eISSN 2005-5447
International Journal of Stem Cells Vol. 18, No. 2, 2025 https://doi.org/10.15283/ijsc24110

ORIGINAL ARTICLE

PML Regulated HIF1AN Ubiquitination and Activated PI3K/AKT Pathway to Promote Bone Marrow Mesenchymal Stem Cells Osteogenic Differentiation

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Osteoporosis (OP) is a metabolic disease caused by osteogenesis and bone resorption disorders. Promyelocytic leukemia protein (PML) was a vital regulator of cellular functions. However, the function of PML in OP remains unknown. Our research aimed to illustrate the molecular mechanism of PML in bone marrow mesenchymal stem cells (BMSCs) osteogenic differentiation. The BMSCs were identified by using flow cytometry analysis. The osteoblast differentiation ability of BMSCs was assessed through using alkaline phosphatase and Alizarin red S stainings. The relationship between hypoxia-inducible factor-1 α (HIF1 α) and superoxide dismutase 3 (SOD3) were confirmed by using chromatin immunoprecipitation and dual-luciferase reporter assays. The binding association between PML and hypoxia-inducible factor 1 α inhibitor (HIF1AN) proteins was verified by using co-immunoprecipitation assay and immunofluorescence staining. Western blot was used for protein detection. PML was up-regulated in osteogenic differentiation of BMSCs. Functionally, PML negatively regulated HIF1AN expression by enhancing HIF1AN ubiquitination degradation. PML knockdown or HIF1AN up-regulation suppressed the osteogenic differentiation of BMSCs. Furthermore, HIF1 α directly bound to the SOD3 promoter region. PML or SOD3 overexpression remarkably promoted the BMSCs osteoblast differentiation under osteogenic medium, which was reversed by LY294002. PML acts as a significant regulator in the BMSCs osteogenic differentiation by regulating the HIF1AN/HIF1 α/SOD3 axis and phosphatidylinositol 3 kinase/protein kinase B pathway.

Keywords: Bone marrow mesenchymal stem cells, Osteogenic differentiation, PML, HIF1AN, SOD3

Received: September 29, 2024, Revised: December 26, 2024,
Accepted: January 7, 2025, Published online: March 10, 2025
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Introduction

Osteoporosis (OP) is a multifactorial systemic skeletal disorder featuring reduced bone mineral thickness and osteopenia, leading to body pain and enhanced risk of fracture (1, 2). It is estimated that OP affects about 200 million people and causes relatively high disability and mortality (3). While the treatment options for OP are primarily confined to physical treatment, exercise regimens, and medication, these approaches often fall short of delivering optimal outcomes (4). Bone marrow mesenchymal stem cells (BMSCs) contain progenitor cells and skeletal stem cells, with self-renewal and multidirectional differentiation potential. Currently, BMSCs have become a promis-

ing cell type for clinical treatment including OP (5). The BMSCs osteogenic differentiation is affected by a variety of factors, including signaling pathways, cytokines, and epigenetic modifications. Recent studies have shown that targeting the miR-203-3p/RUNX2 axis can significantly enhance osteogenic differentiation and help alleviate the progression of OP (6). Therefore, exploring the mechanisms underlying the osteoblastic differentiation of BMSCs in OP, and finding the key regulator that enhances BMSC osteogenic differentiation is of great importance.

The ubiquitin-proteasome system (UPS) is the main approach for protein degradation in cells, which regulates cell cycle progression, apoptosis, inflammatory response and antigen presentation (7). The UPS is composed of ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme, ubiquitin ligase (E3), and 26S proteoplast. E3 ubiquitin ligases have been shown to have an impact on various diseases, including bladder cancer (8), gastric cancer (9) and OP (10). In addition, UPS was evidenced to be an important protein degradation channel in skeletogenous cell divergence and bone formation. Extremely regulation of ubiquitination can cause skeletogenous cell differentiation obstacles, interfere with bone formation, and eventually lead to OP (11). The promyelocytic leukemia protein (*PML*) gene is a vital antioncogene located downstream of the interferon-α pathway to regulate the development of tumors (12). Recent studies indicated that PML was involved in the regulation of bone diseases, as Sun et al. (13) reported, PML overexpression inhibited proliferation and promoted human mesenchymal stem cells (hMSCs). Nevertheless, few investigations have elucidated the regulatory role of PML in OP osteogenic differentiation.

Hypoxia-inducible factor-1α (HIF1α) is a prominent transcription regulator closely associated with cancer (14). Recently, the role of HIF1α in osteoblast differentiation has received increasing attention, indicating its potential influence on bone health and development (15). It was reported that transcriptional activation of factor forkhead box protein 3 by HIF1α promoted osteogenic differentiation of BMSCs (16). Hypoxia-inducible factor 1α inhibitor (HIF1AN) was considered to be a negative regulator of HIF1α to play vital roles in the tissues differentiation, including osteoblast differentiation. For instance, Yin et al. (17) revealed that miR-135-5p promoted MC3T3-E1 cells osteoblast differentiation through regulating HIF1AN. From the prediction of UbiBrowser (http://ubibrowser.ncpsb.org.cn), there are binding sites between PML and HIF1AN. However, whether PML acts as a major regulator of HIF1AN in BMSCs osteoblast differentiation remains unclear.

Superoxide dismutase 3 (SOD3) is a secreted copper-containing antioxidant enzyme that is associated with several oxidative stress-dependent cardiovascular diseases (18). A growing number of reports have demonstrated that SOD3 is a vital regulator in the osteogenesis of BMSCs. Notably, research conducted by Wang et al. (19) has shown that lncRNA MEG3 enhanced BMSCs osteogenic differentiation through modulating the miR-21-5p/SOD3 axis. Xu et al. (20) also found that SOD3 could regulate FLT1 to affect bone metabolism by facilitating osteogenesis via the phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase pathways. In this research, by using the JASPAR database (http://jaspar.genereg.net/), it was anticipated that HIF1α had potential binding sites to SOD3. Nevertheless, the interaction between HIF1α and SOD3 in regulating BMSC osteogenic differentiation is still unknown and deserves further investigation.

Based on these findings, we speculated that PML could promote HIF1AN ubiquitination degradation to up-regulate SOD3, thereby regulating the PI3K/AKT signaling pathway and facilitating the BMSC osteogenic differentiation. Our resulting findings might provide new ideas for the clinical therapies of OP.

