Cellular Signalling 62 (2019) 109343

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

PLEXIN-B2 promotes the osteogenic differentiation of human bone marrow mesenchymal stem cells via activation of the RhoA signaling pathway

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A B S T R A C T

Plexin-B2 (PLXNB2), a transmembrane protein is found in various tissues. Recent studies have indicated the presence of PLXNB2 in large quantity in the growth plates of Sprague-Dawley rats and are believed to be potentially involved in their skeletal development. This study endeavored to analyze the effect of PLXNB2 on the osteogenic differentiation of BMSCs by using gene overexpression and knockdown assays. The results of our study revealed that PLXNB2 was upregulated during BMSCs differentiation into an osteoblastic lineage. By determining the expression levels of specific markers and mineral deposition, the study established that PLXNB2 promotes the osteogenic differentiation of human BMSCs through the activation of the RhoA signaling pathway. In conclusion, the study identified PLXNB2 as a novel regulator that enhanced the osteogenic differentiation of human BMSCs. The enhancing effect of PLXNB2 on osteogenesis of human BMSCs was mediated through activation of RhoA signaling. The results of our study imply that pharmacological targeting of PLXNB2 may initiate a possible improvement in bone formation.

1. Introduction

The subtle balance between bone resorption and formation, which is mediated by osteoblasts and osteoclasts, plays a crucial role in bone homeostasis [1–3]. Earlier studies have gone on to prove that the disrupted bone homeostasis will lead not only to the development of most skeletal diseases, rather also would severely impair the process of fracture healing [4,5]. Bone marrow-derived Mesenchymal Stem Cells (BMSCs) are pluripotent cells derived from the bone marrow, which can be segregated into a variety of tissues, including bone, cartilage, fat, and tendons. By virtue of their strong multipotent differentiation properties, BMSCs are one of the most extensively used elements in cell-based tissue engineering, especially in bone tissue regeneration [6–8]. Researches on the regulation of BMSCs osteogenesis has now become a hot topic, which may help in the understanding and treatment of various skeletal disorders like osteoporosis and fractures [7,9–11].

Plexin family (Plexin A-D) is a group of transmembrane proteins which has a significant role in axon guidance [12,13]. Found to be widely expressed in vertebrates, Plexins transduce the signals mediated by the axon-guiding molecules semaphorins [14–18]. Plexin-B2 (PLXNB2) is a member of the Plexin family, which mediates intracellular RNA processing that contributes to cell growth, survival, and regenerative capabilities of angiogenin [19]. Significantly, PLXNB2 was found to be highly expressed in normal Sprague-Dawley rat growth plate chondrocytes from the perichondral and reserve zones [20], indicating a possible role involved in skeletal development.

This study has established that the PLXNB2 has considerable effect on the osteogenic differentiation of BMSCs. By assessing the expression levels of specific markers and mineral deposition, this study revealed that PLXNB2 promoted osteogenic differentiation of BMSCs partly through the activation of the RHOA signaling pathway in vitro.

2. Materials and methods

2.1. Cell culture

Human BMSCs were purchased from Cyagen Biosciences (Guangzhou, China) and cultured in DMEM/F12 (HyClone, USA) medium supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Sigma USA) and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified atmosphere with 5% CO_2. The complete medium was replaced every 2 days. BMSCs between passage 2 and 5 were used for subsequent experiments.

2.2. Osteogenic differentiation of BMSCs

BMSCs (density: $3 \times 10^3/cm^2$) were plated for osteogenic differentiation. After the BMSCs reached 80% confluence, the DMEM/F12 medium was replaced with osteogenic differentiation medium (Cyagen) containing (Basal Medium, 10% FBS, 0.1 μM dexamethasone, 10 mM β-

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https://doi.org/10.1016/j.cellsig.2019.06.008
Received 3 April 2019; Received in revised form 30 May 2019; Accepted 5 June 2019
Available online 06 June 2019
0898-6568/

glycerophosphate, 1% glutamine, 0.2% ascorbate and 1% penicillin/streptomycin). The medium was replaced every 3 days.

2.3. Antibodies

Anti-PLXNB2 (sc-373,930, 1:1000 in WB analysis and 1:100 in IP assays), anti-RUNX2 (sc-101,145, 1:1000 in Western Blot (WB) analysis), anti-osteocalcin (OCN, sc-390,877, 1:1000 in WB analysis), anti-integrin binding sialoprotein (BSP, sc-73,497, 1:1000 in WB analysis), anti-ras homolog family member A (RHOA, sc-418,1:1000 in WB analysis and 1:100 in IP assays) and anti-β-actin antibodies (sc-517,582, 1:1000 in WB analysis) were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Na^+/K^+ ATPase antibody (05–369, 1:100 in WB analysis) was bought from Merck Millipore. Anti-active RhoA antibody (# 26904, 1:1000 in WB analysis) was bought from NewEast Biosciences (PA, USA).

