# $1,25(OH)_2D_3$ inhibits osteogenic differentiation through activating β-catenin signaling via downregulating bone morphogenetic protein 2

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**Abstract.** The present study explored whether bone morphogenetic proteins (BMPs) and Wnt/β-catenin signaling pathways were involved in the $1,25(OH)_2D_3$-induced inhibition of osteogenic differentiation in bone marrow-derived mesenchymal stem cells (BMSCs). To evaluate the osteogenic differentiation of BMSCs, the expression levels of ossification markers, including BMP2, Runt-related transcription factor 2 (Runx2), Msh homeobox 2 (Msx2), osteopontin (OPN) and osteocalcin (OCN), and the activity of alkaline phosphatase (ALP), as well as the calcified area observed by Alizarin red-S staining, were investigated. Chromatin immunoprecipitation (ChIP) assay was used to detect the effect of $1,25(OH)_2D_3$ on the DNA methylation and histone modification of BMP2, while an immunoprecipitation (IP) assay was performed to assess the crosstalk between Smad1 and disheveled-1 (Dvl-1) proteins. It was observed that $1,25(OH)_2D_3$ significantly decreased the expression levels of BMP2, Runx2, Msx2, OPN and OCN, and reduced ALP activity and the calcified area in BMSCs, whereas these effects were rescued by BMP2 overexpression. ChIP assay revealed that BMSCs treated with $1,25(OH)_2D_3$ exhibited a significant increase in H3K9me2 level and a decrease in the acetylation of histone H3 at the same BMP2 promoter region. In addition, $1,25(OH)_2D_3$ treatment promoted the nuclear accumulation of β-catenin by downregulating BMP2. Furthermore, the β-catenin signaling inhibitor XAV-939 weakened the inhibitory effect of $1,25(OH)_2D_3$ on osteogenic differentiation. Additionally, knockdown of β-catenin rescued the attenuation in Dvl-1 and Smad1 interaction caused by $1,25(OH)_2D_3$. Overexpression of Smad1 also reversed the inhibitory effect of $1,25(OH)_2D_3$ on osteogenic differentiation. Taken together, the current study demonstrated that $1,25(OH)_2D_3$ inhibited the differentiation of BMSCs into osteoblast-like cells by inactivating BMP2 and activating Wnt/β-catenin signaling.

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## Introduction

Osteoporosis is a common bone disease that mainly affects the elderly, and is characterized by low bone density and microarchitectural deterioration of bone tissue (1-3). Osteoporosis is associated with an excessive replacement of osteoblasts with osteoclasts (4). As a result, studying of the osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) has emerged as the main direction in the exploration of the pathogenesis of osteoporosis (5). Since chondrocyte development and bone marrow adipogenesis are based on osteoblast formation (6,7), osteogenic differentiation of BMSCs is favored to osteoblast maturation and contributes to osteoporosis prevention (8,9). The pivotal role of bone morphogenetic protein 2 (BMP2), one of the most well-characterized proteins of the BMP family, in promoting bone formation has been well documented, indicating that factors that regulate BMP2 expression may be considered as important modulators in bone formation.

$1,25(OH)_2D_3$, the most active vitamin D metabolite, is a pleiotropic hormone. Through binding to its intra-nuclear receptor, vitamin D receptor (VDR), $1,25(OH)_2D_3$ has numerous regulatory effects, including calcium homeostasis, cell proliferation and differentiation (10,11). Notably, it is widely accepted that vitamin D is important for bone growth as its deficiency can lead to osteomalacia and rickets (12). Nevertheless, the effects of vitamin D on bone formation remains under debate. In a previous study, Erben *et al* (13,14) reported that $1,25(OH)_2D_3$ administration evidently enhanced new bone remodeling *in vivo*. However, Sooy *et al* (15) found that the knockdown of VDR improved osteogenic potential *in vitro*. Additionally, Yamaguchi and Weitzmann (16) revealed that high-dose administration of $1,25(OH)_2D_3$ significantly inhibited the mineralization of osteoblasts. Fu *et al* (11) further reported that $1,25(OH)_2D_3$

was able to reduce BMP2 expression in BMSCs. However, the mechanisms underlying the action of $1,25(OH)_2D_3$ in osteogenic differentiation suppression remain largely unclear.

The canonical Wnt/β-catenin pathway has been identified to serve an important role in the regulation of the osteogenic differentiation of BMSCs (17). Under normal conditions, β-catenin protein remains at a low level without Wnt ligands through phosphorylation and ubiquitination-mediated degradation. However, β-catenin is released from the 'degradation complex', which contains Axin1/2, APC, casein kinase 1 and glycogen synthase kinase 3β, when Wnt ligands bind to their receptors, thus resulting in the stabilization of β-catenin and its accumulation in the cytoplasm and nucleus. However, to date, the roles of β-catenin in the osteogenic differentiation of stem cells remain paradoxical. For instance, Liu *et al* (18) reported that activation of β-catenin repressed the osteogenic differentiation of periodontal ligament stem cells, a new population of MSCs. By contrast, the studies by Monroe *et al* (17) and Mo *et al* (19) demonstrated that the osteogenic differentiation of BMSCs depends on Wnt/β-catenin signaling activation. Therefore, whether Wnt/β-catenin signaling is involved in the $1,25(OH)_2D_3$-mediated osteogenic differentiation and the exact role of this signaling need to be elucidated.

In the present study, the aim was to explore the mechanism underlying the $1,25(OH)_2D_3$-mediated suppression of osteogenic differentiation *in vitro* by recruiting BMSCs.

## Materials and methods

*BMSC isolation, culture and identification*. In total, three Sprague-Dawley (SD) rats (140±10 g), aged 4-weeks-old, were purchased from Better Biotechnology Co., Ltd. (Nanjing, Jiangsu) and were maintained under specific pathogen-free conditions at 20-26˚C with 55±5% humidity in 12 h light/dark cycle with *ad libitum* access to food and water. After 1 week of accommodation, the rats were humanely euthanized via cervical dislocation. Next, BMSCs were isolated from the femurs and tibias of the SD rats according to the method described in a previous study (20). All animal experiment protocols were approved by the Review Committee for the Use of Human or Animal Subjects of the Shanghai Jiao Tong University (Shanghai, China).