Materials and Methods

BMSCs characterization

BMSCs were provided by American Type Culture Collection. Cells were cultivated at 37℃ under 5% CO_2 and 90% humidity. After 48 hours incubation, the non-adherent cells were washed and treated with fresh culture medium. The medium was changed every 3 days. When the fusion rate of cells reached 90%, cells were trypsinized, centrifuged, and hatched with the CD34, CD44, CD45, and CD90 antibodies for flow cytometry analysis.

Culture of BMSCs

BMSCs were planted into 6-well plates (Corning) and cultivated in α-MEM medium supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin in the incubator at 37℃ with a 5% CO_2. When cells grow to 80% confluence, they were cultivated in the osteogenic medium (OM) (MDADGM; iXCells) which encompassed 1% FBS, 50 mg/mL ascorbic acid, 10 mM b-glycerophosphorus sodium, and 0.1 mg/mL dexamethasone for further incubation for 2 weeks. Afterward, cells were collected on day 7 and 14 for further analysis. After transfection, BMSCs were treated with PI3K inhibitor (50 μM of LY294002, Cat. No. HY-10108; MedChemExpress) for 24 hours.

Cell transfection

Overexpression lentivirus including pcDNA-HIF1AN (oe-HIF1AN), pcDNA-PML (oe-PML), and pcDNA-SOD3 (oe-SOD3), as well as short hairpin RNA (sh)-PML and corresponding negative control (NC) were obtained from GenePharma Inc. BMSCs were cultivated in α-MEM medium until they reached 80% confluence. Cells transfection was performed using Lipofectamine 3000 (L3000001; Thermo Fisher Scientific) for 48 hours on the basis of instructions.

Flow cytometry

BMSCs were digested, washedand resuspended in 2% FBS. After that, the fluorescent antibodies, including anti-CD34-APC (119309; BioLegend), anti-CD45-FITC (157213; BioLegend), anti-CD44-PE (103023; BioLegend), and anti-CD90-FITC (140311; BioLegend) were hatched with BMSCs for 40 minutes. After that, cells were determined by Flow cytometer (Beckman Coulter).

Alkaline phosphatase staining

After the indicated treatments, measurement of alkaline phosphatase (ALP) positive BMSCs was performed with an Alkaline Phophatase Stain Kit (G1480; Solarbio) based on the manufacturer's instructions. BMSCs were collected, washed, and fixed in 4% paraformaldehyde for 15 minutes. Subsequently, they were treated with ALP incubation solution for 30 minutes. Finally, cells were photographed by a light microscope (CX23; Olympus).

Alizarin red S staining

BMSCs were inoculated in 6-well plates (4×10^5 cells/well). After induction, the mineralization of BMSCs was detected by Alizarin red S (ARS) staining kit (C0148S; Beyotime Biotechnology). After washed with PBS, BMSCs were fixed in 4% paraformaldehyde for 10 minutes, and stained with 1% Alizarin red solution for 5～10 minutes. Afterwards, microscope was employed to obtain images.

Co-immunoprecipitation assay

Co-immunoprecipitation (Co-IP) assay was conducted according to the Protein A/G Magnetic Co-IP/IP Kit (K1309; APExBIO). Cells were lysed and centrifuged to obtain cell lysates. Then cell lysates were cultivated with protein A beads for 3 hours at 4℃. Next, they were centrifuged to remove the Sepharose beads, and the IgG (ab172730; Abcam) or HIF1AN antibody (ab237544; Abcam) were cultivated in the prewashed lysate overnight at 4℃. After that, beads were incubated with sample for another 4 hours to catch the antibody-antigen complex. The mixture was centrifuged and washed with Tris HCl buffer salt solution and Tween (TBST) to throw away the supernatant, and the bound proteins were determined using Western blot.

Immunofluorescence staining

BMSCs were fixed in 4% paraformaldehyde for 20 minutes, permeabilized with 0.2% Triton X-100 for 15 minutes, and then closed with 5% bovine serum albumin for 30 minutes. After that, they were incubated in anti-PML antibody (1 : 200, ab179466; Abcam) and anti-HIF1AN antibody (1 : 200, ab92498; Abcam) at 4℃ all night. After washed with PBS, BMSCs were hatched with fluorescent secondary antibody (1 : 500, ab150077; Abcam) at 37℃ for 30 minutes. Finally, nuclei were counterstained with DAPI (4083; Cell Signaling Technology) for 10 minutes and images were obtained by a confocal microscope (Fluoview 500 IX71; Olympus).

Chromatin immunoprecipitation assay

BeyoChIP ChIP Assay Kit (P2080S; Beyotime Biotechnology) was applied for performing chromatin immunoprecipitation (ChIP) assays. BMSCs were treated with 1% formaldehyde for 15 minutes and washed with PBS buffer. Then cells were centrifuged and lysed with SDS lysis buffer, followed by sonication to fragment chromatin. Subsequently, samples were cultivated in anti-HIF1α antibody (SAB2702132; Sigma-Aldrich) or normal rabbit IgG antibody (1 : 100). The purified DNA was identified by reverse transcription polymerase chain reaction analysis.

Dual-luciferase reporter assay

The potential binding site of HIF1α with the SOD3 promoter region was predicted by using the JASPAR database. The wild-type SOD3 (SOD3-WT) and corresponding mutant SOD3 (SOD3-MUT) promotor fragments were constructed and cloned into luciferase vector pmirGLO (Promega) to obtain SOD3-MUT and SOD3-WT reporter vectors. After that, the reporter vectors were co-transfected into 293T cells with oe-HIF1α (or oe-NC) using Lipofectamine 3000 (Invitrogen) for 48 hours. Dual-Luciferase Reporter Assay System (E1910; Promega) was applied to evaluate the luciferase activity.

Western blot assay

BMSCs were lysed using RIPA Lysis Buffer (P0013; Beyotime). After the protein concentration was assessed by BCA Protein Quantification Kit (E112-02; Vazyme), lysates were fractionated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore), after that, the membranes were closed with 5% skimmed milk, washed with TBST, and cultivated overnight at 4℃ with in-

dicated antibodies including: rabbit anti-PML (1 : 500, DF6318; Affinity Biosciences), anti-HIF1AN (1 : 1,000, ab307829; Abcam), anti-HIF1α (1 : 1,000, ab179483; Abcam), anti-SOD3 (1 : 2,000, 14316-1-AP; Proteintech), anti-RUNX2 (1 : 1,000, ab236639; Abcam), anti-OCN (1 : 500, DF12303; Affinity Biosciences), anti-ALP (1 : 2,000, DF6225; Affinity Biosciences), anti-p-PI3K (1 : 1,000, AF3242; Affinity Biosciences), anti-PI3K (1 : 1,000, #4249; Cell Signaling Technology), anti-p-AKT (1 : 3,000, 28731-1-AP; Proteintech), anti-AKT (1 : 1,000, ab38449; Abcam) and GAPDH (1 : 1,000, #2118, Cell Signaling Technology). After that, the membranes were cultivated with corresponding secondary antibody (1 : 5,000, S0001, Affinity Biosciences). Immunoblots were detected by ECL reagent (WP20005; Thermo Fisher Scientific). The relative protein expression was assessed using Image J software.