2.4. In vitro PLXNB2 overexpression or knockdown using lentivirus

Lentiviral particles overexpressing PLXNB2 or short hairpin RNA (shRNA) targeting PLXNB2 or control shRNA were purchased from GenePharma (Shanghai, China). The infection with lentivirus was carried out using 80–90% confluent BMSCs cells at an MOI of 50. We changed the lentivirus-containing culture medium 12 h after the infection. BMSCs were harvested 3 days post infection, which was then confirmed by PCR and WB analyses.

2.5. RNA interference

Small interfering RNAs (siRNA) targeting RhoA and negative control siRNA were purchased from GenePharma. For transient infection, 80%–90% confluence BMSCs were transfected with siRNAs using Lipofectamine 2000 Reagent (Thermo Fisher) and Opti-MEM containing, according to the manufacturer's procedure. BMSCs were harvested 48 h post infection.

2.6. Cell proliferation assay

Cell proliferation of BMSCs was measured using carboxy-fluorescein succinimidyl ester (CFSE) dilution assay (Invitrogen). Briefly, BMSCs (1×10^6 cells/ml) were labeled with 5 μM CFSE for 15 min at 37 °C followed by adding DMEM/F12 medium with 10% FBS and incubation for 10 min to terminate the labeling reaction. CFSE-labeled BMSCs were then seeded into 6-well plates and cultivated with Andro for another 48 h. The cell proliferation was then analyzed by flow cytometry (BD Biosciences, CA, USA). We used FlowJo (Version 7.6.5) software to quantify the data.

2.7. qRT-PCR analysis

We used TRIzol reagent (Invitrogen) to isolate total RNA from BMSCs according to the manufacturer's instructions. Then we reverse transcribed the RNA into cDNA using a PrimeScript Kit (TAKARA, Dalian, China). qRT-PCR analysis were performed to amplify the cDNA using a SYBR Kit (TAKARA) and a CFX PCR Detection System (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. We used the CFX96 system's software (Bio-Rad) to conduct the RT-PCR analysis. Primer pairs used in the PCR analysis are described as follows: PLXNB2: Forward, TCTGGCAGGGTGCTATATGCT; Reverse, GAAGGGCTTGTAG AAGATGTCAC; OCN: Forward, GGCGCTACCTGTATCAATGG; Reverse, GTGGTCAGCCAACTC.

GTCA; RUNX2: Forward, TGGTTACTGTCATGGCGGGTA; Reverse, TCTCAGAT.

CGTTGAACCTTGCTA; BSP: Forward, CCCCACCTTTTGGGAAAA CCA; Reverse, TCCCCGTTCTCACTTTCATAGAT. We used housekeeping gene GAPDH as the internal control and determined relative gene expression as being normalized to GAPDH.

2.8. WB analysis

We washed the BMSCs twice with ice-cold PBS and extracted total proteins using RIPA buffer (Beyotime). Cytoplasmic protein was obtained using a Nuclear and Cytoplasmic Protein Extraction Kit (Kamimi Yasuro Biological Technology, Shanghai, China). Cell membrane protein was obtained using a Cell Membrane Protein Extraction Kit (XY-Bioscience, Shanghai, China). Then we determined and normalized the protein concentrations using a BCA protein assay (Thermo Fisher). We then used 10% SDS-PAGE gels to separate the lysate proteins (30 μg), which was then transferred to PVDF membranes (Millipore, Darmstadt, Germany). After that, we blocked the membranes with 5% nonfat milk in TBST for 1 h and incubated them with specific primary antibodies overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at normal room temperature. Finally, we applied a SuperSignal® WestDura Extended Duration Substrate to visualize the protein bands. According to the manufacture's instructions, the membranes were incubated with 1 ml ECL solution for 1 min at room temperature and sealed with plastic wrap. The bands were visualized under X-ray film exposure for about 5 min. Image J software was used to measure the bands.

2.9. Co-immunoprecipitation (co-IP) assays

Next, we applied co-IP assay to explore the direct interactions between PLXNB2 and RhoA, as described previously [21]. Briefly, BMSCs extracts were obtained using Nonidet P-40 buffer containing protease inhibitors. The lysates were collected and incubated with a anti-RhoA or anti-PLXNB2 antibody overnight at 4 °C. The samples were then immunoprecipitated with protein A/G agarose beads. After centrifugation, proteins were finally detected by Western blotting.

2.10. Alkaline phosphatase staining and quantification

After BMSCs were cultured in osteogenic differentiation medium for 3 days, we applied ALP staining using an ALP reagent (Nanjing Jiancheng Bioengineering Institute, China). For ALP quantification, we applied an ALP assay kit (Beyotime, China). The BMSCs were lysed with RIPA lysis buffer to get the cell supernatants. Then the substrates together with p-nitrophenol were added to the 96-well plates. After a 30-min incubation at 37 °C, we determined the ALP activity at a wavelength of 405 nm. We also determined the total protein content in the same sample by using the BCA method (Thermo Fisher Scientific). We finally normalized the ALP values to the total protein content and determined the relative final ALP activity.