Subsequent to repeated flushing, the BMSCs were cultured in culture medium containing 89% Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), and kept at 37˚C with 5% $CO_2$. The culture medium was refreshed twice per week. BMSCs at the 3rd passage were used in all the experiments.

To identify the BMSCs, cells at the 3rd passage were trypsinized, collected and subjected to flow cytometry analysis (CytoFLEX; Beckman Coulter Commercial Enterprise, Inc.) with antibodies against CD34 (1:50; cat. no. ab81289; Abcam), CD45 (1:10; cat. no. 554878; BD Bioscience, CD44 (1:10; cat. no. 550974; BD Bioscience) and CD90 (1:10; cat. no. 561973; BD Bioscience). Cells identified as BMSCs negatively expressed CD34 and CD45, and positively expressed CD44 and CD90 (19). The results were analyzed using the FlowJo software (version 7.6; FlowJo LLC, Inc.).

*Osteogenic induction and cell treatments*. For osteogenic induction, BMSCs ($1x10^6$ cells/well) were seeded into 6-well plates and cultured in the complete culture medium supplemented with osteogenic induction medium (OIM) containing $10^{-7}$ M dexamethasone, 10 mM β-glycerophosphate and 50 $\mu$M ascorbate-2-phosphate (all purchased from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 14 days (21). Next, the cells were collected and subjected to ossification assessment.

For $1,25(OH)_2D_3$ treatment, BMSCs were incubated with 1, 5, 10, 20 or 50 nM of $1,25(OH)_2D_3$ (Sigma-Aldrich; Merck KGaA) dissolved in ethanol for 48 h. Equivalent volume of 100% ethanol was used in the negative control group. Furthermore, XAV-939 (Selleck Chemicals, Shanghai, China) treatment was used to inhibit Wnt/β-catenin signaling in BMSCs at a concentration of 10 $\mu$M for 1 h, with equal volume of DMSO (Beyotime Institute of Biotechnology) as a negative control. For co-treatment of $1,25(OH)_2D_3$ and XAV-939 (MedChemExpress, Inc.), the cells were first treated with 10 $\mu$M XAV-939 for 1 h at 37˚C, followed by treatment with 10 nM $1,25(OH)_2D_3$ for 48 h at 37˚C.

*Stable transfection cell lines*. In order to induce upregulation of BMP2 and Smad1 levels in BMSCs, overexpressing lentivirus vectors targeting the rat BMP2 (OE-BMP2) and Smad1 (OE-Smad1) genes were designed and synthesized by GenePharma Co., Ltd. Briefly, the BMSCs ($5x10^5$) were seeded in 6-well plates and cultured at 37˚C overnight. Next, the cells were infected with OE-BMP2, OE-Smad1 or OE-NC (serving as the negative control vector) using 5 $\mu$g/ml polybrene (Hanbio Biotechnology Co., Ltd.), followed by incubation with 100 $\mu$g/ml G418 or 7 $\mu$g/ml puromycin for 14 days to select the stably infected cell lines, respectively.

*Small interfering RNAs (siRNAs)*. Three siRNAs targeting the rat β-catenin gene (si-β-catenin) and a negative control vector (si-NC) were purchased from OriGene Technologies, Inc. (Rockville, MD, USA; cat. no. SR500644). The siRNAs (si-1, si-2 and si-3) were applied to knock down β-catenin expression in BMSCs, using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following 6 h of cell transfection, the medium was replaced with fresh DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS. The transfection efficiency was detected by using reverse transcription-quantitative PCR (RT-qPCR) and western blotting assays after 24 and 48 h of cell transfection. The siRNA sequences used were: si-1, 5'-CCAGCAAATCATGCGCCTT-3'; si-2, 5'-GCT GCATAATCTCCTGCTA-3'; and si-3, 5'-CCACTAATGTCC AGCGCTT-3'.

*RT-qPCR*. Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Next, the RNA was reversely transcribed into complementary DNA (cDNA) using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Next, qPCR analysis was performed with SYBR GreenER™ qPCR SuperMix (Thermo Fisher Scientific, Inc.). The qPCR conditions involved pre-denaturation at 95˚C for 5 min,

40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative expressions of mRNAs were calculated by using the $2^{-\Delta\Delta Cq}$ method (22). The primer sequences used in this experiment were as follows: GAPDH forward, 5'-AGTGCCAGCCTC GTCTCATA-3', and reverse, 5'-GATGGTGATGGGTTT CCCGT-3'; BMP2 forward, 5'-GGACATGGTTGTGGAGGG TT-3', and reverse, 5'-TGTTTTCCCAACTTATTTTCGTAG A-3'; Msh homeobox 2 (Msx2) forward, 5'-GGAGATTGC AAGAGGGCGTA-3', and reverse, 5'-GGGCTAGCTGAC TGTGTTGT-3'; Runt-related transcription factor 2 (Runx2) forward, 5'-CGCCTCACAAACAACCACAG-3', and reverse, 5'-TCACTGCACTGAAGAGGCTG-3'; β-catenin forward, 5'-ATCATTCTGGCCAGTGGTGG-3', and reverse, 5'-GAC AGCACCTTCAGCACTCT-3'; Smad1 forward, 5'-TCAATA GAGGAGATGTTCAAGCAGT-3', and reverse, 5'-GGTGGT AGTTGCAGTTCCGA-3'.

*Western blotting*. Total protein was extracted from the BMSCs using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology), while the nuclear and cytoplasmic proteins were extracted from the cells using a CelLytic™ NuCLEAR™ Extraction kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Following the assessment of protein concentrations with a BCA kit (Thermo Fisher Scientific, Inc.), 25 $\mu$g protein from each sample was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer onto PVDF membranes (Thermo Fisher Scientific, Inc.). The membranes were then blocked with 5% non-fat milk for 1 h at room temperature and probed overnight at 4˚C with the following primary antibodies: GAPDH (1:10,000 dilution; ProteinTech), BMP2 (1:1,000 dilution; cat. no. ab14933; Abcam), Runx2 (1:1,000 dilution; cat. no. ab76956; Abcam), Msx2 (1:2,000 dilution; cat. no. HPA005652; Sigma-Aldrich), osteocalcin (OCN; 1:2,000 dilution; cat. no. ab93876; Abcam), osteopontin (OPN; 1:2,000 dilution; cat. no. ab8448; Abcam), β-catenin (1:2,500 dilution; cat. no. 9562; Cell Signaling Technology), disheveled-1 (Dvl-1; 1:2,000 dilution; cat. no. D3570; Sigma-Aldrich), Smad1 (1:1,000 dilution; cat. no. 9743; Cell Signaling Technology), tubulin (1:1,000 dilution; cat. no. 2148; Cell Signaling Technology) and histone (1:5,000 dilution; cat. no. ab1791; Abcam). Subsequently, incubation with a horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. SA00001-1/ SA00001-2; ProteinTech) was performed for 1 h at room temperature. GAPDH, histone and tubulin were used as the internal references for the total, nuclear and cytoplasmic protein expression, respectively. The band signals were enhanced by chemiluminescence (ECL reagent; EMD Millipore, Billerica, MA, USA) and visualized with a FluorChem Q system (SelectScience, Waltham, MA, USA). Protein expression quantification was performed using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