Statistical analysis

Data were given as means±SD. Comparisons between two groups were analyzed by Student's t-test, and differences among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. All experiments were conducted in triplicate. All statistical analyses were performed by using GraphPad Prism 7 software. A p-value <0.05 was deemed statistically obvious.

Results

PML was up-regulated in BMSCs osteogenic differentiation

The purity of BMSCs was detected by flow cytometry. As presented in Fig. 1A, 99.93% and 99.60% of the cells were positive for CD44 and CD90, while only 0.16% and

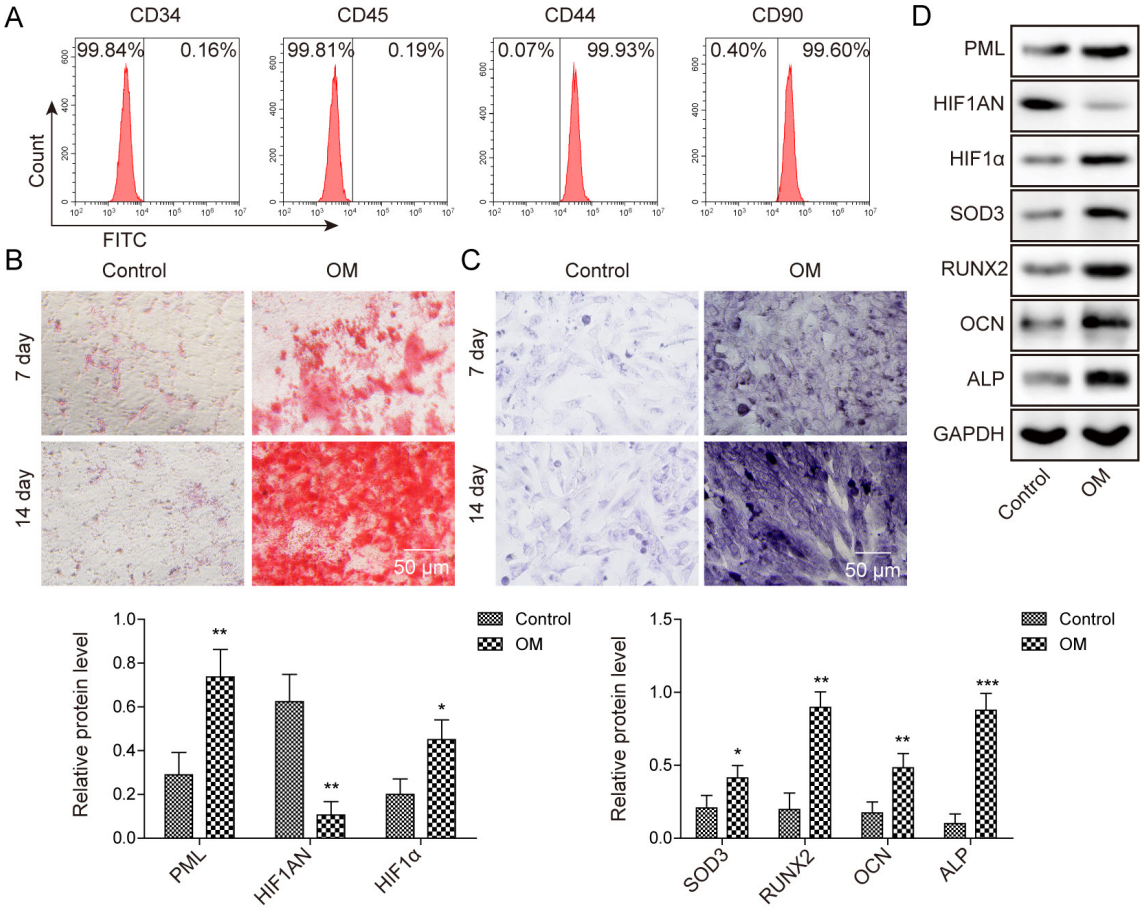

Fig. 1. Promyelocytic leukemia protein (PML) was up-regulated in osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). (A) Flow cytometry was used to determine BMSCs surface markers (CD44 and CD90) and hematopoietic cells surface markers (CD34 and CD45). BMSCs were cultivated in an osteogenic medium (OM) for 7 days or 14 days, and cells were divided into Control and OM groups. (B, C) The osteogenic differentiation potential of BMSCs was measured by Alizarin red S (ARS) and alkaline phosphatase (ALP) stainings (scale bar=50 μm), ARS staining was used to check the calcium deposition in BMSCs, and the ALP staining was employed to detect the number of ALP-positive BMSCs. (D) The protein expressions of PML, HIF1α, HIF1AN, SOD3, RUNX2, OCN, and ALP were determined by using Western blot. Data were exhibited as mean±SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

0.19% BMSCs positively expressed CD34 and CD45, demonstrating that the BMSCs had high purity. Due to the ability of ARS and ALP stainings to detect calcium deposition in BMSCs and the number of ALP positive BMSCs, respectively, ARS and ALP stainings were used to detect osteogenic differentiation of BMSCs. it was found that the levels of ALP activity and calcification were enhanced in BMSCs after osteogenic differentiation induction for two weeks (Fig. 1B, 1C). Furthermore, the levels of PML, HIF1α, SOD3 and osteogenic differentiation markers (RUNX2, OCN and ALP) were notably increased after BMSCs incubated with OM, while the HIF1AN expression was downregulated (Fig. 1D). Collectively, our findings revealed that the dysregulation of PML, HIF1AN, HIF1α, and SOD3 might be associated with BMSCs osteogenic differentiation.