2.11. Alizarin red staining and quantification

We used Alizarin red (pH 4.2; Sigma-Aldrich) staining to assess the effect of PLXNB2 on calcium deposition. BMSCs cultured in osteogenic differentiation medium for 14 days. Cells were washed by PBS in triplicate and fixed in 4% paraformaldehyde. Then we applied 1% Alizarin red for 20 min at room temperature, to finish the staining. For quantify the degree of mineralization, we applied 10% cetylpyridinium chloride (Sigma) to dissolve the stain. We measured the absorbance at 570 nm and calculated the Alizarin red staining intensity relative to the control treatment after normalization to the protein content.

2.12. Statistical analysis

SPSS 17.0 software was used for the statistical analysis. We repeated all experiments independently for at least three times. The results of this study are presented as means ± SD. We used the two-tailed Student's *t*-test and ANOVA analysis with post-hoc tests when

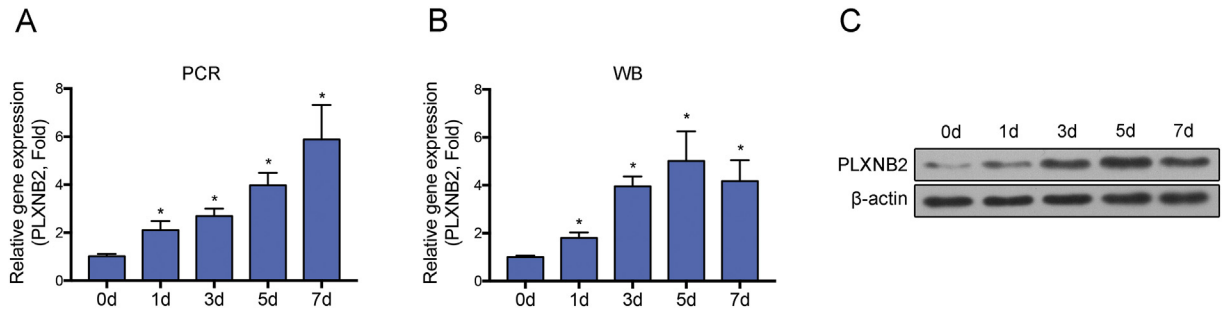

Fig. 1. PLXNB2 is upregulated during the osteogenesis of human BMSCs. (A) The mRNA expression of PLXNB2 was by qRT-PCR detection at various time points (0~7d) during BMSCs osteogenesis. (B—C) The protein expression of PLXNB2 was by WB analysis at various time points (0~7d) during BMSCs osteogenesis. *$p < .05$ when compared with the results of 0d.