*Alkaline phosphatase (ALP) activity*. To assess ALP activity, BMSCs at a density of $1x10^5$ cells/well were seeded into 24-well plates and cultured for 14 days with OIM. Next, the ALP activity was measured with an ALP activity kit (Shanghai Suer Biological Technology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. In brief, cells were lysed with RIPA lysis buffer on ice, and then the lysates were mixed with a working solution for 15 min at 37˚C. The optical density values at 520 nm were subsequently measured, and the ALP activity was normalized to the total intracellular protein concentration.

In order to assess whether $1,25(OH)_2D_3$ affected the osteogenic differentiation of BMSCs, after 12 days of incubation with OIM, BMSCs ($5x10^5$/well in 6-well plates) were cultured in OIM containing $1,25(OH)_2D_3$ (10 nM) for a further 48 h and the ALP activity was determined. In addition, to assess the effects of BMP2 and Smad1 on osteogenic differentiation, the OE-BMP2/OE-Smad1 stable expressing cells were cultured in OIM for 14 days, followed by ALP detection.

*Alizarin red-S staining*. Cells at a density of $5x10^6$/well in 6-well plates treated with $1,25(OH)_2D_3$ (10 nM) for 48 h or OE-BMP2/OE-Smad1 stable expression cell lines were washed with PBS and then fixed with 75% ethanol at 4˚C for 30 min. Next, the cells were stained with Alizarin red-S (40 mM, pH 6.2) at room temperature for a total of 30 min. Any excess stain was removed by distilled water, and images of the plates were captured to visualize cell calcification. Quantification was performed using Image Pro Plus software (version 6.0; Media Cybernetics, Inc.).

*Cell Counting Kit-8 assay (CCK-8)*. A CCK-8 assay was used to detect cell proliferation. In brief, BMSCs were seeded in 96-well plates at a density of $3x10^3$ cells/well and cultured at 37˚C overnight. Subsequently, the cells were subjected to different treatments, including $1,25(OH)_2D_3$ (10 nM)/ethanol (10 nM) and/or XAV-939 (10 $\mu$M)/DMSO (10 $\mu$M). Following incubation at 37˚C for 48 h, the cell culture medium was replaced with 10 $\mu$l CCK-8 reagent (Beyotime Institute of Biotechnology) and 90 $\mu$l fresh medium, and incubated at 37˚C for another 4 h. The absorbance at 450 nm was finally measured with a plate reader (model 680; Bio-Rad Laboratories, Inc.).

*Luciferase gene reporter assay*. The fragment of rat BMP2 promoter region C that contains the VDR binding sites was amplified by PCR using the followed primers: Forward primer, 5'-ATTTGCCCTAAACTCGGGCATCTG-3', and reverse primer, 5'-TTCGTCCCGAGCTGCCAAT-3' (11). Next, the fragment was cloned into the pGL3 promoter vector (Promega Corporation) containing a SV40 promoter upstream of the luciferase gene. The pSV40-BMP2-Luciferase vector was then transfected into the BMSCs cells with or without 10 nM $1,25(OH)_2D_3$ treatment. Cells were harvested 48 h after the aforementioned treatments, and the relative luciferase activities were measured using the Dual-Glo luciferase assay kit (Promega Corporation).

*Chromatin immunoprecipitation (ChIP) assay*. ChIP assay was performed as previously described (23). Briefly, the crosslinked chromatins were immunoprecipitated with antibodies against histone H3 dimethylated lysine 9 (H3K9me2; cat. no. ab1220; Abcam) and acetyl-histone H3 (histone H3-Ac; cat. no. ab4729; Abcam). The enrichment of the specific amplified region was analyzed by RT-qPCR. Normal IgG was used as a negative control. The primers for amplifying the fragments of the BMP2 promoter in region C were

as follows: Forward, 5'-CGCCCCGCCCCGCCCCG-3', and reverse, 5'-ATTTGCCCTAAACTCGGGCATCTG-3' (11).

*Immunoprecipitation (IP).* An IP assay was used to evaluate the interaction between Dvl-1 and Smad1 proteins. Briefly, cells were lysed in 5 ml lysis buffer (containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5% Nonidet P40 and protease inhibitor cocktail) for 30 min at 4˚C. After 1 h of incubation with 50 $\mu$l Dynabeads protein A (Invitrogen; Thermo Fisher Scientific, Inc.), the supernatants containing 200 $\mu$g proteins were incubated with antibody against Smad1 (2 mg; cat. no. 9743; Cell Signaling Technology) at 4˚C overnight. Next, the Dynabeads were washed with lysis buffer for five times, followed by resuspension in SDS-PAGE loading buffer and assessment by western blotting using antibody against Dvl-1 (1:2,000; cat. no. D3570; Sigma-Aldrich; Merck KGaA).

*Statistical analysis.* Each experiment was repeated at least three times, and the data are represented as the mean ± standard deviation. Data analysis was performed with SPSS software, version 21.0 (IBM Corporation, Armonk, NY, USA), using the Student's t-test for comparisons between two groups, or one-way analysis of variance followed by Bonferroni post-hoc test for comparisons of more than two groups. $P<0.05$ was considered to denote a statistically significant difference.