PML knockdown inhibited the BMSCs osteogenic differentiation

To further explore the regulatory role of PML in BMSCs

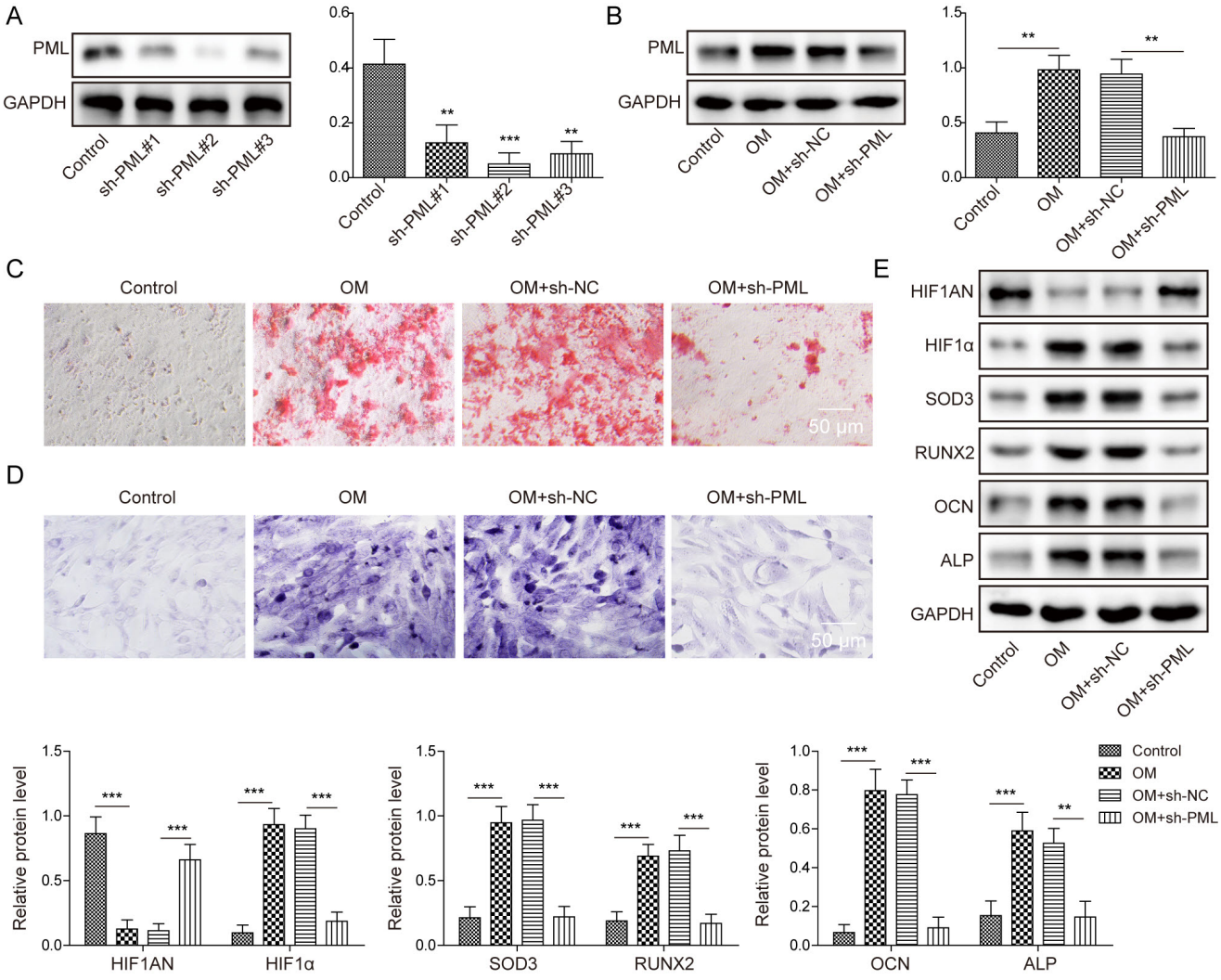

Fig. 2. Promyelocytic leukemia protein (PML) knockdown inhibited the osteoblast differentiation of bone marrow mesenchymal stem cells (BMSCs). Short hairpin RNA (sh)-PML was transfected into BMSCs for PML knockdown, and sh-NC served as the negative control. (A) The transfection efficiency was assessed by Western blot. Cells incubated with osteogenic medium (OM) and divided into four groups: Control, OM, OM+sh-NC, and OM+sh-PML. (B) The PML expression was determined using Western blot. (C, D) The osteogenic differentiation potential of BMSCs was measured by Alizarin red S (ARS) and alkaline phosphatase (ALP) stainings (scale bar=50 μm), ARS staining was used to check the calcium deposition in BMSCs, and the ALP staining was employed to detect the number of ALP-positive BMSCs. (E) Western blot was used to detect the HIF1AN, HIF1α, SOD3, RUNX2, OCN, and ALP expressions. Data were exhibited as mean±SD from three independent experiments. **p<0.01 and ***p<0.001.

osteogenic differentiation, sh-PML was transfected into BMSCs for PML knockdown. The results showed that sh-PML#2 had the best efficiency in reducing PML levels in BMSCs and was selected for subsequent experiments (Fig. 2A). As presented in Fig. 2B, after BMSCs were cultured with OM to induce the osteogenic differentiation in BMSCs, it was found that the level of PML was up-regulated, but was reversed by PML knockdown. Moreover, knockdown of PML inhibited the generation of calcium nodules in BMSCs under OM (Fig. 2C). Further experiment demonstrated that PML down-regulation remarkably reduced the ALP positive rate in OM-induced BMSCs (Fig. 2D). Besides, PML knockdown could increase HIF1AN expression, while reduce HIF1α, SOD3, RUNX2, OCN, and ALP levels in OM-treated BMSCs (Fig. 2E). All these results suggested that the down-regulation of PML suppressed the osteogenic differentiation of BMSCs.