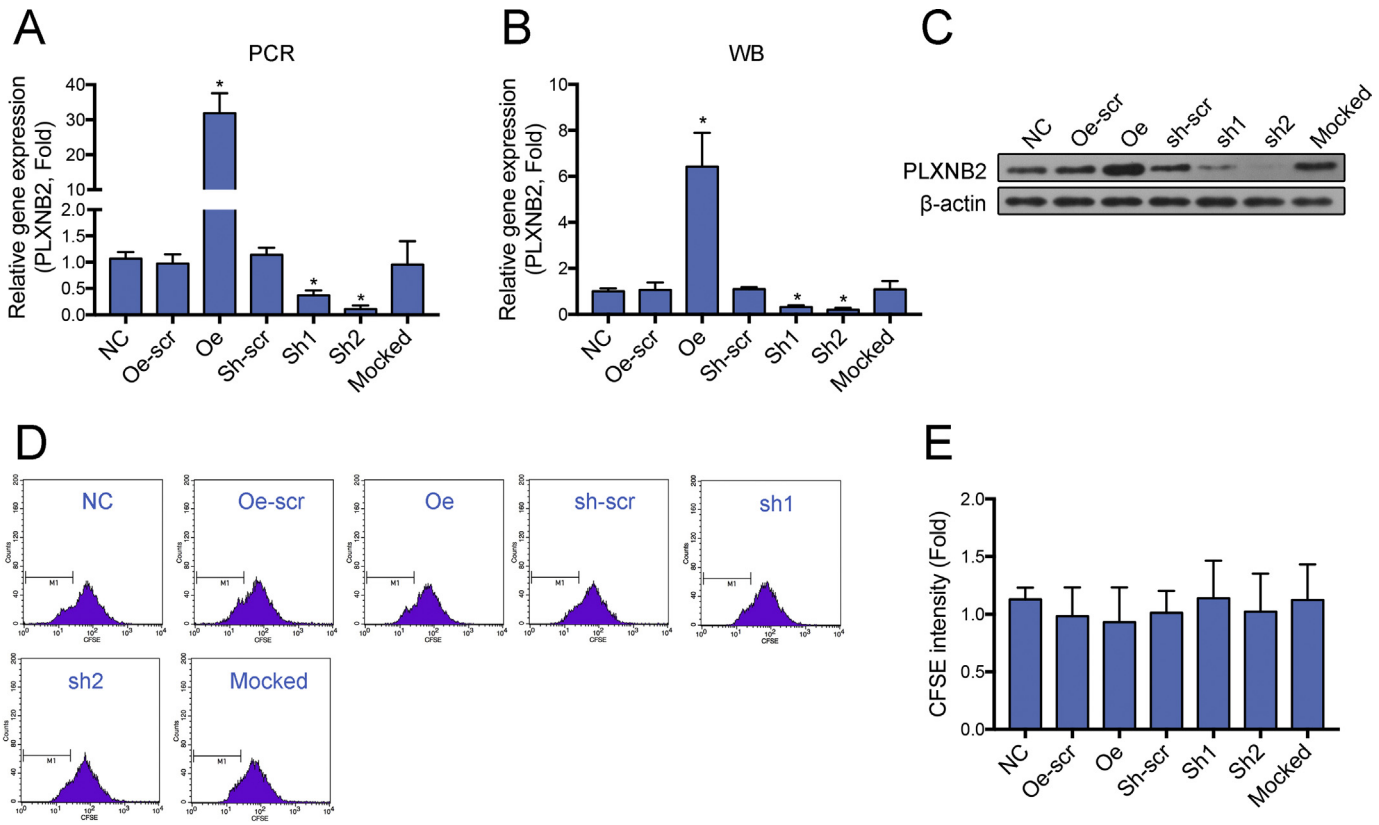

Fig. 2. Confirmation of successful transfection. (A) qRT-PCR analysis of the PLXNB2 mRNA expression levels after BMSCs were transfected with PLXNB2 overexpression or knockdown lentiviral vector, scramble vector, mock vector or a NC empty vector. (B—C) WB analysis of the PLXNB2 protein expression levels after transfection. (D) CFSE dilution assay was applied to evaluate the influence of PLXNB2 overexpression or knockdown on the proliferation of BMSCs. (E) Quantification of the CFSE dilution assay. NC: normal control; Oe-scr: the scramble vector of the Oe group; Oe: overexpression; Sh: short hairpin RNA; Sh-scr: the scramble vector of the knockdown group. *$p < .05$ when compared with the results of NC group.

appreciated. A P values below 0.05 was defined as statistically significant.

3. Results

3.1. PLXNB2 is upregulated during the osteogenesis of human BMSCs

As is shown in Fig. 1A, we determined the mRNA expression of PLXNB2 was by qRT-PCR at various time points (0~7d) during BMSCs osteogenesis. The result showed that PLXNB2 mRNA expression was upregulated after the induction of osteogenesis (Fig. 1A). We also applied WB analysis and confirmed similar results (Fig. 1B-C).

3.2. PLXNB2 stimulates the osteogenesis of human BMSCs in vitro

We used lentivirus to overexpress or knock down PLXNB2 expression in BMSCs. The results of PCR confirmed successful transduction that the mRNA expression level of PLXNB2 was almost 30-fold increased in the overexpression group (Oe) and almost 60–80% decreased in the knockdown group (Sh1 and Sh2), compared with the normal control group (NC) (Fig. 2A). We also applied WB analysis and confirmed similar results (Fig. 2B-C). To evaluate the influence of PLXNB2 overexpression or knockdown on the proliferation of BMSCs, we applied CFSE dilution assay. We found that PLXNB2 overexpression or knockdown doesn't impair the proliferation of BMSCs.

We next applied PCR and WB analyses at various time points to investigate the role of PLXNB2 in BMSCs osteogenesis. The results of PCR analysis showed that the mRNA expression of PLXNB2 was still

