMSCs on days 0, 7 and 14 after induction ($n=3$). Scale bar: 100 μm. *$P<0.05$

osteogenic differentiation of hBMSCs under the induction conditions. In addition, CCK-8 assay revealed cell proliferation during the process of osteogenic differentiation (Fig. 1F). ALP staining and activity assays showed that the expression and activity of ALP increased by day 7 and further elevated by day 14 (Fig. 1G). Consistent with the ALP activity, the data of Alizarin Red Staining (ARS) showed the increase of calcium deposits in hBMSCs during the process of osteogenic differentiation (Fig. 1H), reinforcing the successful osteogenic differentiation of hBMSCs.

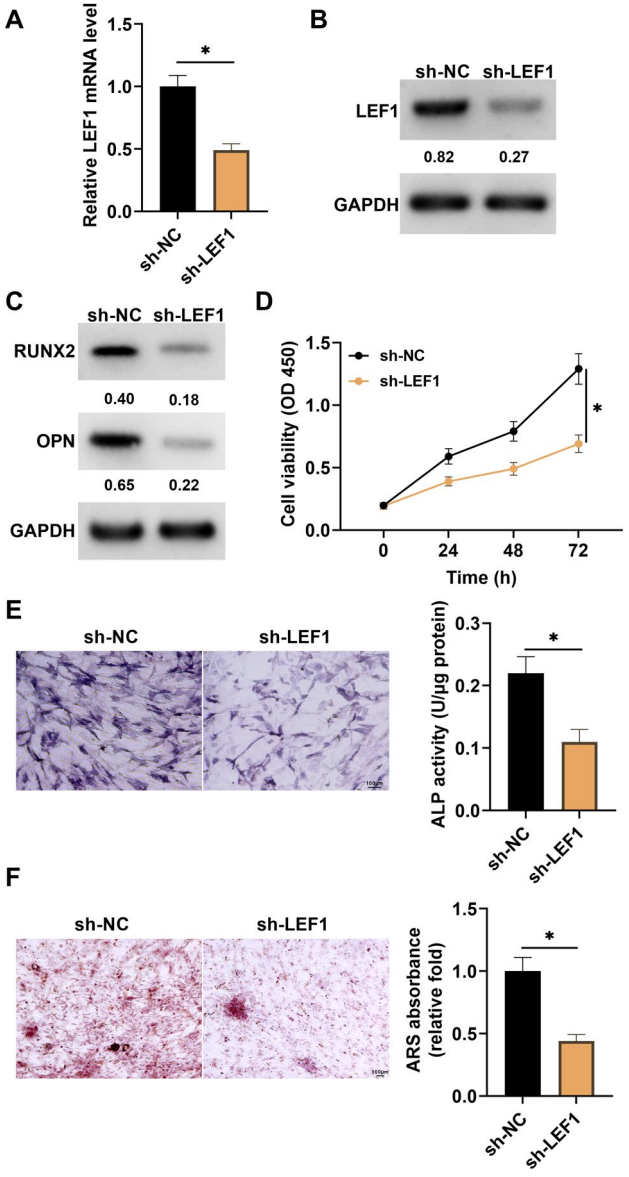

Fig. 2 LEF1 depletion reduces the proliferation and osteogenic differentiation of hBMSCs. (**A**) Quantitative PCR showing the knockdown of LEF1 mRNA in sh-LEF1-transfected hBMSCs ($n=3$). (**B**) Immunoblot analysis revealing the depletion of LEF1 protein in sh-LEF1-transfected hBMSCs. (**C-F**) hBMSCs were subjected to sh-NC transfection or sh-LEF1 introduction and then exposed to the specific medium to induce osteogenic differentiation for 7 days. (**C**) Immunoblot analysis of RUNX2 and OPN protein levels in LEF1-depleted hBMSCs under the induction conditions. (**D**) CCK-8 assay showing the reduced proliferation of LEF1-depleted hBMSCs ($n=3$). (**E**) Microscopic images depicting ALP expression reduction in LEF1-depleted hBMSCs and quantification of ALP activity by ALP activity assay ($n=3$). Scale bar: 100 μm. (**F**) Microscopic pictures revealing the reduced calcium deposition in LEF1-depleted hBMSCs as presented by Alizarin Red Staining (ARS) and absorbance measurement ($n=3$). Scale bar: 100 μm. *$P<0.05$

LEF1 Inhibition diminishes the proliferation and osteogenic differentiation of hBMSCs

We then investigated its functional role using loss-of-function studies with a "phenocopy" approach to deplete LEF1 expression in hBMSCs prior to differentiated induction. Knockdown of LEF1 expression in hBMSCs using the shRNA specific for LEF1 (sh-LEF1) was confirmed at the mRNA and protein levels (Fig. 2A and B). Depletion of LEF1 expression in induced hBMSCs resulted in reduced RUNX2 and OPN levels (Fig. 2C). We also observed that the proliferation under the induction conditions was reduced in LEF1-silenced hBMSCs compared to the sh-NC cells (Fig. 2D). Moreover, LEF1 silencing resulted in decreased ALP expression, ALP activity, and calcium deposition in hBMSCs under the induction conditions (Fig. 2E and F).