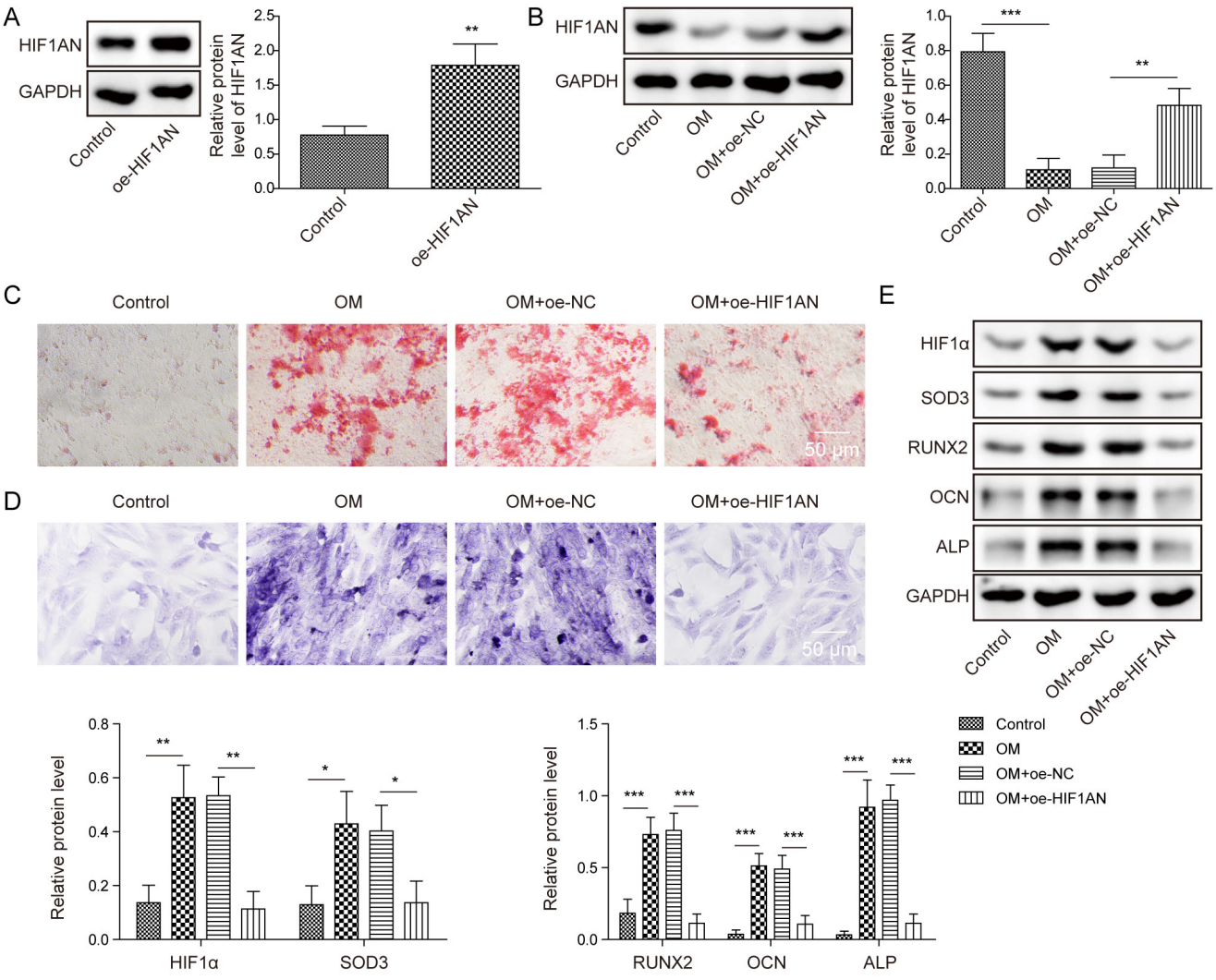

Fig. 3. HIF1AN overexpression suppressed the osteoblast differentiation of bone marrow mesenchymal stem cells (BMSCs). pcDNA (oe)-HIF1AN was transfected into BMSCs for HIF1AN overexpression, and oe-NC served as the negative control. Cells were divided into the Control, osteogenic medium (OM), OM+oe-NC, and OM-oe-HIF1AN groups. (A) Western blot was conducted to analyze the expression of HIF1AN. The overexpressed BMSCs were incubated with OM, and the sh-NC as the negative control. (B) HIF1AN expression was evaluated by using western blot. (C, D) The osteogenic differentiation potential of BMSCs was measured by Alizarin red S (ARS) and alkaline phosphatase (ALP) stainings (scale bar=50 μm), ARS staining was used to check the calcium deposition in BMSCs, and the ALP staining was employed to detect the number of ALP-positive BMSCs. (E) Protein expressions of HIF1α, SOD3, and osteogenesis-related markers (RUNX2, OCN, and ALP) were assessed by western blot. Data were exhibited as mean±SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

HIF1AN overexpression suppressed the osteoblast differentiation of BMSCs

Our UbiBrowser prediction showed that PML could bind with HIF1AN, and HIF1AN was evidenced to be involved in the osteoblast differentiation of BMSCs. Next, we further illustrated the role of HIF1AN in osteoblast differentiation of BMSCs. First, the HIF1AN level was elevated after oe-HIF1AN transfection (Fig. 3A). HIF1AN was inhibited in OM-induced BMSCs, while HIF1AN overexpression relieved the inhibitory effect of OM on the HIF1AN level (Fig. 3B). Further ARS and ALP staining assays uncovered that up-regulation of HIF1AN reduced the osteogenic differentiation of BMSCs under OM incubation (Fig. 3C, 3D). In addition, the up-regulation of HIF1AN inhibited the levels of HIF1α, SOD3, and osteogenic differentiation marker proteins in BMSCs under OM condition (Fig. 3E). In summary, these results revealed that HIF1AN up-regulation could block the osteoblast differentiation of BMSCs.

PML promoted HIF1AN ubiquitination degradation and facilitated BMSCs osteoblast differentiation

Next, we lucubrated the molecular mechanism of HIF1AN in osteoblast differentiation of BMSCs, as revealed by the Co-IP assay, PML could bind to HIF1AN in BMSCs (Fig. 4A). Moreover, PML overexpression increased the ubiquitination of HIF1AN and inhibited HIF1AN expression, and the proteasome inhibitor MG-132 treatment was further enhanced by HIF1AN ubiquitination level, which certificated that PML accelerated HIF1AN degradation by promoting HIF1AN ubiquitination (Fig. 4B). We also found that the up-regulation of PML remarkably shortened the half-life of the HIF1AN protein (Fig. 4C). Results presented in Fig. 4D indicated that the up-regulation of PML significantly enhanced the endogenous degradation of HIF1AN, while this PML-mediated degradation of HIF1AN can be effectively reversed by MG-132. Immunofluorescence staining further confirmed that PML and HIF1AN co-localized in the cytoplasm and nucleus of BMSCs (Fig. 4E). Our observations indicated that the overexpression of PML not only increased PML level in OM-induced BMSCs, but also further suppressed the levels of HIF1AN. oe-HIF1AN had no effect on PML level in BMSCs but reversed the inhibitory effect of PML upregulation on HIF1AN expression (Fig. 4F). Overexpression of PML further enhanced the BMSCs osteoblast differentiation under OM, but this effect was alleviated after the up-regulation of HIF1AN (Fig. 4G, 4H). Western blot further suggested that the protein levels of HIF1α, SOD3 and osteogenic differentiation markers were elevated by oe-PML, while this trend was reversed by HIF1AN overexpression (Fig. 4I). Based on these findings, we found that PML facilitated BMSCs osteoblast differentiation by regulating HIF1AN ubiquitination degradation.

