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LETTER TO EDITOR

B-cell lymphoma-3 controls mesenchymal stem cell commitment and senescence during skeletal aging

Dear Editor,

Skeletal aging is characterized by progressive bone loss and increased marrow adiposity.[1,2] Age-related bone loss impairs the exercise activity of patients and increases the risk of fracture. Bone marrow mesenchymal stem cells (BMSCs) could differentiate into osteoblasts and adipocytes.[3,4] The senescence and differentiation shift of BMSCs plays a critical role in skeletal aging and osteoporosis. In our study, we demonstrated that B-cell lymphoma 3 (Bcl-3), a member of the inhibitor of κB (IκB),[5,6] attenuated BMSCs senescence and regulated BMSCs differentiation fate through manipulating Wnt signalling.[7,8]

First, we showed that the level of Bcl-3 from 1 to 18 months in mice was significantly decreased with aging (Figure 1A). Then, we generated Bcl-3$^{-/-}$ mice which displayed slightly smaller size than that of wild-type (WT) mice and results of WB and qRT-PCR confirm that Bcl-3 was deleted in Bcl-3$^{-/-}$ mice (Figure S1A–C). Micro-computed tomography (μCT) analysis (Figure 1B,C and Figure S1D) and H&E staining (Figure S1E) showed a significant bone loss in 10-week-old Bcl-3$^{-/-}$ mice compared with control. Bcl-3 knockout also significantly increased bone loss following ovariectomy (OVX) by μCT (Figure 1D and Figure S1F). Calcein double labelling verified Bcl-3$^{-/-}$ mice had less endosteal, periosteal and trabecular bone formation than that of the WT counterparts, mineral apposition and bone formation was lower in Bcl-3$^{-/-}$ group (Figure 1E,F). In addition, the number of trabecular OCN-positive cells in Bcl-3$^{-/-}$ mice was less than that in WT mice, while the number of FABP4-positive adipocytes and trap-positive cells were increased in Bcl-3$^{-/-}$ mice (Figure 1G–I and Figure S1G).

Then, we examined the differentiation potential, namely osteogenesis and adipogenesis of BMSCs after Bcl-3 was silenced in vitro. BMSCs were isolated from 5-week-mice and identified by flow cytometry (Figure S2A,B). The osteogenesis of BMSCs was decreased when Bcl-3 was knockdown verified by staining of alkaline phosphatase (ALP) and calcium nodules stained by Alizarin Red S (ARS) and the decreased mRNA levels of *Ocn*, *Osterix* and *Runx2* (Figure 2A,B). On the contrary, adipogenesis was increased in Bcl-3-knockdown BMSCs validated by oil red staining and the mRNA levels of *Fabp4, Adipoq* and *Pparγ* (Figure 2C,D). When Bcl-3 was knocked down in BMSCs, the expression of FABP4 was increased, accompanied by decreased OSTERIX expression (Figure 2E). On the other hand, we found that Bcl-3 overexpression could promote osteoblastic differentiation and inhibit adipogenic differentiation in senescent BMSCs treated by H_2O_2 (Figure S3A–F).

In aging BMSCs, the expression level of Bcl-3 was decreased (Figure 2F and Figure S4A). For self-renewable capacities, BMSCs of Bcl-3$^{-/-}$ mice showed fewer colony-forming unit-fibroblasts (CFU-Fs) (Figure 2G,H). The number of SA-β-gal-positive blue cells in Bcl-3 knockdown BMSCs was significantly increased than control BMSCs (Figure 2I). The expression of aging makers, *P21* and *P16*, was significantly upregulated when Bcl-3 was knockdown in BMSCs (Figure 2J). Moreover, Bcl-3 overexpression reduced senescent cells numbers and the expression of aging makers after treated with H_2O_2 (Figure S4B,C). To sum up, Bcl-3 was decreased during BMSCs senescence, and the supply of Bcl-3 could protect BMSCs from aging.