USP2 is upregulated in hBMSCs during osteogenic differentiation and affects the differentiation

Considering deubiquitinating enzymes (DUBs) are known to critically regulate Wnt/β-catenin signaling through post-translational modifications [41, 42], we postulated that identifying the specific DUB mediating LEF1 deubiquitination would elucidate a key mechanism controlling its protein abundance in osteogenic differentiation. Using the computational algorithm Ubibrowse, we found several deubiquitinases that have the potential to modulate LEF1 deubiquitination (Fig. 3A). Among these, USP2 has attracted significant attention in the research of the osteogenic differentiation and proliferation of hBMSCs [36, 37]. We thus focused on USP2 for further investigation. In contrast to the normal controls, USP2 mRNA and protein levels were strongly downregulated in bone marrow samples of osteoporosis patients (Fig. 3B and C). Furthermore, during the process of hBMSC osteogenic differentiation, USP2 expression was gradually augmented in hBMSCs at both mRNA and protein levels (Fig. 3D and E).

To determine the exact action of USP2 on hBMSC osteogenic differentiation, we knocked down USP2 expression using a USP2-shRNA (sh-USP2), which effectively decreased USP2 abundance at both mRNA and protein levels (Fig. 3F and G). Inhibition of USP2 reduced the levels of RUXN2 and OPN proteins in hBMSCs (Fig. 3H) and hindered their proliferation (Fig. 3I) under the induction conditions. In addition, USP2 deficiency