## Results

*BMP2 upregulation reverses the $1,25(OH)_2D_3$-induced inhibition of osteogenic differentiation.* The flow cytometry results demonstrated that the isolated cells positively expressed CD44 and CD90, while they negatively expressed CD34 and CD45 (Fig. 1A), suggesting that BMSCs were successfully isolated. The present study then attempted to explore whether BMP2 was involved in $1,25(OH)_2D_3$-mediated osteogenesis inhibition of BMSCs. First, stable overexpression of BMP2 was induced in the BMSCs, and the expression of BMP2 was found to be significantly elevated at the mRNA and protein levels in the OE-BMP2 group, compared with the OE-NC group (Fig. 1B and C). Next, the ALP activity of cells treated with $1,25(OH)_2D_3$ was assessed. Compared with the negative control (ethanol) group, $1,25(OH)_2D_3$ treatment decreased ALP activity in a dose-dependent manner (Fig. 1D). Significant reduction in ALP activity was observed at a minimum concentration of 10 nM (Fig. 1D), and thus this concentration was selected for use in subsequent assays. No evident changes in BMSC viability were observed when cells were treated with $1,25(OH)_2D_3$ or/and overexpressed BMP2 (Fig. 1E).

When cells were treated with $1,25(OH)_2D_3$ (10 nM) for 48 h, the ALP activity and calcified area of BMSCs were significantly decreased, whereas BMP2 overexpression rescued these results (Fig. 1F and G). Furthermore, $1,25(OH)_2D_3$ treatment significantly decreased the expression levels of ossification-associated proteins, including BMP2, OPN, OCN, Runx2 and Msx2 (Fig. 1H), and reduced the mRNA levels of BMP2, Runx2 and Msx2 in BMSCs (Fig. 1I). However, these effects of $1,25(OH)_2D_3$ were all neutralized when BMP2 was overexpressed in BMSCs (Fig. 1H and I). These results indicated that $1,25(OH)_2D_3$ repressed the osteogenic differentiation of BMSCs via downregulating BMP2 expression.

*$1,25(OH)_2D_3$ decreases BMP2 expression through DNA methylation and histone modification.* Next, the current study explored the mechanism underlying $1,25(OH)_2D_3$-induced BMP2 downregulation in BMSCs. Compared with the control (ethanol) group, $1,25(OH)_2D_3$ treatment significantly decreased the activity of the pSV40-BMP2-Luciferase promoter (Fig. 2A), and enhanced the combination between BMP2 promoter and H3K9me2 (Fig. 2B), while it weakened the interaction between BMP2 promoter and histone H3-Ac (Fig. 2C). These results suggested that $1,25(OH)_2D_3$ downregulated BMP2 expression at the epigenetic level.

*$1,25(OH)_2D_3$ represses the osteogenic differentiation through activating $\beta$-catenin signaling.* Next, the study explored the role of β-catenin signaling in $1,25(OH)_2D_3$-induced osteogenesis repression. The results revealed that $1,25(OH)_2D_3$ treatment enhanced the nuclear expression level of β-catenin and decreased its cytoplasmic expression level, whereas BMP2 overexpression reversed the effects of $1,25(OH)_2D_3$ (Fig. 3A). These findings suggested that the activation of β-catenin signaling may be involved in the $1,25(OH)_2D_3$-induced repression of the osteogenic differentiation of BMSCs. To further explore the role of β-catenin, the inhibitor of β-catenin, XAV-939, was recruited to repress β-catenin levels. The results demonstrated that XAV-939 treatment had no evident influence in BMSC viability (Fig. 3B). XAV-939 significantly blunted the role of $1,25(OH)_2D_3$ in the inhibition of ALP activity (Fig. 3C) and in the reduction of the calcified area (Fig. 3D), as well as reversed the $1,25(OH)_2D_3$-induced decrease in the expression levels of Runx2, Msx2, BMP2, OPN and OCN (Fig. 3E). The aforementioned findings illustrated that $1,25(OH)_2D_3$ repressed the osteogenic differentiation of BMSCs through activating β-catenin signaling.

*$1,25(OH)_2D_3$ represses the osteogenic differentiation through impairing the interaction between Dvl-1 and Smad1 proteins via $\beta$-catenin.* As Wnt/β-catenin signaling closely interacts with BMP signaling pathway (24), the present study further explored the role of the interaction between β-catenin and BMP2 signaling pathways in the $1,25(OH)_2D_3$-mediated inhibition of the osteogenic differentiation of BMSCs. Following transfection with siRNAs targeting the β-catenin gene, si-2 exhibited the highest knockdown efficiency among the three siRNAs at the mRNA and protein levels (Fig. 4A and B); therefore, si-2 was selected for use in further experiments. IP assay revealed that $1,25(OH)_2D_3$ treatment evidently reduced the crosstalk between Dvl-1 and Smad1 protein, while knockdown of β-catenin significantly impaired this effect (Fig. 4C). This suggested that the weakness in Dvl-1 and Smad1 interaction may serve a vital role in $1,25(OH)_2D_3$-induced osteogenic differentiation repression. To further explore this interaction, BMSCs were transfected with OE-Smad1 to upregulate Smad1 expression. As shown in Fig. 4D and E, the mRNA and protein expression levels of Smad1 were significantly increased when BMSCs were transfected with OE-Smad1, while it had no evident influence on cell viability (Fig. 4F). In contrast to the $1,25(OH)_2D_3$ treatment, Smad1 upregulation significantly increased the ALP activity and calcified area in BMSCs, and impaired the effect of $1,25(OH)_2D_3$ on osteogenic

