



Original Article

Circ_0134944 inhibits osteogenesis through miR-127-5p/PDX1/SPHK1 pathway

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ABSTRACT

Introduction: Osteoporosis, a common skeletal disorder mainly affecting postmenopausal women, is characterized by the imbalance between osteogenesis and osteoclastogenesis. Circ_0134944 has been recently found to be upregulated in postmenopausal osteoporosis (PMOP) patients. However, its role in osteogenesis remains unknown. Here we aimed to explore the role of circ_0134944 in osteogenesis and reveal the underlying mechanism.

Methods: qRT-PCR was used to determine the expression of circ_0134944, miR-127-5p, PDX1 and SPHK1 in the blood mononuclear cells (BMCs) of PMOP patients. Bone marrow mesenchymal stem cells (BMSCs) were used as the cellular model. Western blotting and qRT-PCR were used to determine the expression of osteogenesis-related genes (Runx2, OPN, OCN). ALP and Alizarin Red S staining were performed to evaluate osteogenic differentiation. The interactions between circ_0134944 and miR-127-5p, miR-127-5p and PDX1, PDX1 and SPHK1 were determined by dual-luciferase reporter and ChIP assay.

Results: Circ_0134944, PDX1 and SPHK1 were upregulated while miR-127-5p was downregulated in PMOP patients. Enhanced expression of circ_0134944 suppressed osteogenesis, which was then reversed by miR-127-5p overexpression. The binding between circ_0134944 and miR-127-5p, PDX1 and miR-127-5p were confirmed by dual-luciferase reporter assay. Moreover, PDX1 was enriched in the promoter region of SPHK1, and SPHK1 overexpression prevented the promotion of osteogenesis induced by miR-127-5p overexpression.

Conclusions: Taken together, these results demonstrate that circ_0134944 inhibit osteogenesis via miR-127-5p/PDX1/SPHK1 axis. Thus, the present study offered evidence that circ_0134944/miR-127-5p/PDX1/SPHK1 axis could be a potential therapeutic target for PMOP.

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1. Introduction

Bone undergoes constant renovation by maintaining a balance between bone resorption by osteoclasts and bone formation by osteoblasts [1,2]. Postmenopausal osteoporosis (PMOP) is a common bone disease characterized insufficient bone formation by osteoblasts, resulting in decreased bone mineral density and low trauma fractures [3]. According to Mayo Clinic, from 2000 to 2011, the medical cost of osteoporotic fractures in US women over 55 was \$5.1 billion, remarkably greater than that of stroke, breast cancer and myocardial infarction [4]. BMSCs play a critical role in bone repair and remodeling with the ability to differentiate into osteoblasts [5,6]. Therefore, elucidating the regulatory network

underlying the osteogenic differentiation of BMSCs is of great significance for the treatment of PMOP.

S1P (sphingosine-1-phosphate) is a lipid mediator that regulates various biological actions, such as vascular homeostasis and immune cell trafficking partially by modulating its receptors (S1PR) [7]. In most cells, sphingolipids are metabolized and converted to sphingosines, which are then phosphorylated into S1P by sphingosine kinase 1 and 2 (SPHK1 and SPHK2) [8]. Mounting evidence has suggested the important role of SPHK1–S1P–S1PR in bone remodeling [9]. For example, S1P signaling controlled the mobilization of osteoclast precursors between the blood and bone by upregulating receptor activator of NF- κ B ligand (RANKL), thus regulating bone homeostasis [10]. In bone marrow-derived macrophage (BMM)/osteoblast coculture systems, exogenous S1P remarkably increased osteoclastogenesis by increasing RANKL expression [11]. Moreover, osteoclast-derived S1P coupled bone formation to bone resorption by activating JAK/STAT3 and FAK/PI3K/AKT [12]. Thus, identifying regulatory networks of S1P signaling might shed light on the therapy for PMOP.

Circular RNAs, or circRNAs, are circular-shaped non-coding RNAs which exert various biological functions. Numerous studies underlined the essential role of circRNAs in PMOP. For example, circ_0016624 promoted osteogenesis via its interaction with miR-98/BMP2 axis [13]. Circ_0024097 alleviated osteoporosis by sponging miR-376 [14]. Two studies have revealed that circ_0134944 was upregulated in PMOP patients [13], indicating a potential role of circ_0134944 in PMOP. However, few information is available about the role of circ_0134944 in osteogenesis. According to bioinformatics analysis on circinteractome, circ_0134944 was predicted to bind with miR-127-5p. Combined with the fact that circ_0134944 was upregulated while miR-127-5p was downregulated in PMOP patients, we hypothesized that circ_0134944 might play an important role in osteogenesis of BMSCs via its interaction with miR-127-5p.