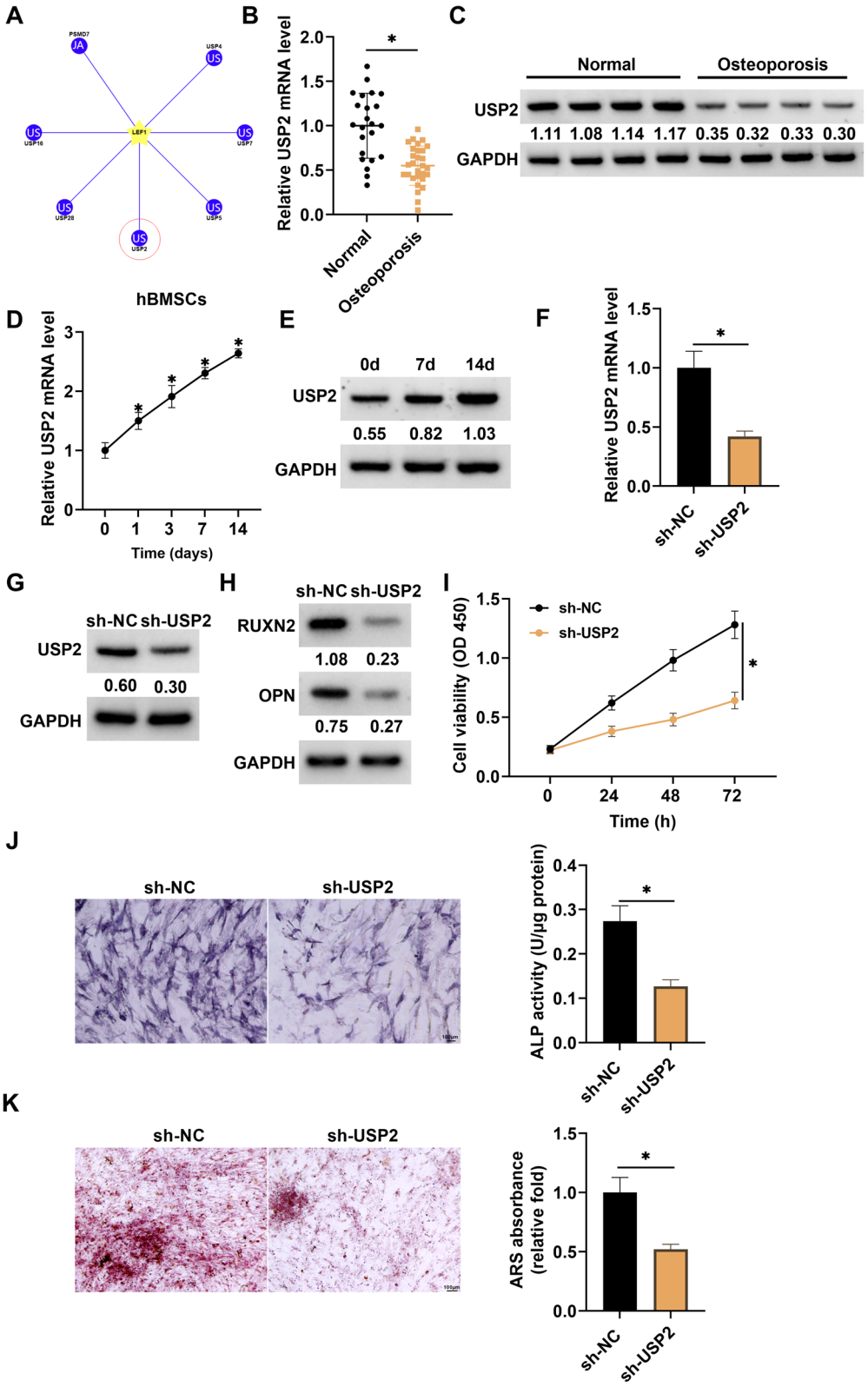

Fig. 3 (See legend on next page.)

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Fig. 3 USP2 is upregulated in hBMSCs during osteogenic differentiation, and its depletion diminishes osteogenic differentiation of hBMSCs. (**A**) Ubibrowse algorithm showed several deubiquitinases with the potential to modulate LEF1 deubiquitination. (**B**) Quantitative PCR of USP2 mRNA expression in bone marrow samples from patients with osteoporosis ($n=29$) and healthy participants ($n=23$). (**C**) Immunoblotting of USP2 protein abundance in bone marrow samples from patients with osteoporosis ($n=4$) and healthy participants ($n=4$). (**D** and **E**) USP2 mRNA (**D**) and protein (**E**) expression in hBMSCs exposed to the specific medium to induce osteogenic differentiation for 14 days. (**F**) Quantitative PCR showing the knockdown of USP2 mRNA in sh-USP2-transfected hBMSCs ($n=3$). (**G**) Immunoblot analysis revealing the deficiency of USP2 protein in sh-USP2-transfected hBMSCs. (**H-K**) hBMSCs were subjected to sh-NC transfection or sh-USP2 introduction and then exposed to the specific medium to induce osteogenic differentiation for 7 days. (**H**) Immunoblot analysis of RUNX2 and OPN protein levels in sh-USP2-transfected hBMSCs under the induction conditions. (**I**) CCK-8 assay showing the reduced proliferation of sh-USP2-transfected hBMSCs ($n=3$). (**J**) Microscopic images depicting ALP expression reduction in sh-USP2-transfected hBMSCs and quantification of ALP activity by ALP activity assay ($n=3$). Scale bar: 100 μm. (**K**) Microscopic pictures revealing the reduced calcium deposition in sh-USP2-transfected hBMSCs as presented by Alizarin Red Staining (ARS) and absorbance measurement ($n=3$). Scale bar: 100 μm. $^*P<0.05$

resulted in diminished ALP expression, ALP activity and calcium deposition in hBMSCs under the induction conditions, as examined by ALP staining, ALP activity assay and ARS detection, respectively (Fig. 3J and K).

USP2 promotes LEF1 protein stability by deubiquitination

Next, we identified whether USP2 can modulate LEF1 in a deubiquitination way. Although USP2 depletion did not affect LEF1 mRNA expression, however, reduced LEF1 protein levels (Fig. 4A and B). Under CHX treatment, USP2 depletion by sh-USP2 could shorten the half-life of LEF1 protein, indicating that USP2 depletion weaken the stabilization of LEF1 protein in hBMSCs (Fig. 4C). Through Co-IP assays using an anti-USP2 or anti-LEF1 antibody, we demonstrated the interaction between USP2 and LEF1 in hBMSCs, as indicated by LEF1 enrichment in USP2-associating precipitates and USP2 enrichment in LEF1-related precipitates (Fig. 4D). Importantly, through Co-IP experiments using the anti-LEF1 antibody and the subsequent immunoblotting with an anti-Ub antibody, we observed that the knockdown of USP2 led to a distinct augmentation in ubiquitinated LEF1 level and LEF1 degradation (Fig. 4E).