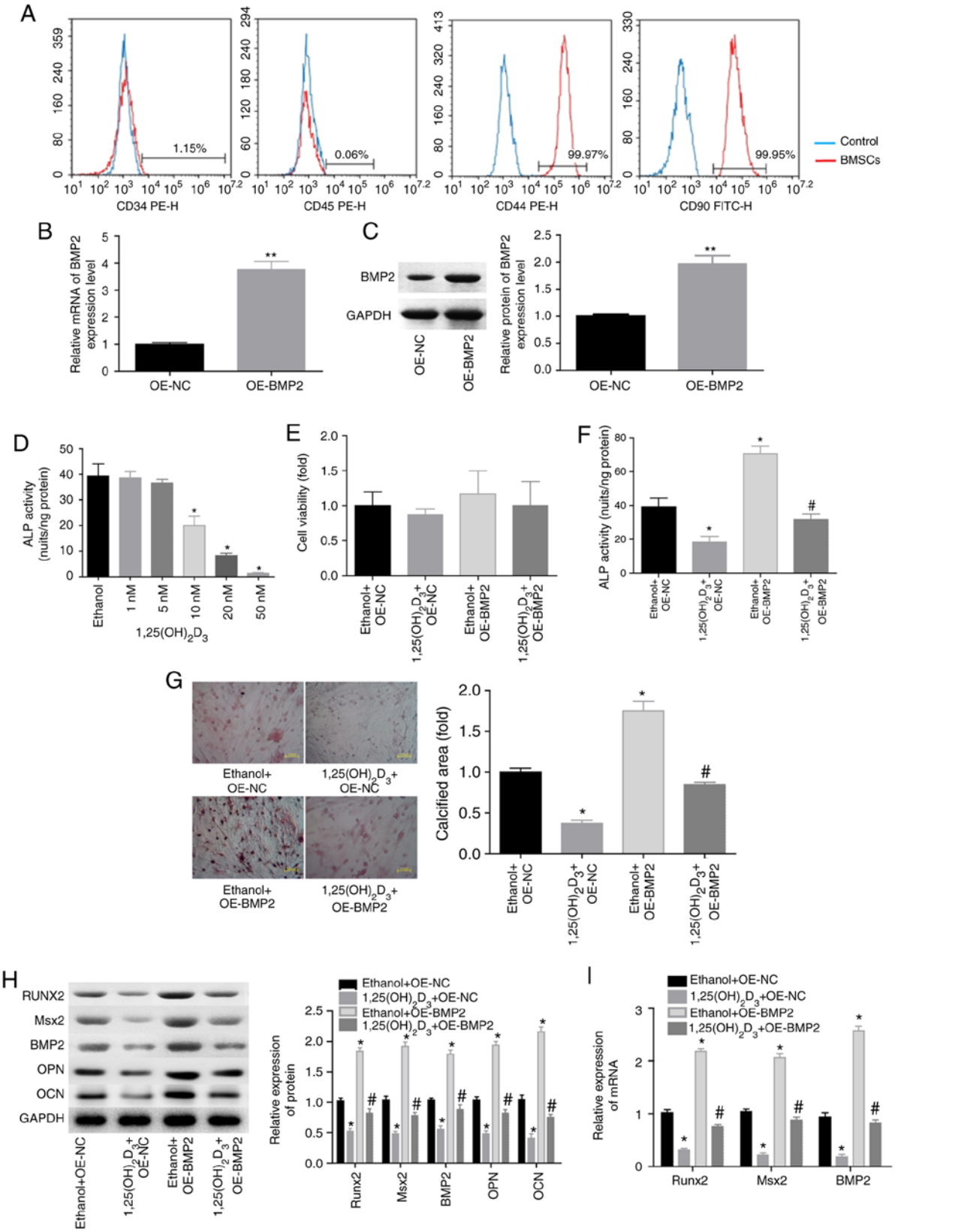


Figure 1. $1,25(OH)_2D_3$ repressed the osteogenic differentiation of BMSCs via BMP2 downregulation. (A) Flow cytometry was conducted to test the proportion of $CD34^+$, $CD44^+$, $CD45^+$ and $CD80^+$ in the isolated BMSCs. (B) mRNA and (C) protein levels of BMP2, determined by RT-qPCR and western blotting subsequent to stable infection of BMSCs with OE-BMP2 or OE-NC (n=3). (D) The effect of different concentrations of $1,25(OH)_2D_3$ (1, 5, 10, 20 or 50 nM) on ALP activity in BMSCs, with ethanol serving as a negative control (n=3). (E) Cell viability determined by Cell Counting Kit-8 assay and (F) ALP activity in BMSCs treated with $1,25(OH)_2D_3$ and/or OE-BMP2. (G) Alizarin red-S was used to assess the calcified nodules of BMSCs treated with $1,25(OH)_2D_3$ and/or OE-BMP2. Magnification, x200. (H) Western blotting of the protein levels of BMP2, Runx2, Msx2, OPN and OCN in BMSCs with different treatments. (I) RT-qPCR analysis of the mRNA levels of Runx2, BMP2 and Msx2 in differently treated BMSCs (n=3). $^*P<0.05$ and $^{**}P<0.01$, vs. corresponding control group (OE-NC, ethanol or ethanol + OE-NC group); $^{\#}P<0.05$ vs. $1,25(OH)_2D_3$+OE-NC group. BMSCs, bone marrow-derived mesenchymal stem cells; BMP2, bone morphogenetic protein 2; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; Msx2, Msh homeobox 2; OPN, osteopontin; OCN, osteocalcin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; OE, overexpressing vector; NC, negative control.

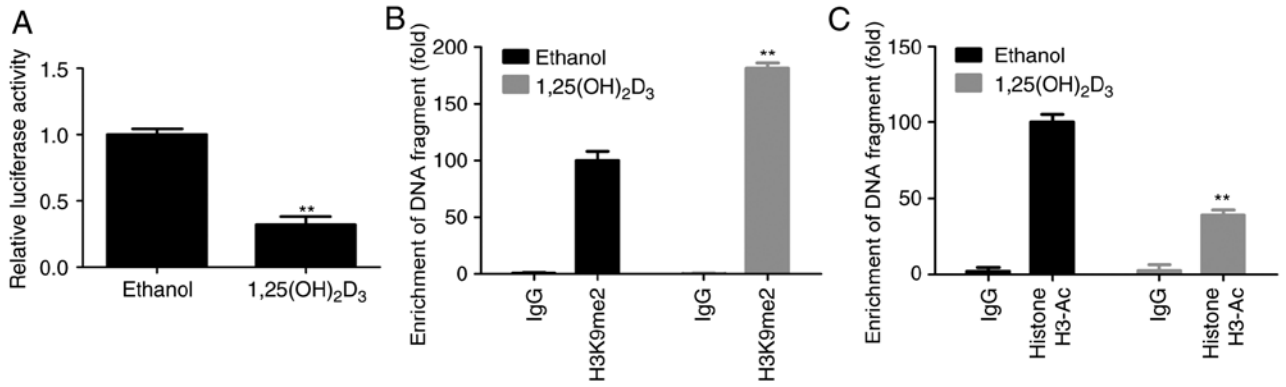


Figure 2. $1,25(OH)_2D_3$ decreased BMP2 expression through DNA methylation and histone modification. (A) The effect of $1,25(OH)_2D_3$ (10 nM) on the transcriptional activity of BMP2 was assessed by luciferase report assay with pSV40-BMP2-Luciferase plasmid transfection. Chromatin immunoprecipitation assays were used to detect the crosstalk of BMP2 promoter with (B) histone H3-Ac or (C) H3K9me2 (n=3). $^{**}P<0.01$ vs. control (ethanol) group. BMP2, bone morphogenetic protein 2; histone H3-Ac, acetyl-histone H3; H3K9me2, histone H3 dimethylated lysine 9.