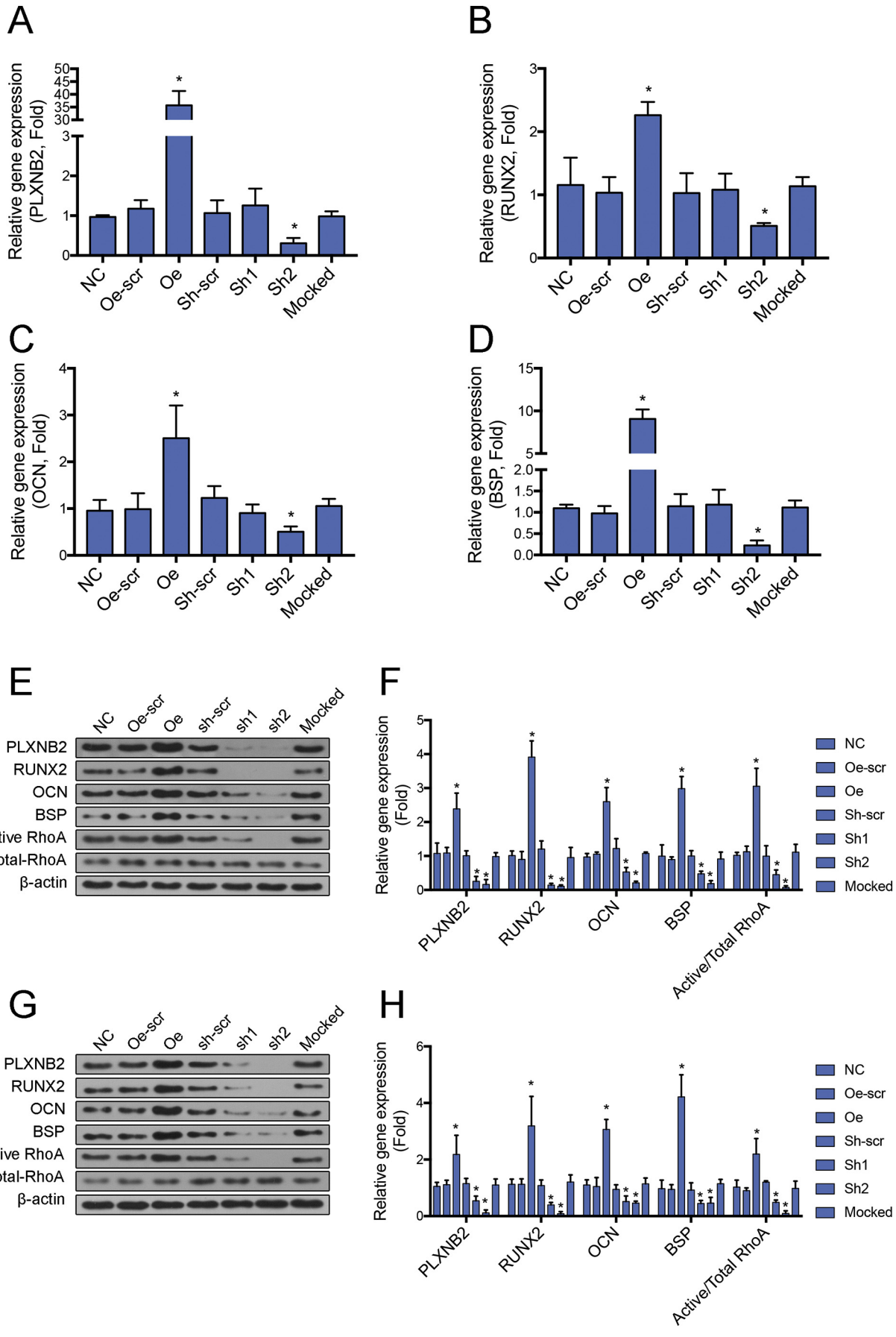

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Fig. 3. PLXNB2 stimulates the osteogenesis of human BMSCs in vitro. (A) qRT-PCR analysis of the PLXNB2 mRNA expression levels at 7 days after the induction of osteogenic differentiation. (B-D) qRT-PCR analysis of the RUNX2, OCN and BSP mRNA expression levels at 7 days after the induction of osteogenic differentiation. (E-F) WB analysis of the PLXNB2, RUNX2, OCN, BSP, active RhoA, and total RhoA at 3 days after the induction of osteogenic differentiation. (G-H) WB analysis of the PLXNB2, RUNX2, OCN, BSP, active RhoA, and total RhoA at 7 days after the induction of osteogenic differentiation. NC: normal control; Oe-scr: the scramble vector of the Oe group; Oe: overexpression; Sh: short hairpin RNA; Sh-scr: the scramble vector of the knockdown group. *$p < .05$ when compared with the results of NC group.