Increased expression of LEF1 reverses USP2 knockdown-imposed suppression on proliferation and osteogenic differentiation of hBMSCs

We further elucidated if the influence of USP2 on hBMSC osteogenic differentiation is LEF1-dependent. To address this question, we elevated LEF1 expression using a LEF1 ORF construct (oe-LEF1) in USP2-silenced hBMSCs because USP2 depletion led to a reduction in LEF1 expression (Fig. 5A). Restored LEF1 expression exerted a counteracting impact on USP2 silencing-mediated downregulation in RUNX2 and OPN proteins (Fig. 5B), proliferation defect (Fig. 5C), as well as reduction in ALP expression, ALP activity and calcium deposition (Fig. 5D and E) in hBMSCs under the induction conditions.

Upregulation of USP2 reduces bone loss in OVX mice

Finally, we explored its function in affecting osteoporosis development in vivo by generating an OVX-induced mouse model of osteoporosis with or without administration of lentivirus expressing USP2 or nontarget control. H&E staining showed that trabecular bone was markedly less in the femurs of OVX mice compared with that in sham mice (Fig. 6A). Moreover, administration of oe-USP2 lentivirus strongly diminished trabecular bone loss in OVX mice (Fig. 6A). Through immunohistochemistry with sections of the femurs, we found that OVX mice had reduced expression of RUNX2 protein compared to sham controls, while oe-USP2 lentivirus treatment remarkably abated the reduction (Fig. 6B). Furthermore, immunoblot results showed that the expression levels of USP2 and LEF1 were decreased in OVX mice (Fig. 6C). Additionally, oe-USP2 lentivirus-mediated USP2 upregulation led to a clear elevation in the expression of LEF1 protein in the femurs of OVX mice (Fig. 6C).

Discussion

Osteoporosis is a prevalent skeletal disorder that significantly impairs patients' quality of life worldwide [2]. BMSCs have been uncovered to have the potential to diminish trabecular bone loss and ameliorate bone structure and fracture healing in osteoporosis by differentiating into osteogenesis [43]. Enhancing osteogenic differentiation of BMSCs is beneficial to their efficacy in osteoporosis therapy. Here, we have confirmed that LEF1 can promote the osteogenic differentiation and proliferation of hBMSCs. Notably, the deubiquitinase USP2 is demonstrated to stabilize LEF1 protein via deubiquitination, highlighting a novel mechanism underlying hBMSC osteogenic differentiation. Our findings elucidate the interplay between LEF1 and USP2, providing insights that may facilitate the development of BMSCs as therapeutic agents for osteoporosis.

LEF1, a member of the TCF/LEF family, has been extensively studied in various diseases, particularly in malignancy, where it plays a crucial role in Wnt signaling-mediated tumorigenesis [44]. In recent years, LEF1 has garnered attention due to its involvement in the regulation of bone homeostasis and remodeling [45, 46]. Previous studies have elucidated the close association between LEF1 expression and osteogenic differentiation of BMSCs, bone marrow stromal cells and human