differentiation repression (Fig. 4G and H). These findings demonstrated that $1,25(OH)_2D_3$ repressed the osteogenic differentiation of BMSCs through regulating the interaction between BMP and β-catenin signaling pathways.

## Discussion

The imbalance in bone formation and bone resorption induced by the inactivity of osteoblasts and the hyperactivation of osteoclasts is considered as the main reason of osteoporosis (21,25). Therefore, the presence of a sufficient number of osteoblasts is beneficial for osteoporosis prevention or even treatment (26). In the present study, the effects of BMPs and Wnt/β-catenin signaling on $1,25(OH)_2D_3$-mediated repression of the osteogenic differentiation of BMSCs was investigated. The study results demonstrated that $1,25(OH)_2D_3$ treatment induced a significant inhibition in the osteogenic differentiation of BMSCs, including reduced the expression levels of several ossification markers (27), including Runx2, Msx2, BMP2, OPN and OCN. Furthermore, $1,25(OH)_2D_3$ decreased ALP activity and the calcified area of BMSCs, and these effects were induced by downregulating BMP2 and activating β-catenin signaling.

It is well documented that Wnt/β-catenin and BMP signaling pathways are essential for BMSCs to differentiate into osteoblasts (17,28). To further comprehend the role of β-catenin and BMP signaling pathways in $1,25(OH)_2D_3$-induced osteogenic differentiation repression, the present study initially explored the effects of $1,25(OH)_2D_3$ on the expression of BMP2, which serves as an osteogenic activation factor through stimulating osteoblast differentiation and osteogenic nodule formation (28,29). The current study results indicated that $1,25(OH)_2D_3$ negatively regulated BMP2 expression, not only at the transcriptional level, but also at the translational level. The decrease in the expression levels of ossification markers (Runx2, Msx2, OPN, OCN), ALP activity and the calcified area of BMSCs induced by $1,25(OH)_2D_3$ treatment were rescued when BMP2 expression was upregulated, suggesting that BMP2 repression was strongly implicated in the $1,25(OH)_2D_3$-induced repression of the osteogenic differentiation of BMSCs.

Previous evidence demonstrated that the $1,25(OH)_2D_3$ role depends on its combination with its receptor VDR. Once $1,25(OH)_2D_3$ binds to VDR, this receptor will heterodimerize with the retinoid X receptor and translocate into the nucleus to combine with vitamin D3 responsive elements (VDREs) in the promoter regions of the target genes of VDR, leading to upregulation or downregulation of gene transcription (30). Tagami *et al* (31) revealed that certain corepressors were able to block the VDRE in VDR target genes and deacetylate histones in the absence of $1,25(OH)_2D_3$, suggesting that $1,25(OH)_2D_3$ can regulate target gene expression through VDREs. Notably, a study by Fu *et al* (11) demonstrated that inhibition of histone deacetylase using trichostatin A and DNA methyltransferase using 5-aza-29-deoxycytidine increased BMP2 expression. In addition, $1,25(OH)_2D_3$ treatment significantly increased the levels of H3K9me2 and reduced the acetylation of histone H3 at the same BMP2 promoter region in UMR-106 cells (11). Consistently, the present study also demonstrated that $1,25(OH)_2D_3$ treatment decreased the acetylation of histone H3 and increased the expression of its methylated modification H3K9me3, suggesting that $1,25(OH)_2D_3$ negatively regulates BMP2 expression at the epigenetic level.

The present study further investigated the effects of the $1,25(OH)_2D_3$/BMP2 axis on Wnt/β-catenin signaling activation. It was observed that $1,25(OH)_2D_3$ treatment evidently enhanced the nuclear accumulation of β-catenin protein, while BMP2 overexpression reversed this effect, suggesting that $1,25(OH)_2D_3$ activated β-catenin signaling through downregulating BMP2. However, the results of the present study were the opposite from those reported by Larriba *et al* (32), who indicated that $1,25(OH)_2D_3$ functioned as a multilevel repressor of Wnt/β-catenin signaling in cancer cells, particularly in colon cancer. This divergence may be caused by the different cell contents and different usage concentrations. In detail, both Palmer *et al* (33) and Larriba *et al* (34) used a $1,25(OH)_2D_3$ concentration of $10^{-7}$ M in colon cancer cells, while the present study applied a concentration of $10^{-8}$ M $1,25(OH)_2D_3$ in BMSCs. Furthermore, in the current study, it was observed that inhibition of β-catenin signaling with XAV-939 significantly weakened the effects of $1,25(OH)_2D_3$ on the inhibition of ALP activity and calcification formation,