To explore the molecular mechanisms, we performed RNA sequencing in shBcl-3 and shCtrl BMSCs. The results of gene ontology (GO) revealed that the downregulated genes following Bcl-3 knockdown were mainly enriched in ossification, bone mineralization, bone growth, etc. Kyoto encyclopaedia of genes and genomes (KEGG) revealed that Bcl-3-knockdown BMSCs influenced Wnt/β-catenin signalling pathways (Figure 3A,B). Heat map showed that relative expression levels of genes in the Wnt pathway were decreased in shBcl-3 BMSCs compared to shCtrl BMSCs (Figure 3C). In addition, we found that Wnt/β-catenin signalling was downregulated in Bcl-3 knockdown BMSCs indicated by gene set enrichment

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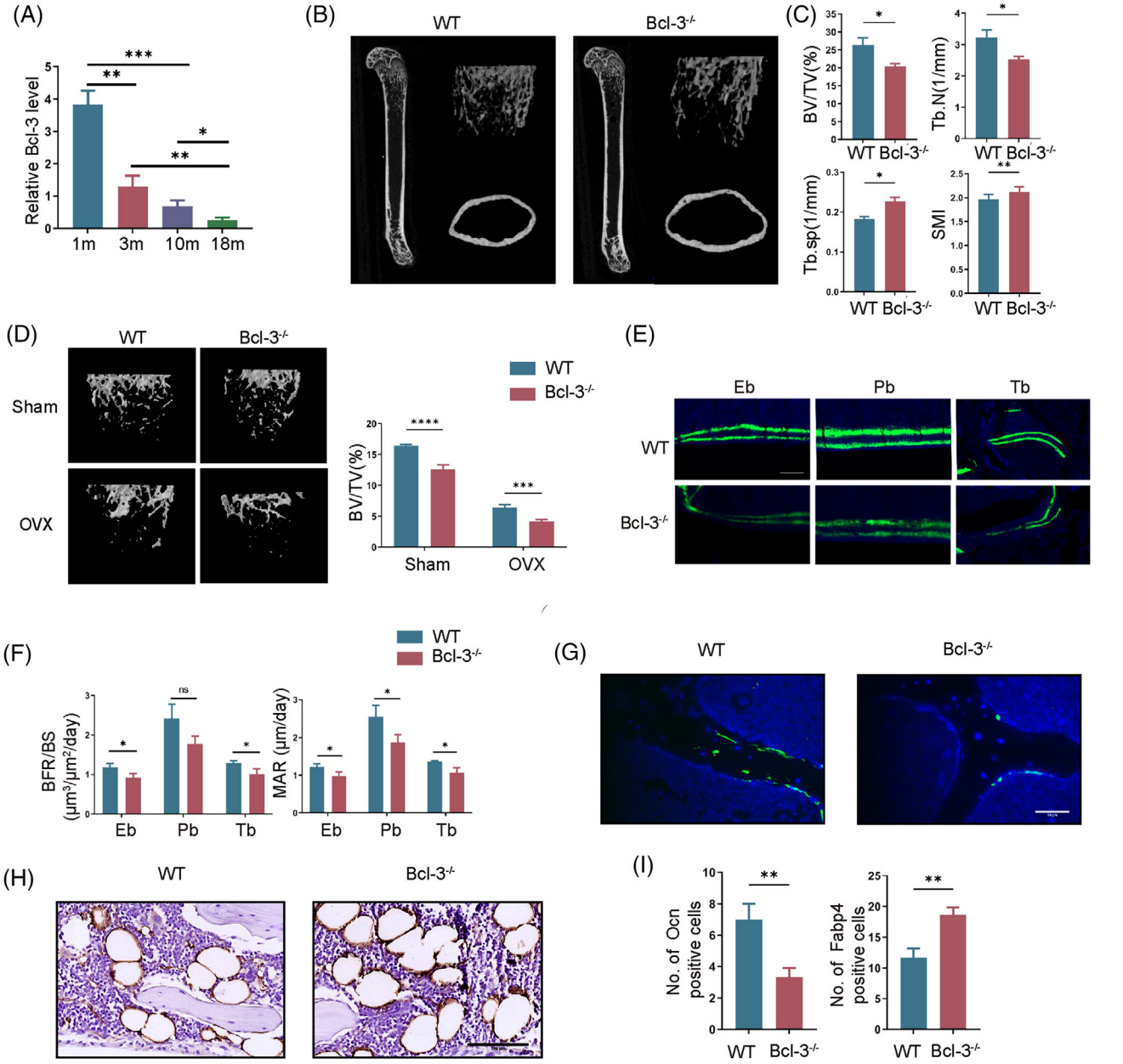