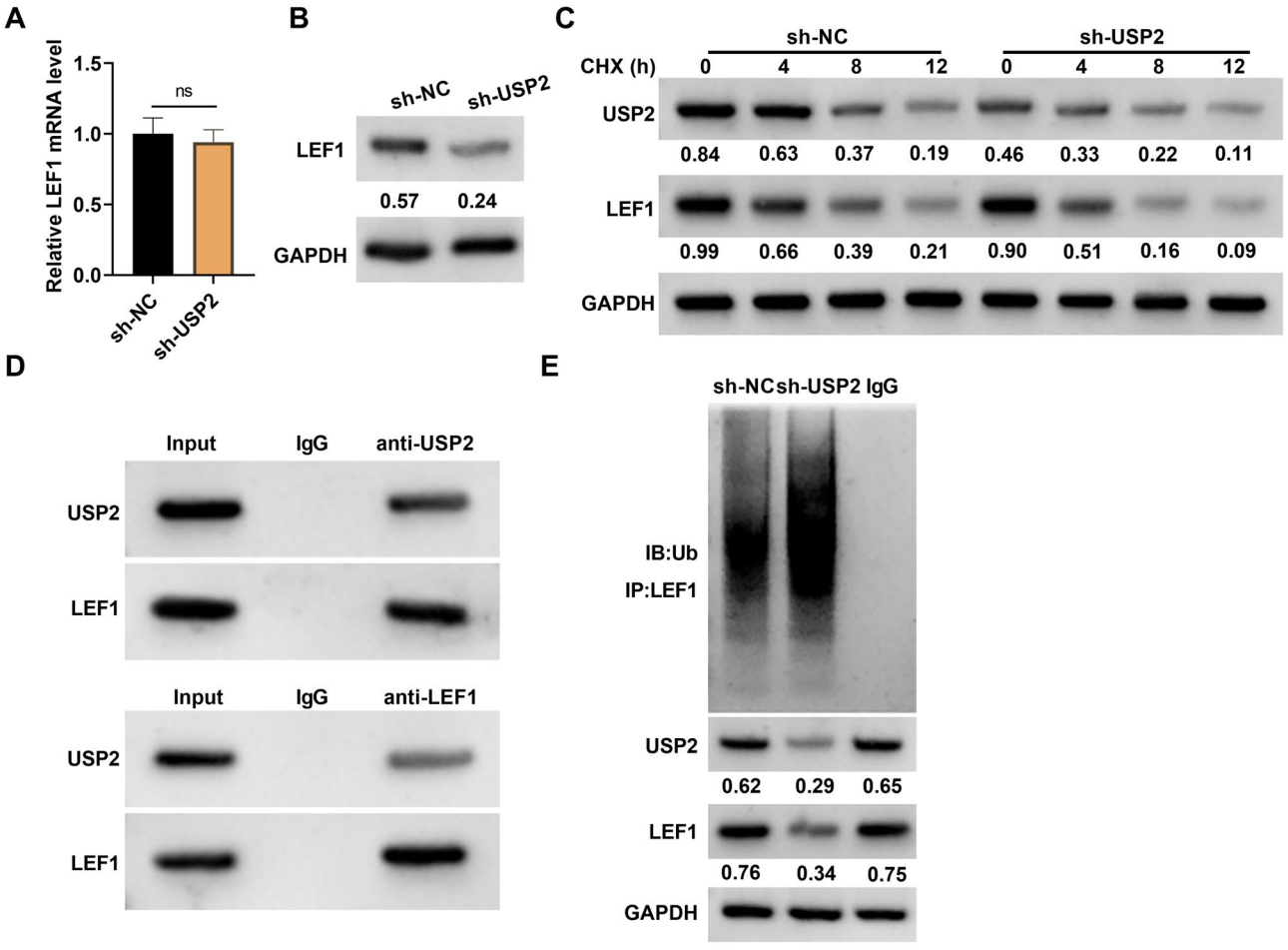

Fig. 4 USP2 deubiquitinates and stabilizes LEF1 protein. (**A**) LEF1 mRNA expression in sh-NC- or sh-USP2-transfected hBMSCs. (**B**) Immunoblot analysis validated the reduced expression of LEF1 protein in USP2-depleted hBMSCs. (**C**) Sh-NC- or sh-USP2-transfected hBMSCs were exposed to CHX for the indicated time frames and checked for USP2 and LEF1 protein levels by immunoblotting. (**D**) hBMSCs were homogenized and subjected to Co-IP assays using an anti-USP2 or anti-LEF1 antibody, followed by immunoblotting for LEF1 or USP2 enrichment abundance. (**E**) Sh-NC- or sh-USP2-transfected hBMSCs were lysed and subjected to Co-IP assays using an anti-LEF1 antibody, with the subsequent immunoblotting with an anti-Ub antibody

adipose-derived stem cells, where it controls the expression of genes crucial for bone formation and maintenance [31, 32, 47, 48]. Moreover, LEF1 actively participates in the processes of osteogenesis and fracture healing in osteoporosis [28, 29]. In our study, we have discovered that LEF1 expression exhibits a gradually increasing trend during the osteogenic differentiation of hBMSCs. Furthermore, our data show that inhibiting LEF1 results in a marked reduction in both the proliferation and osteogenic differentiation of hBMSCs. These findings suggest that LEF1 acts as a positive regulator of hBMSC osteogenic differentiation. Thus, LEF1 may contribute to the development of novel strategies for preventing and managing bone loss associated with osteoporosis.