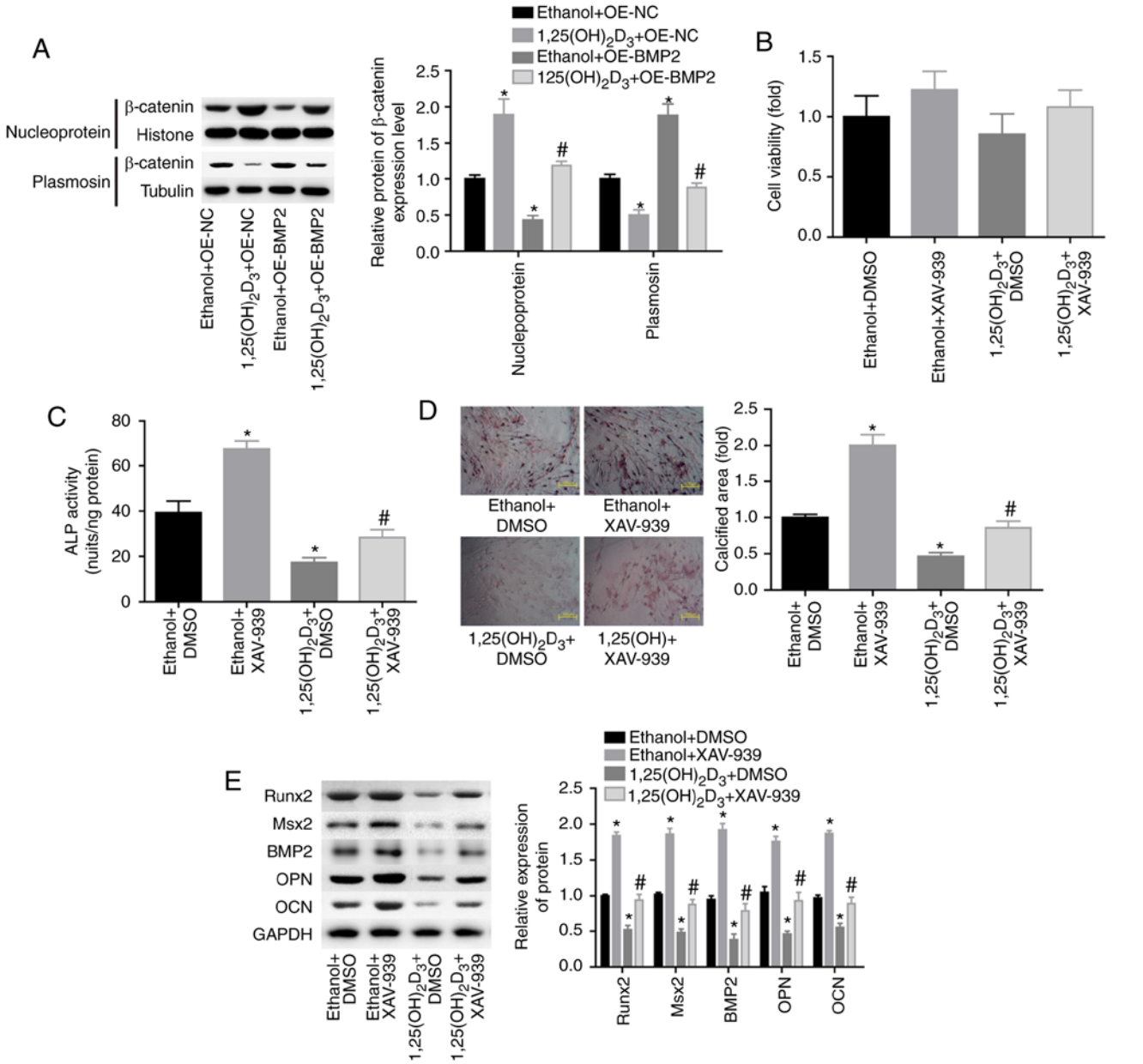


Figure 3. Inhibition of β-catenin signaling rescued the repression of the osteogenic differentiation of BMSCs induced by $1,25(OH)_2D_3$. (A) Western blotting of the expression of β-catenin in the nucleus and cytoplasm of BMSCs treated with ethanol plus OE-NC or OE-BMP2, or with $1,25(OH)_2D_3$ (10 nM) plus OE-NC or OE-BMP2. *P<0.05 vs. ethanol + OE-NC group, #P<0.05 vs. $1,25(OH)_2D_3$ + OE-NC group. (B) Cell viability determined by Cell Counting Kit-8 assay and (C) ALP activity in differently treated BMSCs (ethanol plus DMSO or XAV-939, and $1,25(OH)_2D_3$ plus DMSO or XAV-939). (D) Alizarin red-S was used to assess the calcified nodules of BMSCs. Magnification, x200. (E) Western blotting of the protein expression levels of BMP2, Runx2, Msx2, OPN and OCN after BMSCs were treated with ethanol plus DMSO or XAV-939, and with $1,25(OH)_2D_3$ plus DMSO or XAV-939. (n=3). C-E, *P<0.05 vs. ethanol + DMSO group; #P<0.05 vs. $1,25(OH)_2D_3$ + DMSO group. BMSCs, bone marrow-derived mesenchymal stem cells; BMP2, bone morphogenetic protein 2; ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; Msx2, Msh homeobox 2; OPN, osteopontin; OCN, osteocalcin; OE, overexpressing vector; NC, negative control.

indicating that $1,25(OH)_2D_3$ inhibited the differentiation of BMSCs to osteoblasts through activating β-catenin signaling pathway. Although a number of studies have demonstrated that enhanced Wnt/β-catenin signaling promotes bone formation (35), there are also researchers who reported the opposite effect, that is, activation of the Wnt/β-catenin pathway weakens osteogenic differentiation (36,37).

Increasing evidence has suggested that Wnt/β-catenin signaling closely interacts with the BMP signaling pathway. For instance, Haramis *et al* (24) demonstrated that inhibition of BMP2/4 signaling in the intestine increased polyp formation with β-catenin upregulation. Derfoul *et al* (38) further reported that BMP2 reversed Wnt3a-induced inhibition of OCN and OPN expression in C3H10T1/2 cells. In addition, BMP2 markedly reduced Wnt3a-induced β-catenin nuclear accumulation and BMSC proliferation through enhancing the binding of Smad1 and Dvl-1, which is required for β-catenin activation (39-41). As reported in the present study, repression of β-catenin signaling by XAV-939 treatment neutralized the $1,25(OH)_2D_3$-mediated reduction in BMP2 expression, suggesting that $1,25(OH)_2D_3$ negatively regulated BMP2 expression via activating β-catenin. Furthermore, BMP2 was