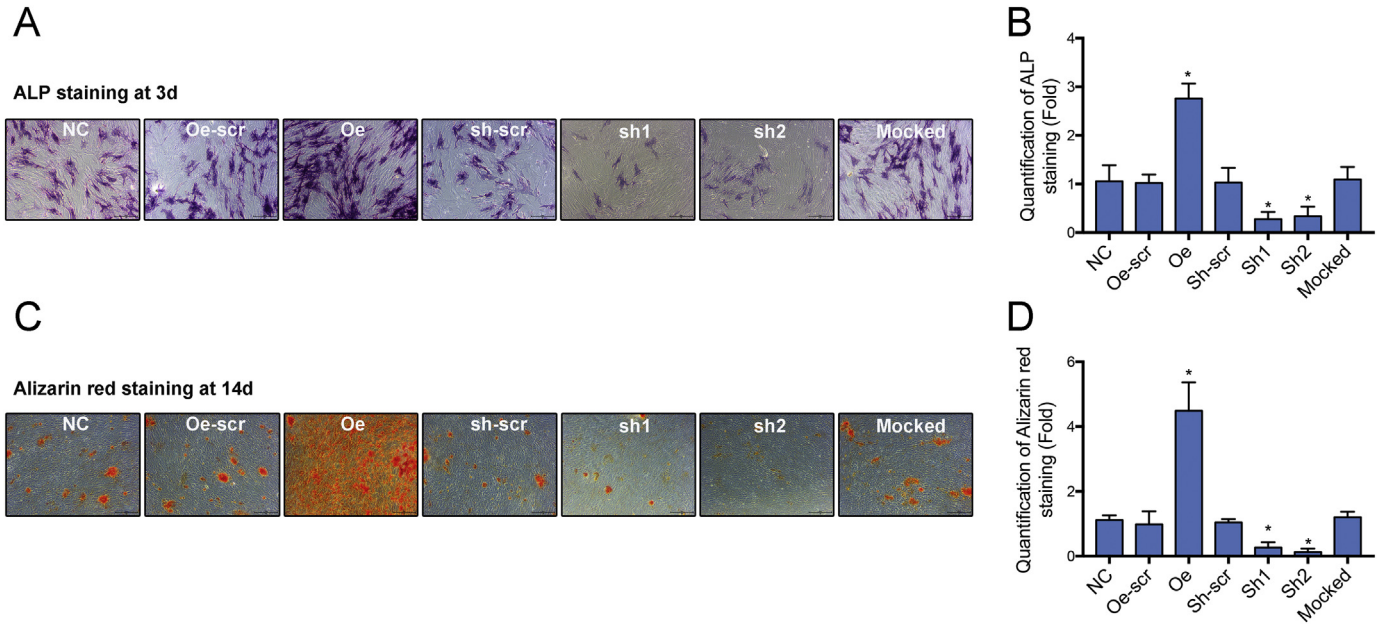

Fig. 4. PLXNB2 increased the ALP activity and calcium deposition of human BMSCs. (A) ALP staining was performed on day 3 after the induction of osteogenic differentiation. (B) Quantification of the ALP activity. (C) Alizarin red staining was performed on day 14 after the induction of osteogenic differentiation. (D) Quantification of the Alizarin red staining. NC: normal control; Oe-scr: the scramble vector of the Oe group; Oe: overexpression; Sh: short hairpin RNA; Sh-scr: the scramble vector of the knockdown group. *p < .05 when compared with the results of NC group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stable at 7 days after the induction of osteogenic differentiation (Fig. 3A). At the same time point, the mRNA expression levels of osteogenic markers RUNX2, osteocalcin (OCN) and bone sialoprotein (BSP) were remarkably upregulated in the PLXNB2 overexpression group and down regulated in the PLXNB2 knockdown group (Fig. 3B-D). We also conducted WB analysis at 3 (Fig. 3E-F) and 7 (Fig. 3G-H) days after the induction of osteogenic differentiation. The results of WB analysis showed that PLXNB2 overexpression effectively stimulated the osteogenesis of human BMSCs, while PLXNB2 knockdown significantly inhibited BMSCs osteogenesis.

Moreover, we applied ALP staining and Alizarin red staining and found that PLXNB2 overexpression enhanced the ALP activity and calcium deposition of BMSCs, while PLXNB2 knockdown showed opposite effects (Fig. 4).

3.3. Overexpression of PLXNB2 promotes the RHOA translocation from cell membrane to cytoplasm

To investigate the underlying mechanisms of PLXNB2-mediated regulation of osteogenesis, RHOA, a key signaling pathway involved in osteogenic differentiation was studied. We first applied WB analysis and found that PLXNB2 overexpression significantly increased the protein expression of active RHOA, which was dramatically decreased in PLXNB2 knockdown groups (Fig. 3E-H). To further confirm the role of RHOA in PLXNB2-mediated regulation of osteogenesis, we found that PLXNB2 overexpression significantly decreased the protein level of cell membrane RHOA and increased cytoplasm RHOA protein level. Opposite tendency was found in the PLXNB2 knockdown groups. Taken together, these results indicated that PLXNB2 promoted the RHOA translocation from cell membrane to cytoplasm.

3.4. Knockdown of RHOA compromises the effect of PLXNB2 in BMSCs osteogenesis

Next, we used siRNA to knock down RHOA in human BMSCs. We found that knock down of RHOA had no influence on PLXNB2 expression (Fig. 5C-D). We also found that knock down of RHOA dramatically inhibited the protein expression level of RUNX2, OCN and BSP in BMSCs from the control groups including the NC group and the Oe-scr group. Most importantly, the protein expression level of RUNX2, OCN and BSP in PLXNB2 overexpression group was also decreased, showing that knockdown of RHOA compromises the effect of PLXNB2 in BMSCs osteogenesis (Fig. 5C-D).

3.5. Direct targeting of RHOA by PLXNB2

Finally, we conducted Co-IP analysis to identify the potential direct interactions between PLXNB2 and RHOA. The cell lysates from different groups (NC, Oe-scr, Oe) were immunoprecipitated with anti-RHOA or anti-PLXNB2 antibody and WB analysis were performed after that. The results of Co-IP showed that overexpression of PLXNB2 notably enhanced the binding between RHOA and PLXNB2 (Fig. 5E-H).