MiRNAs have been reported to exert biological functions by regulating the levels of their target genes [15]. So bioinformatics analysis on StarBase were conducted to identify potential targets of miR-127-5p. It was suggested that, PDX1 (pancreatic and duodenal homeobox 1) might be a downstream target of miR-127-5p. PDX1 is a well-established regulator of cell differentiation [16,17], but few studies has linked PDX1 with osteogenesis. A recent study reported that PDX1 mediated the osteogenic effects of miR-1249-5p in adipose-derived stem cells [18]. Also, we found that PDX1 was aberrantly expressed in PMOP patients. Altogether, these lines of evidence reminded us to investigate the role of PDX1 in PMOP.

In the current study, we used BMSCs as the cellular model to test our hypothesis. Our results demonstrate that circ_0134944 suppresses osteogenesis of BMSCs by regulating miR-127-5p. We also identified PDX1/SPHK1 as the underlying mechanism of the regulatory role of circ_0134944/miR-127-5p. These results highlight the essential role of circ_0134944 in osteogenic differentiation of BMSCs, which could provide insights into the therapy of PMOP.

2. Materials and methods

2.1. Isolation of BMCs and BMSCs

This study was approved by Research Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University. 25 cases of PMOP women aged 50–60 years and 25 cases of postmenopausal non-osteoporotic women were recruited for this study. All participants had a natural menopausal history of 5–10 years and PMOP patients had a BMD T-score of < -2.5 at the lumbar spine. Written informed consent was obtained from all subjects. Blood mononuclear cells (BMCs) were extracted from each subject. The blood samples were diluted (1:1, PBS) and layered on a Ficoll–Paque gradient (GE

Healthcare, Madison, WI, USA). The gradient was centrifuged at 400 g for 30 min. Subsequently, mononuclear cell layer was collected, resuspended in PBS and centrifuged twice at 200 g for 10 min. Finally, the cells were maintained in α -MEM (Gibco, Grand Island, NY, USA).

Human BMSCs were isolated from discarded femoral head tissues from osteoporosis patients and cultured in α -MEM containing 10% FBS (Gibco), 100 U/mL penicillin (Hyclone, South Logan, UT, USA) and 100 μ g/mL streptomycin (Hyclone). To induce osteogenic differentiation, 10 mM β -glycerophosphate, 50 mg/mL ascorbic acid and 0.1 mg/mL dexamethasone were added into the culture medium. The cells were cultured in this differentiation buffer for 2 weeks, and the medium was replaced every 3 days.

2.2. Transfection

Plasmids containing circ_0134944, miR-127-5p mimics, PDX1, SPHK1, wild type PDX1 (WT-PDX1), mutated SPHK1 (MUT-SPHK1) and shRNA against PDX1 (shPDX1) were purchased from ThermoFisher Scientific (Waltham, MA, USA). Transfection was performed with lipofectamine 3000 (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were plated into a 12-well plate at the density of 1×10^5 cells/mL. After reaching 30–40% confluence, transfection of plasmids was performed with Lipofectamine 3000. After 48 h, cells were used for further experiments.

2.3. ALP staining and activity assay

Osteogenic differentiation was measured by ALP staining and activity assay. The protocol for ALP staining was as follows: after discarding the culture medium, BMSCs were washed with PBS and immobilized in 4% PFA. Then the cells were stained with BCIP/NBT liquid substrate (Beyotime, Zhejiang, China) for 0.5 h, washed with PBS, and counterstained with hematoxylin. Finally, the images were observed under a light microscope (Leica DMIRB, Germany).

For quantitative ALP activity detection, Alkaline Phosphatase Assay Kit from Beyotime was used. Briefly, the cells were washed with PBS and lysed with 1% Triton X-100 (Beyotime). Protein concentration in each sample was determined by BCA assay (Beyotime). The samples were mixed with substrate and detection buffer according to the manufacturer's instructions. The absorption at 405 nm was monitored for 30 min, and the ALP activity was calculated based on the rate of para-nitrophenol formation, and normalized to protein amount in the samples. Thus, the results were expressed as moles of para-nitrophenol/min/mg protein.

2.4. Alizarin red S staining (ARS)

BMSCs were washed in PBS without magnesium chloride and calcium chloride, and immobilized in 4% PFA. Then, cells were stained with 40 mM alizarin red (Sigma–Aldrich) for 12 min in dark. Afterwards, cells were washed with dH₂O and visualized under a light microscope (Leica DMIRB, Germany). For the quantification of ARS, a commercial kit from Sigma–Aldrich was used. First, cells were stained with ARS per the protocol described above. Then cells were collected with a scraper in a centrifuge tube, incubated with 10% HAc, and heated at 85 °C for 10 min. Then the tube was cooled on ice, centrifuged, and the supernatant was collected and neutralized with 10% NH₃.H₂O. Subsequently, samples and standards are added into a 96-well plate, and absorption at 405 nm was read by a plate reader. The ARS concentration was then calculated based on the standard curve, and then normalized to protein levels which was determined by BCA assay.