FIGURE 1 Deletion of Bcl-3 exacerbates bone loss and reduces bone formation. (A) Bcl-3 mRNA in femurs from 1 m, 3 m, 10 m and 18 m wild-type (WT) male mice was assessed by qRT-PCR. ($N = 3$ independent experiments). (B) Representative micro-computed tomography (μCT_ images of femurs from 10-week-old Bcl-3$^{-/-}$ and WT male mice. ($N = 3$ mice/group). (C) Quantitative measurements of BV/TV, Tb.N, Tb.Sp and SMI in 10-week-old Bcl-3$^{-/-}$ and WT mice by μCT. ($N = 3$ mice/group). (D) Representative μCT images of femurs from 10-week-old Bcl-3$^{-/-}$ and WT female mice following sham and ovariectomy (OVX). ($N = 3$ mice/group). (E,F) Representative images of calcein double labelling of endosteal bone (Eb), periosteal bone (Pb) and trabecular bone (Tb) with quantification of BFR/BS and MAR in the femurs of 10-week-old WT and Bcl-3$^{-/-}$ male mice. (G) Representative immunostaining of OCN in 10-week-old Bcl-3$^{-/-}$ and WT male mouse femurs. Scale bar, 50 μm. ($N = 3$ mice/group). (H) Representative immunostaining of FABP4 in 6-month-old Bcl-3$^{-/-}$ and WT male mouse femurs. Scale bar, 100 μm. ($N = 3$ mice/group). (I) Quantification of immunostaining of OCN and FABP4. The data are presented as the mean ± SD. *P <.05; **P <.01; ***P <.005; ****P <.0001 vs. control group. Statistical analysis was performed using Student's t-test (A, C, F and I) and two-way ANOVA test (D). Primary antibodies: OCN, Abcam(ab93876); FABP4, Abcam(ab92501)