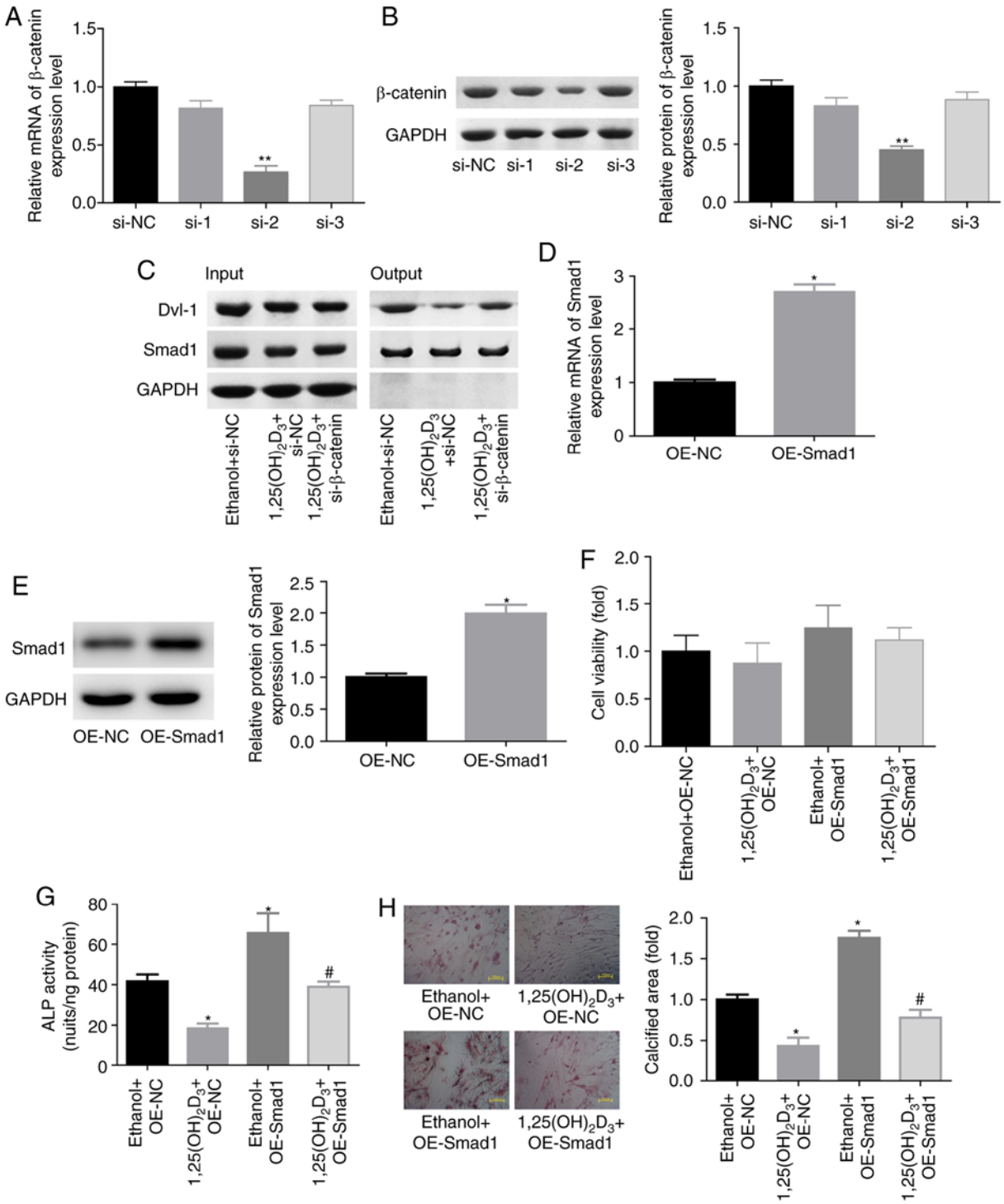


Figure 4. 1,25(OH)$_2$D$_3$/β-catenin repressed the osteogenic differentiation of BMSCs through impairing the interaction between Dvl-1 and Smad1 proteins. (A) mRNA and (B) protein levels of β-catenin in BMSCs transfected with siRNAs were assessed by RT-qPCR and western blotting, respectively. $^{**}$P<0.01 vs. si-NC group. (C) Immunoprecipitation assay, evaluating the crosstalk between Dvl-1 and Smad1 protein in BMSCs treated with 1,25(OH)$_2$D$_3$ (10 nM, 48 h) with or without si-β-catenin transfection. (D) mRNA and (E) protein expression levels of Smad1 in BSMCs stably infected with OE-Smad1 or OE-NC were determined by RT-qPCR and western blotting, respectively. $^{*}$P<0.05 vs. OE-NC group. (F) BMSC viability determined by Cell Counting Kit-8 assay, and (G) ALP activity in BMSCs. $^{*}$P<0.05 vs. ethanol + OE-NC group, $^{\#}$P<0.05 vs. 1,25(OH)$_2$D$_3$ + OE-NC group. (H) Alizarin red-S was used to assess the calcified nodules of BMSCs. Magnification, x200. $^{*}$P<0.05 vs. ethanol + OE-NC group, $^{\#}$P<0.05 vs. 1,25(OH)$_2$D$_3$ + OE-NC group. (n=3). BMSCs, bone marrow-derived mesenchymal stem cells; Dvl-1, disheveled-1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si-/siRNA, small interfering RNA; ALP, alkaline phosphatase; OE, overexpressing vector; NC, negative control.

able to enhance the interaction between Smad1 and Dvl-1, and promote the activation of β-catenin (39); it can thus be speculated that 1,25(OH)$_2$D$_3$ may modulate the interaction between Dvl-1 and Smad1 via β-catenin. In accordance with our predictions, it was observed that knockdown of β-catenin neutralized the 1,25(OH)$_2$D$_3$-mediated decrease in the interaction between Dvl-1 and Smad1 proteins. This result suggested that the weakness in the interaction between Dvl-1 and Smad1

proteins may be involved in $1,25(OH)_2D_3$-mediated repression of osteogenic differentiation, which was further confirmed by the ALP activity detection and Alizarin red-S staining assays in BMSCs with Smad1 overexpression.

In conclusion, the present study revealed that $1,25(OH)_2D_3$ inhibited the differentiation of BMSCs into osteoclast-like cells through inactivating BMPs and activating Wnt/β-catenin signaling. The study provides a deeper understanding on the mechanisms of vitamin D in the inhibition of osteogenic differentiation, as well as reconsiders the role of vitamin D in osteoporosis treatment.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

GC contributed to the design of the study and revised the manuscript. XH performed the experiments and data analysis, and wrote the manuscript. NZ and YW performed the experiments. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Experiments in this study involving animals were approved by the Review Committee for the Use of Human or Animal Subjects of Shanghai Jiao Tong University and were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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