4. Discussion

BMSCs play crucial roles in the development and treatment of various skeletal disorders. The differentiation potential and function of BMSCs are of vital importance and their regulation remains largely imprecise. The present study, examined the role of PLXNB2 in the osteogenic differentiation of BMSCs. It revealed that PLXNB2 was upregulated during BMSCs differentiation into an osteoblastic lineage. The

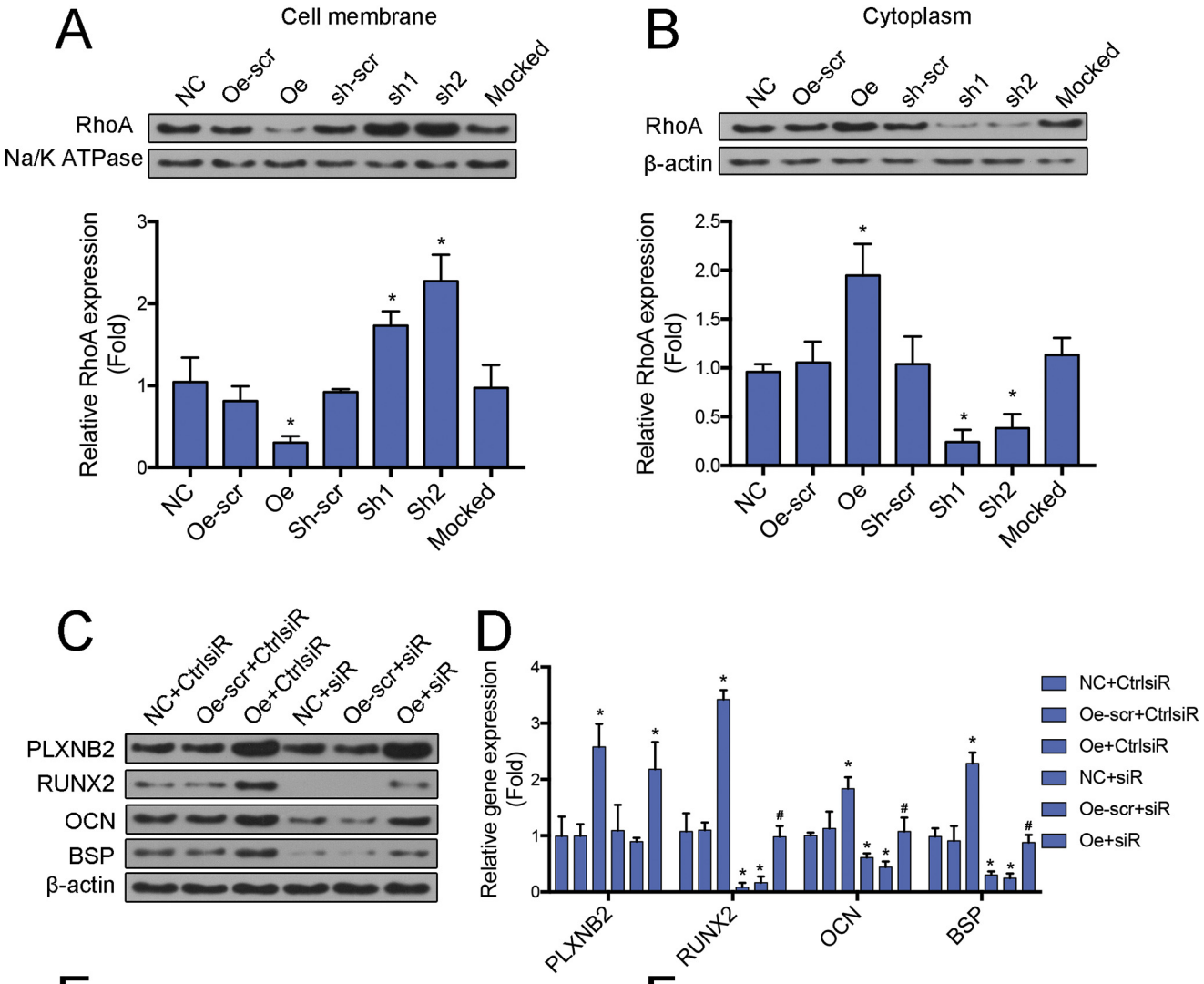

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Fig. 5. PLXINB2 promotes the osteogenic differentiation of BMSCs via activation of the RhoA signaling pathway. (A) WB analysis of the PLXNB2 showed that overexpression of PLXNB2 reduced the RHOA protein level of the cell membranes. (B) WB analysis of the PLXNB2 showed that overexpression of PLXNB2 increased the RHOA protein level of the cell cytoplasm. *p < .05 when compared with the results of NC group. (C-D) WB analysis showed that knock down of RHOA using siRNA had no influence on PLXNB2 expression, but inhibited the protein expression level of RUNX2, OCN and BSP in BMSCs from the control groups and Oe group. *p < .05 when compared with the results of NC + CtrlsiR group. (*E*-H) The results of Co-IP analysis showed that overexpression of PLXNB2 notably enhanced the binding between RHOA and PLXNB2 (Fig. 5E-H). NC: normal control; Oe-scr: the scramble vector of the Oe group; Oe: overexpression; Sh: short hairpin RNA; Sh-scr: the scramble vector of the knockdown group. *p < .05 when compared with the results of NC group.

overexpression and knockdown experiments were applied to indicate that PLXNB2 promotes the osteogenic differentiation of human BMSCs through the activation of the RhoA signaling pathway.