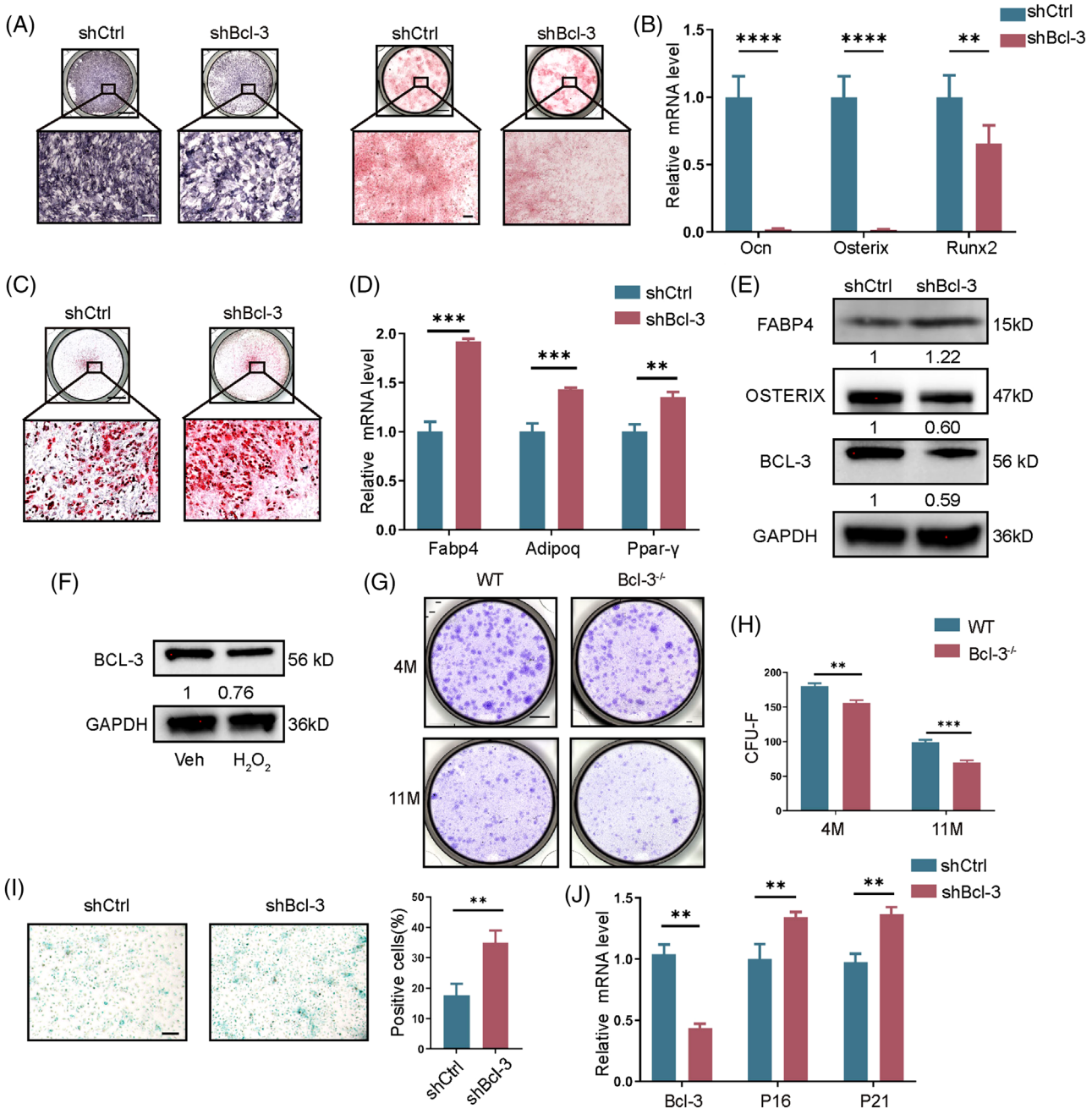

FIGURE 2 Bcl-3 regulates adipo-osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) in vitro and protects BMSCs from senescence. (A) Bcl-3 was knockdown in BMSCs and cultured in differentiation medium. Cell differentiation was assessed by alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining after14 days of osteogenic induction. Scale bar, 5 mm (above) and 200 μm (below). (B) The expression of osteogenesis markers (OCN, Osterix and Runx2) were analysed 14 days after osteogenic induction of Bcl-3 knock-out BMSCs. ($N = 4$ independent experiments). (C) Bcl-3 was knockdown in BMSCs and cultured in differentiation medium. Cell differentiation was assessed by oil red staining after 21 days of adipogenic induction. Scale bar, 5 mm (above) and 200 μm (below). (D) The expression of adipogenic markers (Fabp4, Adipoq and PPARγ) was analysed 6 days after adipogenic induction of Bcl-3 knock-out BMSCs. ($N = 4$ independent experiments). (E) Western blot detection of GAPDH, Bcl-3, Osterix and Fabp4 protein in BMSCs. The data are presented as the mean ± SD. (F) Western blot detection of GAPDH and Bcl-3 protein levels in H_2O_2 treated BMSCs. (G,H) Representative images and quantification of CFU-Fs formed by cells from Bcl-$3^{-/-}$ and wild-type (WT) mice. Scale bar, 0.5 mm. ($N = 3$ independent experiments). (I) SA-β-gal staining of Bcl-3 knockdown BMSCs and quantitative analysis of SA-β-gal staining. Scale bar, 200 μm. ($N = 3$ independent experiments). (J) Bcl-3, P16 and P21 mRNA in Bcl-3 knockdown BMSCs was assessed by qRT-PCR. ($N = 4$ independent experiments). The data are presented as the mean ± SD. *P <.05; **P <.01; ***P < .005; ****P < .0001 vs. control group. Statistical analysis was performed using Student's t-test (B, D, I and J) and two-way ANOVA test (H). Primary antibodies: GAPDH, Abcam(ab181602); BCL-3, Abcam(ab259832); FABP4, Abcam(ab92501); OSTERIX, Abcam(ab209484)