USP2 is a key deubiquitinating enzyme that has emerged as a critical player in various diseases, including cancer, metabolic disorders, and neurodegenerative disorders, where it functions as a modulator of protein stability and signaling pathways [49–52]. A previous document proves that USP2 is required for osteoblast proliferation induced by PTH [36]. Furthermore, the USP2-activated Wnt/β-catenin pathway by mediating deubiquitination of β-catenin protein contributes to the osteogenic differentiation of hBMSCs [37]. We observed that USP2 is upregulated during hBMSC osteogenic differentiation. Notably, USP2 not only enhances the osteogenic differentiation of hBMSCs in vitro but also demonstrates an increased influence on osteogenesis-associated protein RUNX2 expression in OVX mice. Mechanistically, our findings indicate that USP2 promotes the stability of LEF1 through deubiquitination, thereby preventing its degradation. Furthermore, increased expression of LEF1 is able to reverse the suppressive impact on hBMSC proliferation and osteogenic differentiation caused by USP2 knockdown. These findings reveal a novel mechanism by which USP2 and LEF1 interact to regulate hBMSC osteogenic differentiation. Additionally, the observed suppression of adipocyte

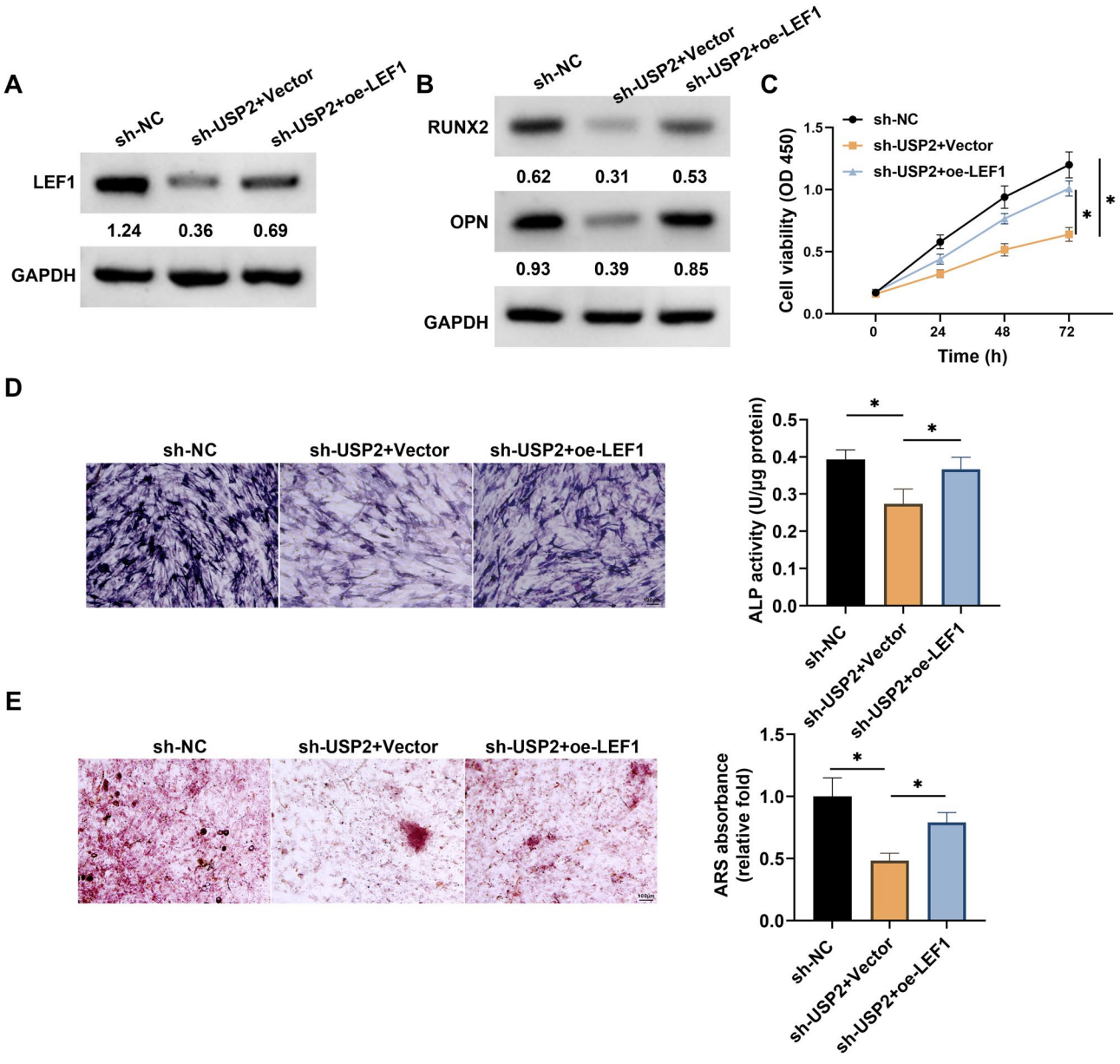

Fig. 5 USP2 affects hBMSC osteogenic differentiation partially through LEF1. (**A**) Immunoblot analysis of LEF1 protein in hBMSCs transfected with sh-NC, sh-USP2 + Vector, or sh-USP2 + oe-LEF1. (**B-E**) hBMSCs transfected with sh-NC, sh-USP2 + Vector, or sh-USP2 + oe-LEF1 were exposed to the specific medium to induce osteogenic differentiation for 7 days. (**B**) Immunoblot analysis of RUNX2 and OPN protein levels in hBMSCs transfected as indicated under the induction conditions. (**C**) CCK-8 assay showing the proliferation alterations of transfected hBMSCs ($n = 3$). (**D**) Microscopic images depicting ALP expression