PML promoted HIF1α to activate SOD3 and PI3K/AKT pathway to enhance BMSCs osteoblast differentiation

HIF1AN was evidenced to be a negative regulator of HIF1α in osteoblast differentiation of BMSCs, then we explored the regulatory mechanism of HIF1α in BMSCs. The potential binding site of HIF1α on the SOD3 promoter region was predicted by JASPAR, and the result was presented in Fig. 5A. As displayed in Fig. 5B, the up-regulation of HIF1α significantly enhanced the luciferase activity of the SOD3-WT plasmid, but the luciferase intensity of the SOD3-MUT plasmid revealed no notable changes, which indicated that HIF1α directly bound to SOD3 in 293T cell. Further ChIP assay revealed that HIF1α had a strong affinity in the promoter regions of SOD3 (Fig. 5C). We further studied whether the regulation of SOD3 by PML on osteoblast differentiation involves the PI3K/AKT signaling pathway, the PI3K inhibitor LY294002 was applied to evaluate the role of PI3K/AKT pathway in BMSCs osteoblast differentiation. After transfected with oe-SOD3, the level of SOD3 in BMSCs incubated with OM was further upregulated but had no effect on PML expression. However, when PML was knocked down, the expression of SOD3 and PML in SOD3 overexpression treated cells were significantly decreased, while these expressions did not affected by LY294002 (Fig. 5D). ARS and ALP staining further showed that SOD3 over-expression remarkably promoted the BMSCs osteoblast differentiation under OM, which was reversed by sh-PML. However, after further LY294002 treatment, the above results were inhibited (Fig. 5E, 5F). In addition, the up-regulation of SOD3 increased RUNX2, OCN, ALP, p-PI3K, and p-AKT levels in BMSCs incubated with OM, which were abolished by PML knockdown. Meanwhile, all these results were further reduced in LY294002-treated BMSCs under OM conditions. Besides, in BMSCs incubated with OM, oe-SOD3 or LY294002 treatment did not affect the levels of HIF1AN and HIF1α, and PML downregulation promoted HIF1AN expression, but HIF1α was inhibited (Fig. 5G). Besides, the levels of PML and SOD3 in BMSCs incubated with OM were enhanced, PML overexpression could upregulate PML and SOD3 levels in OM-treated BMSCs, while SOD3 overexpression only increased the SOD3 expression, and had no effect on PML level. However, regardless of the upregulation of PML or SOD3 in OM-induced BMSCs,