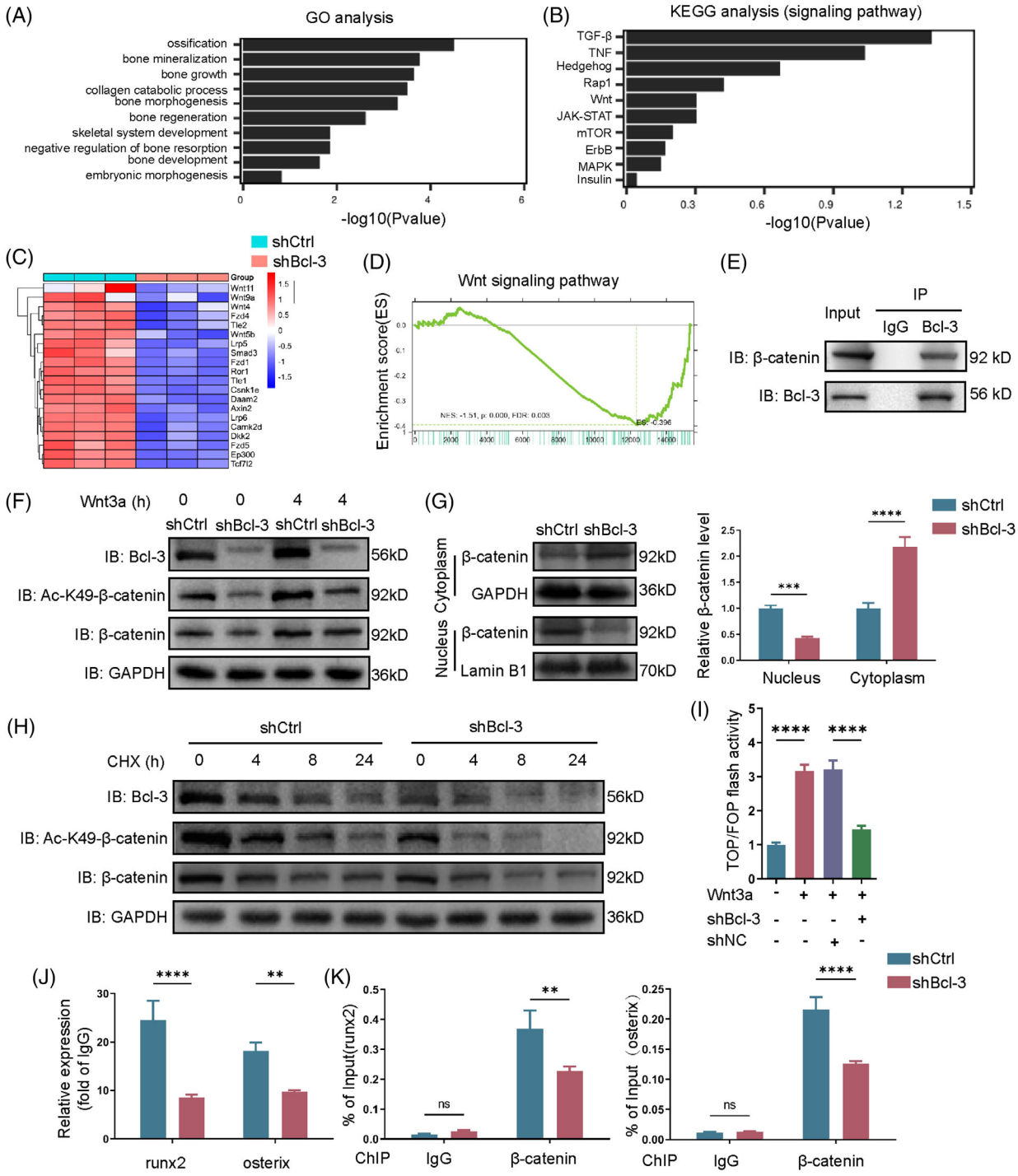

FIGURE 3 Bcl-3 activates Wnt/β-catenin signalling pathway and maintains β-catenin functions in vitro. (A,B) Gene ontology (GO, Left) and Kyoto encyclopedia of genes and genomes (KEGG, Right) analysis of downregulated genes with Bcl-3 deletion in bone marrow mesenchymal stem cells (BMSCs). (C) Heat map showed relative genes expression in Wnt pathway of shCtrl and shBcl-3 BMSCs. (D) Gene set enrichment analysis (GSEA) showed a significant decrease of Wnt/β-catenin gene signatures in shBcl-3 BMSCs. (E) Lysates from BMSCs were used, and anti-Bcl-3 antibody was used in IP followed by immunoblot using the indicated antibodies. (F) Bcl-3, Ac-K49-β-catenin and β-catenin were analysed by immunoblot in control and Bcl-3-knockdown BMSCs after treated with Wnt 3a for 0 h or 4 h. (G) The levels of Bcl-3 in nuclear and cytoplasmic. (H) Bcl-3, Ac-K49-β-catenin and β-catenin were analysed by immunoblot in control and Bcl-3-knockdown BMSCs after treated with cycloheximide (CHX, 50 mg/ml). (I) BMSCs were cotransfected with the indicated siRNA and TOP/FOP Flash reporter plasmid. (J) ChIP assays on the promoter regions of the Runx2 and Osterix genes were performed in control and Bcl-3-silenced BMSCs. The data are presented as the mean ± SD. $^{**}P < .01$; $^{****}P < .0001$ vs. control group. Statistical analysis was performed using two-way ANOVA. Primary antibodies: GAPDH, Abcam(ab181602); Lamin B1, Abcam(ab133741); β-catenin, Proteintech (51067-2-AP); K49 acetyl-β-catenin, Cell Signalling Technology(9030); BCL-3, Abcam(ab259832)