in transfected hBMSCs and quantification of ALP activity by ALP activity assay ($n = 3$). Scale bar: 100 μm. (**E**) Microscopic pictures revealing the calcium deposition in transfected hBMSCs as presented by Alizarin Red Staining (ARS) and absorbance measurement ($n = 3$). Scale bar: 100 μm. *$P < 0.05$

differentiation and increased RUNX2 expression upon USP2 overexpression in OVX mice align with our proposed USP2/LEF1 regulatory axis in BMSC osteoblastic differentiation. The inhibition of the Wnt/β-catenin pathway can promote adipogenesis by upregulating the adipogenic regulator PPARγ [53]. Thus, in OVX-induced bone loss, diminished Wnt signaling may shift BMSC differentiation toward adipogenesis. USP2 overexpression likely counteracts this imbalance by amplifying LEF1-mediated Wnt signaling, thereby redirecting BMSCs toward osteoblastic differentiation.

In our study, we have found that both USP2 and LEF1 are downregulated in the bone marrow of patients with osteoporosis, suggesting that disruptions in the expression of these proteins may contribute to impaired osteogenic activity and bone loss. However, due to the limited sample size in our analysis, future research will require a larger cohort to better evaluate the potential of USP2

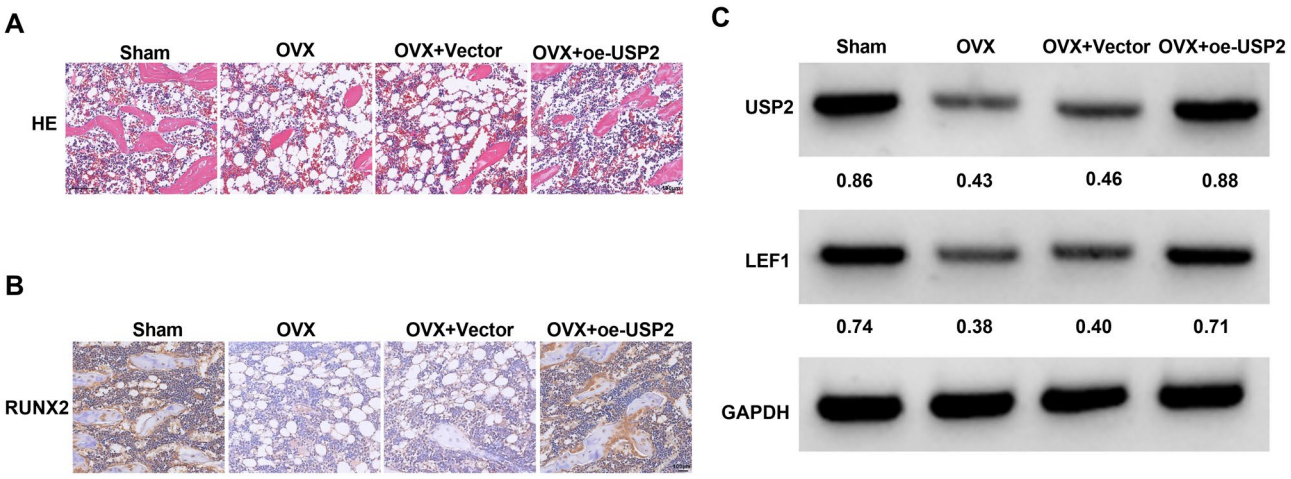

Fig. 6 USP2 and LEF1 are underexpressed in OVX mice, and increasing USP2 reduces bone loss and enhances osteogenic differentiation in vivo. (**A-C**) An OVX-induced mouse model of osteoporosis was generated on C57BL/6J mice, which was then administrated with lentivirus expressing USP2 or nontarget sequence. $n = 6$ for each group. (**A**) Histological evaluation of sections of the femurs of OVX mice or sham mice by H&E staining. (**B**) Immunohistochemistry analysis of sections of the femurs for RUNX2 probing. (**C**) Immunoblot assay of the femurs of OVX mice or sham mice for the assessment of USP2 and LEF1 protein levels