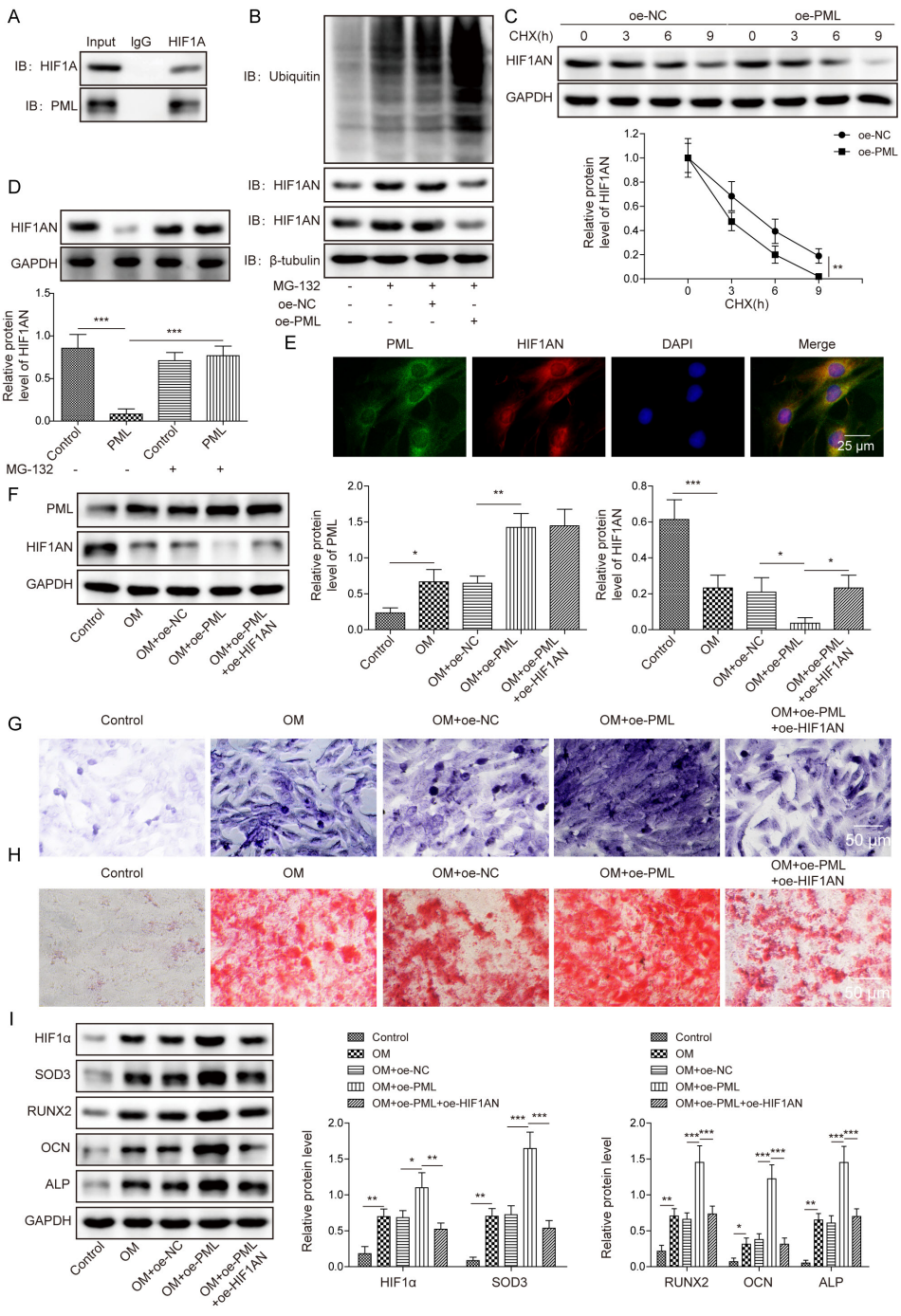

Fig. 4. Promyelocytic leukemia protein (PML) promoted HIF1AN ubiquitination degradation and facilitated bone marrow mesenchymal stem cells (BMSCs) osteoblast differentiation. (A) The interaction between PML and HIF1AN was verified by co-immunoprecipitation (Co-IP) assay. (B) PML overexpression BMSCs were treated with a proteasome inhibitor (MG-132), and Co-IP was employed to detect HIF1AN ubiquitination. PML overexpression BMSCs treated with cycloheximide (CHX) or MG-132. (C) Analysis of HIF1AN the half-life and degradation rate on PML up-regulation in CHX treated BMSCs. (D) Western blot is used to assess the level of HIF1AN protein. (E) The location of PML and HIF1AN was evaluated by immunofluorescence staining (scale bar=25 μm). (F) BMSCs were transfected with pcDNA (oe)-PML and oe-HIF1AN, and PML and HIF1AN expressions were measured by Western blot. (G, H) Osteogenic differentiation of BMSCs was detected by Alizarin red S (ARS) and alkaline phosphatase (ALP) staining (scale bar=50 μm), ARS staining was used to check the calcium deposition in BMSCs, and the ALP staining was employed to detect the number of ALP-positive BMSCs. (I) The expressions of HIF1α, SOD3, RUNX2, OCN, and ALP were analyzed by using Western blot. Data were exhibited as mean±SD from three independent experiments. OM: osteogenic medium, NC: negative control. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

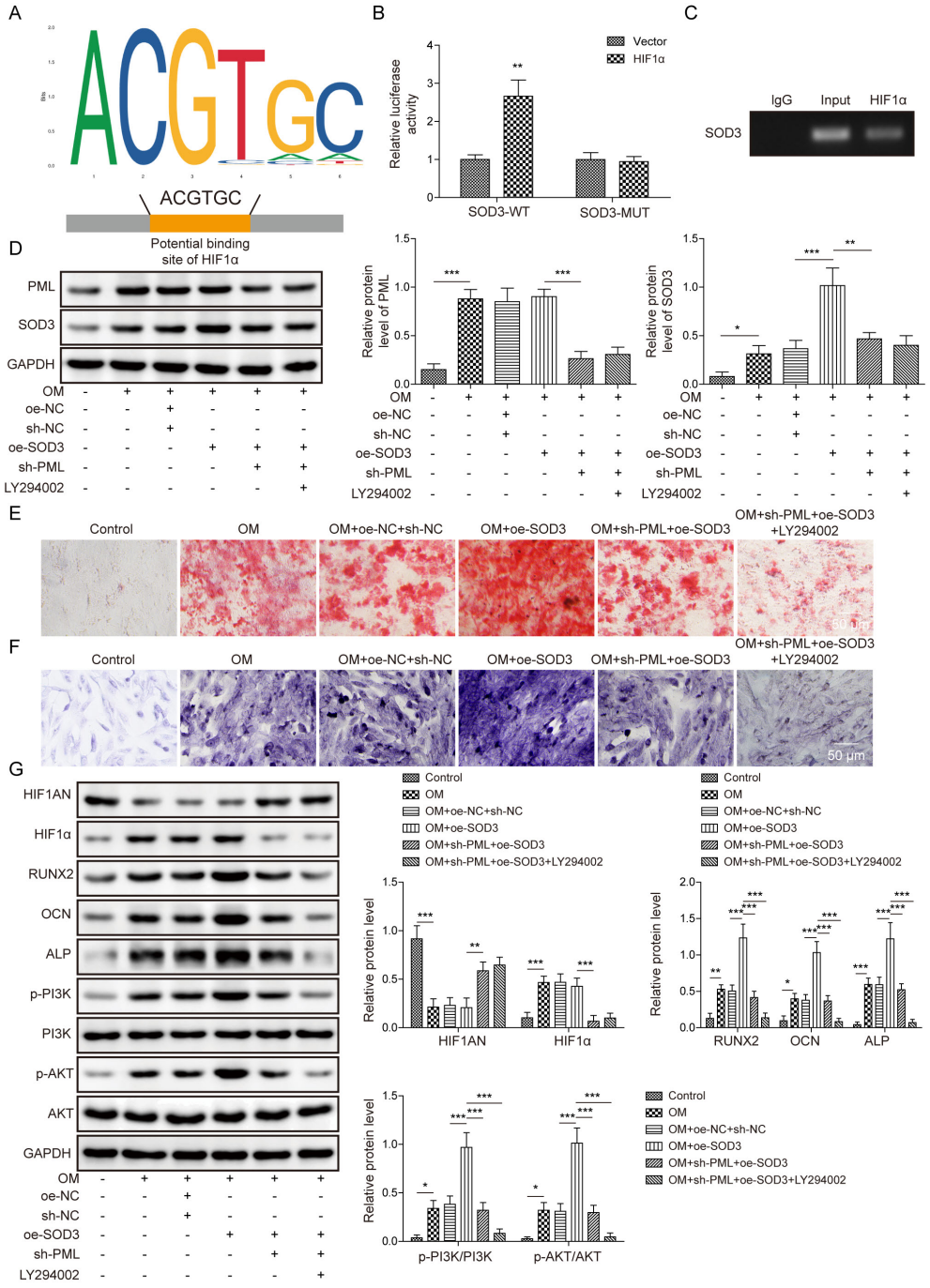

Fig. 5. Promyelocytic leukemia protein (PML) promoted HIF1α to activate SOD3 and PI3K/AKT pathway to enhance bone marrow mesenchymal stem cells (BMSCs) osteoblast differentiation. BMSCs were transfected with pcDNA (oe)-SOD3 and/or short hairpin RNA (sh)-PML, and then induced with osteogenic medium (OM). (A) The potential HIF1α binding site on the SOD3 promoter region was predicted JASPAR database. (B, C) The relationship between HIF1α and SOD3 was confirmed by dual luciferase reporter and chromatin immunoprecipitation assays. BMSCs were transfected with oe-SOD3 and/or sh-PML, and then induced with LY294002 under OM. (D) The expressions of SOD3 and PML were measured by Western blot. (E, F) The osteogenic differentiation potential of BMSCs was measured by Alizarin red S (ARS) and alkaline phosphatase (ALP) stainings (scale bar=50 μm), ARS staining was used to check the calcium deposition in BMSCs, and the ALP staining was employed to detect the number of ALP-positive BMSCs. (G) HIF1AN, HIF1α, RUNX2, OCN, ALP, p-PI3K/PI3K, and p-AKT/AKT expressions were determined by Western blot. Data were exhibited as mean±SD from three independent experiments. WT: wild-type, MUT: mutant, NC: negative control. *p<0.05, **p<0.01, and ***p<0.001.