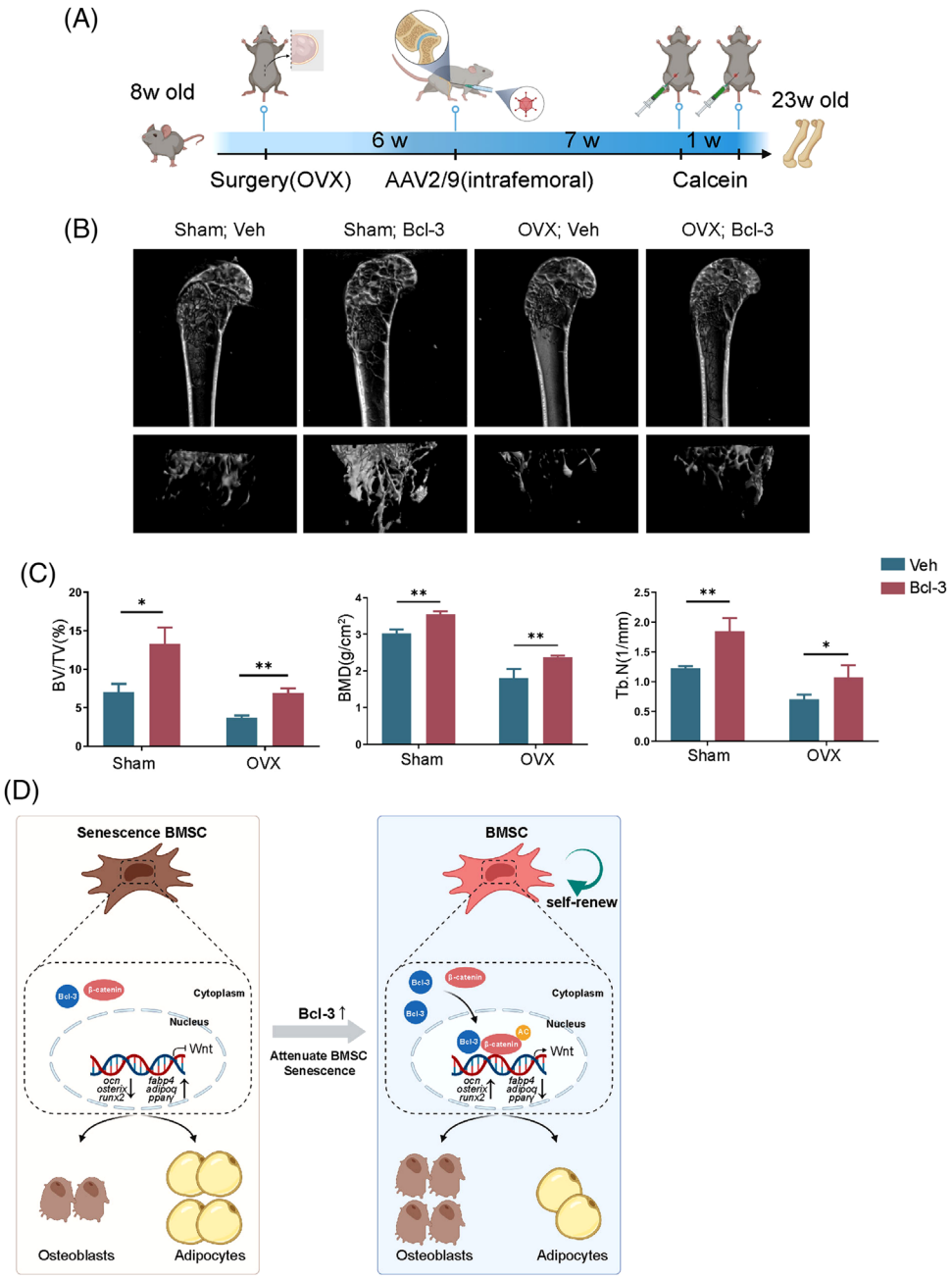

FIGURE 4 AAV2/9-mediated Bcl-3 overexpression prevents ovariectomy (OVX)-induced bone loss. (A) Diagram of the study and treatment methods. (B) Representative micro-computed tomography (μCT) images of femurs from 23-week-old Bcl-3 overexpressed and WT female mice following induced OVX and sham surgery. ($N = 3$ mice/group). (C) Quantitative measurements of BV/TV, BMD and Tb.N in 23-week-old Bcl-3 overexpressed and wild-type (WT) female mice by μCT. ($N = 3$ mice/group). (D) Graphical abstract. The data are presented as the mean ± SD. *P <.05; **P <.01 vs. control group. Statistical analysis was performed using two-way ANOVA

analysis (GSEA, Figure 3D). The expression levels of genes in Wnt signalling were decreased when Bcl-3 was knockdown in BMSCs (Figure S5A,B) and increased in Bcl-3-overexpressed BMSCs (Figure S5C,D).

Next, we explored the molecular mechanisms of Bcl-3 regulating β-catenin. The Co-immunoprecipitation (Co-IP) analysis revealed that Bcl-3 was bound to endogenous β-catenin in BMSCs (Figure 3E). Depletion of Bcl-3 leaded to decreased protein level of β-catenin and Ac-K49 β-catenin after treated with Wnt 3a for 4 h (Figure 3F and Figure S5E). Moreover, nuclear translocation of β-catenin was inhibited when Bcl-3 was silenced (Figure 3G and Figure S5F), while Bcl-3 overexpression in BMSCs could significantly activate β-catenin translocation (Figure S5G).

The degradation of Ac-K49 β-catenin was significant in Bcl-3-silenced BMSCs after treated with cycloheximide (CHX, Figure 3H and Figure S5H). In addition, TOP/FOP flash assays showed that Bcl-3 depletion reduced the transcriptional activity of β-catenin and decreased the combination of β-catenin with promotors of *Runx2* and *Osterix* (Figure 3I–K).