Plexin-B receptors (B1-B3) are transmembrane proteins involved in regulating the cellular processes [14–18]. Among them, PLXNB2 is presumed to be critically involved in the development of the nervous system and cell differentiation [22]. Nicolas et al. reported that Plexin-B1 and −B2 played redundant roles during forebrain development, while Plexin-B3 was restricted to postnatal oligodendrocytes. In their study, Plexin-B1 and −B2 double deletion mutants were found to influence the impairment of the cortical neurogenesis [23]. PLXNB2 is also an important regulator of vessel formation. Using a zebrafish model, loss of PLXNB2 was found to delay the intersegmental vessel sprouting [24]. Recently, PLXNB2 was confirmed to be the functional receptor for angiogenin in multiple cells including endothelial and hematopoietic stem/progenitor cell, etc.. Antibodies against the angiogenin binding site on PLXNB2 not only impairs the angiogenin-induced neurogenesis, but also inhibits the levels of pro-self-renewal transcripts in hematopoietic cells [19]. Moreover, PLXNB2 influences in various ways the development and prognosis of various human malignancies including breast cancer, glioblastoma, and leukemia. In breast cancer, Muhammad et al. reported that PLXNB2 was markedly reduced in tumors with local recurrence, compared to those remaining disease-free, signifying that reduced expression of PLXNB2 in breast cancer is associated with poorer prognosis [25]. Principally, Mingliang et al. applied microarray analysis of growth plate zones of Sprague-Dawley rats and identified significantly high expression of PLXNB2, which was indicative of the possible involvement of PLXNB2 in the process of cartilage development, ossification, and bone remodeling [20]. In the present study, it was observed that PLXNB2 was upregulated during the osteogenesis of BMSCs, which confirmed the possible role of PLXNB2 during skeletal development.

As is well known, RhoA, a prototypical member of the Rho family, plays the part of a molecular switch in maintaining the actin cytoskeleton and several cellular responses including the osteogenic differentiation of BMSCs [26–28]. Basem et al. submitted that CRMP4 inhibits bone formation by inhibiting RhoA signaling [29]. ABBAS et al. stated that inhibition of protein kinase G1 improves the osteogenesis of BMSCs through activation of RhoA pathway [30]. It was proved that Dimethyloxalylglycine also promotes BMSCs osteogenesis through RhoA signaling [31]. On the other side, the disruption of RhoA will lead to reduced osteoblastogenesis.

and enhanced adipogenesis [32]. In consonance with the previous studies, this study could determine that when the osteogenesis of BMSCs was enhanced by PLXNB2, the RhoA signaling was notably activated. Also, PLXNB2 promoted RhoA translocation to the plasma and resulted in its activation. Moreover, the results of Co-IP analysis showed that during this process, the binding of RhoA and PLXNB2 were significantly increased, which again confirmed that involvement of RhoA during the promoted osteogenesis of BMSCs that induced by PLXNB2.

It is worth noting that the interactions between class B plexins and Rho-GTPases are very complicated and remains controversial, which may due to different cell types or the involvement of the immune system. Also, although we found PLXNB2 promoted the osteogenic differentiation of BMSCs via activation of the RhoA signaling pathway, it is likely that other members of GTPases may also be involved in this process. Thus, further studies with different cell types as well as focusing on different GTPases will be helpful in confirming our findings.

5. Conclusion

In conclusion, this study established PLXNB2 as a novel regulator that enhanced the osteogenic differentiation of human BMSCs. The effect of enhancing of PLXNB2 on osteogenesis of human BMSCs is mediated through activation of RhoA signaling. The results of our study established that pharmacological targeting of PLXNB2 may represent a possible approach to improve bone formation.

Acknowledgments

This study is funded by the National Science Foundation of China (No. 81774348 and 81874477), the Science and Technology Project of Henan Province (No. 162102310364) and Special Project of Traditional Chinese Medicine Research in Henan Province (No. 2016zy2027).

Authors' contributions

Conception and design of the research: WL and YL; acquisition of data: YZ, SS, and PL; analysis and interpretation of data: PL, YF, and LZ; statistical analysis: YF and LZ; drafting the manuscript: YZ and SS; revision of manuscript for important intellectual content: WL and YL.

Conflicts of interests

The authors declare that they have no competing interests.

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