LY294002 has no effect on their expressions (Supplementary Fig. S1A). ARS and ALP staining further showed that PML or SOD3 overexpression remarkably promoted the BMSCs osteoblast differentiation under OM, which was reversed by LY294002 (Supplementary Fig. S1B, S1C). In addition, the up-regulation of PML or SOD3 increased RUNX2, OCN, ALP, p-PI3K, and p-AKT levels in BMSCs which were incubated with OM. However, these results were abolished by LY294002 (Supplementary Fig. S1D). The findings further confirmed the effect of PML or SOD3 on osteogenic differentiation was related to regulating the PI3K/AKT pathways. In conclusion, our results demonstrated that PML up-regulated HIF1α to activate SOD3 and PI3K/AKT pathways during the osteoblast differentiation of BMSCs.

Discussion

OP is a systemic disease featured with declined bone mass, bone mineral density, and bone microarchitecture (21). Increasing evidence demonstrated that BMSC depletion and defective osteoblast differentiation are vital factors resulting in OP (22). However, the precise mechanisms underlying the osteogenic differentiation of BMSCs in OP remain largely elusive and warrant further investigation. In this study, we explored the mechanism of BMSCs osteoblast differentiation *in vitro*. Our report demonstrated for the first time that PML was up-regulated in BMSCs osteoblast differentiation and activated HIF1α by regulating HIF1AN ubiquitination degradation, and then elevated SOD3 level and promoted PI3K/AKT pathway to accelerate osteogenic differentiation of BMSCs.

PML, one of the TRIM family members, is required for UPS to degrade proteins (23). PML enhanced the hMSCs osteogenic differentiation. Under osteogenic or non-osteogenic differentiation conditions, PML-overexpressing hMSCs showed an obvious enhance in mineralized matrix production and ALP activity on day 7 (13). In this study, our findings revealed that PML played significant roles in regulating the BMSCs osteoblast differentiation. The knockdown of PML could block the BMSCs osteoblast differentiation, which was revealed by reduced ALP-positive BMSCs and calcium nodules, as well as down-regulated RUNX2, OCN, and ALP expressions in BMSCs.

Multiple investigations have verified that HIF1AN is involved in osteoblast differentiation. For example, Zhou et al. (24) confirmed that up-regulation of miRNA-1-3p promoted MC3T3-E1 cells osteoblast differentiation via targeting HIF1AN. For the first time to our knowledge, we found the functions of HIFAN in the osteoblast differentiation of BMSCs. Our data demonstrated that HIF1AN was down-regulated in OM-induced BMSCs. Besides, up-regulation of HIF1AN inhibited the osteoblast differentiation of BMSCs. Our data further illustrated that PML overexpression promoted the ubiquitination degradation of HIF1AN in BMSCs induced with OM, while up-regulation of HIF1AN reversed the effects of PML overexpression on BMSCs osteoblast differentiation. It is worth noting that HIF1AN was reported to be an inhibitor of HIF1α stabilization and transcriptional activity (25). Increasing evidence suggests that increasing HIF1α could promote the osteoblast differentiation of BMSCs (26). A report from Li et al. (27) indicated that roxadustat promoted osteoblast differentiation by stabilizing HIF1α and activating the Wnt/β-catenin pathway. In our investigation, PML degraded HIF1AN through ubiquitination and activated HIF1α to promote BMSCs osteoblast differentiation.

According to the above research, we also explored the downstream mechanism of HIF1α in the BMSCs osteoblast differentiation. We successfully predicted the binding site of HIF1α within the promoter region of SOD3 and subsequently validated the relationship between HIF1α and SOD3. As previous research suggested that, SOD3 participated in osteogenic differentiation, hMSCs specifically secreted SOD3 to promote cell survival after oxidative stress by inhibiting cellular toxic reactive oxygen species (28). Besides, lncRNA MEG3 promoted the hBMSCs osteogenesis and bone regeneration and increased the expression of SOD3 and COL2A1 through miR-21-5p/SOD3 axis (19). In OP, the PI3K/AKT signaling pathway was evidenced to be linked with the activation of osteoclasts and the differentiation of osteoblasts (29). Previous research has reported that SOD3 was involved in the regulation of the PI3K/AKT pathway (20, 30), which was consistent with our observations. In our investigation, we observed that SOD3 overexpression significantly promoted the osteoblast differentiation of BMSCs, which was reversed by sh-PML. Moreover, SOD3 was up-regulated in OM-induced BMSCs, and HIF1α promoted the PI3K/AKT pathway by transcriptionally activating SOD3. Inhibiting the PI3K/AKT signaling pathway could directly interfere with the effects of PML and SOD3 overexpressions in the BMSCs osteogenic differentiation.

In summary, our research revealed a previously unknown function of PML in regulating osteoblast differentiation of BMSCs. Our findings explained the regulatory mechanism of PML and HIF1α/SOD3 axis in the differentiation of BMSCs, which may provide novel target genes for the treatment of OP. However, the exact mechanism of PML in promoting osteogenesis needs further

elucidation. Currently, our research is based solely on cell experiments, and we have yet to validate our results in animal models. In future studies, we plan to conduct animal experiments to confirm and expand upon our observations, providing a more comprehensive understanding of PML's role in bone formation.

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Funding

This work was supported by Scientific Research Project of Education Department of Hunan Province (21C0259).

Potential Conflict of Interest

There is no potential conflict of interest to declare.

Authors' Contribution

Conceptualization: XPZ, YL. Data curation: QWL. Formal analysis: XPZ. Funding acquisition: XPZ, YL. Investigation: XPZ. Methodology: XPZ. Project administration: XPZ, YL. Resources: ZZS. Software: ZZS. Supervision: XPZ, YL. Validation: XPZ. Visualization: XPZ. Writing – original draft: XPZ, YL. Writing – review and editing: XPZ, YL.

Supplementary Materials

Supplementary data including one figure can be found with this article online at https://doi.org/10.15283/ijsc24110

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