and LEF1 as diagnostic markers for osteoporosis. Additionally, while we have established the role of USP2 in regulating osteogenesis-associated protein RUNX2 expression in OVX mice, the validation of its influence on BMSC osteogenic differentiation in vivo remains insufficient. This gap highlights the need for comprehensive in vivo studies to better understand the functional significance of USP2 in bone health. Furthermore, the lack of in vivo evidence demonstrating USP2's regulatory effects through LEF1 is a critical limitation in our current study. Future endeavors should prioritize addressing these knowledge gaps to deepen our comprehension of the mechanisms underlying osteoporosis and to explore potential therapeutic interventions.

By injecting the virus into the bone marrow cavity via the femoral trochanteric fossa, our study specifically aimed to investigate the local effects of the virus on bone metabolism within the femoral region. The lentiviral vector, carrying the oe-USP2 construct, was injected locally to assess its direct impact on the local bone microenvironment. The choice of this route of administration was deliberate, as it allowed for a focused investigation of the virus's effects on bone metabolism within the injected site, minimizing systemic interference. While the OVX model induces generalized bone loss, our study design was not intended to evaluate systemic changes but rather to elucidate the localized mechanisms by which the virus may affect bone. We hypothesize that the lentiviral-mediated gene transfer may locally modulate osteogenic or osteoclastogenic pathways, potentially influencing bone formation and resorption specifically within the injected femoral region. However, further studies would be required to comprehensively assess any potential systemic effects of this localized treatment approach. In addition, we performed immunohistochemistry to analyze the expression and distribution of RUNX2 and immunoblotting to quantify the overall protein expression levels of USP2 and LEF1 in the animal models. Although immunohistochemistry can detect and localize specific antigens within tissue sections, immunoblotting is capable of offering a more sensitive and quantitative assessment of protein abundance, crucial for understanding the underlying mechanisms of osteoporosis. By studying USP2 and LEF1 expression alterations, we observed their roles in osteoporosis pathogenesis.

Collectively, our study provides the first demonstration of the USP2/LEF1 cascade that enhances the osteogenic differentiation of hBMSCs, as illustrated in Fig. 7. Our findings could pave the way for innovative therapeutic strategies targeting this axis and broaden the field for the development of BMSCs as effective agents in osteoporosis therapy.

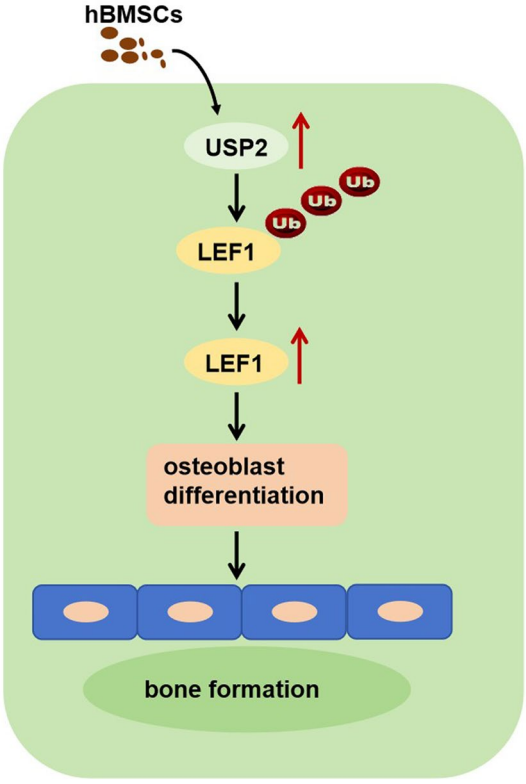

Fig. 7 Schematic of the USP2/LEF1 axis in regulating osteogenic differentiation of hBMSCs. During osteogenic differentiation, USP2 is upregulated in hBMSCs and leads to an increase in USP2 protein level through deubiquitination, thereby promoting BMSC osteogenic differentiation

Supplementary Information

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Supplementary Material 1

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None.

Author contributions
Zhihong Zhang designed and performed the research; Hanwen Xing, Jing Liu, Linshuo Li, Yue Zhang analyzed the data; Zhihong Zhang and Jie Cao wrote the manuscript. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Written informed consents were obtained from all participants and this study was permitted by the Ethics Committee of Kailuan General Hospital.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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