Lastly, we performed OVX surgery on 8-week-old female mice which were intrafemorally injected with Bcl-3 AAV2/9 or control AAV (5×10^{12} GC/kg) 6 weeks post-surgery (Figure 4A). As shown in μCT, AAV2/9-mediated Bcl-3 overexpression prevented OVX-induced bone loss (Figure 4B,C).

Osteoporosis is common among aged individuals, which could be attributed to the senescence and bone-fat imbalance of BMSCs. During osteogenetic differentiation in BMSCs, Wnt/β-catenin signalling functions positively. In the previous study, Bcl-3 was proved to interact with β-catenin and regulate Wnt signalling in colorectal tumour cells.[9] However, the potential correlations between Bcl-3 and Wnt /β-catenin in osteoporosis are unclear. Bcl-3 has been proven to activate the matrix metalloproteinase 1 expression in chondrocytes and synovial fibroblast, being the limited research on the skeletal system.[10] Our study revealed that loss of Bcl-3 led to bone loss, while the overexpression could be therapeutic, unveiling the substantial role of Bcl-3 in skeletal aging. Nevertheless, the investigations into BMSCs transcriptional alterations could reflect the regulative effects of Bcl-3 partially, calling for further explorations in the future.

Our results demonstrated that Bcl-3 inhibited BMSCs senescence, promoted osteogenesis and decreased adipogenesis. For mechanisms, Bcl-3 maintained Wnt/β-catenin signalling and remained a potential target to treat age-related osteoporosis (Figure 4D).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPORTING INFORMATION

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