2.5. Quantitative real-time reverse transcription PCR (qRT-PCR)

PCR was performed with a PCR kit from Takara Bio (Dalian, China). Briefly, RNA was extracted and reversely transcribed into cDNA. Then qRT-PCR was carried out using SYBR-Green. The relative expression of a target gene to that of GAPDH was calculated using $2^{-\Delta\Delta Ct}$ method. The primers used are as follows: circ_0134944, forward 5'-GAAGATTCGGATCTGGCTCA-3' and reverse 5'-CCCA-GAAGCTACGAGGACTTT-3'; PDX1, forward 5'-CGGACATCTCCCA-TACG-3' and reverse 5'-GGCAGACCTGGCGGTTTACAT-3'; SPHK1, forward 5'-GGTGGCGGCTCTATTCT-3' and reverse 5'-GGTGCCACTGTGAAAAC-3'; Runx2, forward 5'-CGAATAA-CAGCAGCTATTAA-3' and reverse 5'-GTCGCCAAACAGATT CATCCA-3'; OPN, forward 5'-GGAGTTGAATGGTGCATACAAGG-3' and reverse 5'-CCACGGCTGTCCCAATCAG-3'; OCN, forward 5'-GGCGTACTGT-TATCAATGG-3' and reverse 5'-GTGGTCAGCCAACTCGTCA-3'; GAPDH, forward, 5'-CCAGTGGTCTCTCTGA-3' and reverse, 5'-GCTGTAGCCAAATCGTTGT-3'; miR-127-5p, forward 5'-CTCTTCAAGCTCCAAACAAAC-3' and reverse 5'-GTATCC ACCA-GAACCACCAGG-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTACGAATTTGCGT-3'. Relative expression of circ_0134944 and mRNA was normalized by GAPDH, and the relative expression miR-127-5p was normalized to U6.

2.6. Western blotting

Cells were washed with cold PBS and lysed in RIPA buffer containing 1% protease and phosphatase inhibitors (Cwbio, Beijing, China). Lysates were sonicated and protein concentrations were determined by BCA kit (ThermoFisher Scientific). 30 ng of protein lysates were loaded and separated through a 10% SDS-PAGE gel, and then transferred onto NC membranes (GE Healthcare, Madison, WI, USA). Membranes were blocked with 5% BSA for 2 h at room temperature, and then incubated with primary antibodies anti-SPHK1 (ab260073, Abcam, Cambridge, MA, USA), anti-PDX1 (ab240084, Abcam), anti-OPN (ab63856, Abcam), anti-Runx2 (ab92336, Abcam), and anti-OCN (sc-365797, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:2000 at 4 °C overnight. GAPDH (ab8254, Abcam) was used as an internal control. Subsequently, the membranes were incubated with secondary antibodies at room temperature for 1 h. Finally, the blots were visualized using an ECL detection kit (Beyotime).

2.7. Animals

12 female C57BL/6 mice (6 weeks) were purchased from SLRC Laboratory (Shanghai, China). These mice were randomly divided into sham and OVX groups. Mice in OVX group underwent bilateral ovariectomy (OVX) operations to establish a PMOP model. In brief, the mice were anesthetized and a small dorsal midline incision was made. An abdominal cavity was created using a blunt puncture and the ovary was pulled out from the cavity. The same procedure was repeated on the other side of the body. For the sham group, the same abdominal cavities on both side of the body were performed, without removing the ovary. 6 weeks after the OVX operation, all mice were sacrificed and bone samples were taken for further analysis.

2.8. Dual-luciferase reporter assay

Segments of circ_0134944 and the 3'UTR of PDX1 containing miR-127-5p binding sites were synthesized by Genepharma (Shanghai, China). These fragments were linked to the luciferase reporter gene vector (Promega, Madison, WI, USA). Mutagenesis of the binding sites in circ_0134944 and PDX1 were achieved using

the Site-Directed Mutagenesis Kit (Toyobo, Japan). HEK 293T cells were cotransfected with various combinations of reporter plasmids with miR-127-5p or NC or wild type/mutated circ_0134944 or PDX1 using Lipofectamine 3000 (Invitrogen, USA). 3 days later, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA). The operation method of PDX1 binding to SPHK1 promoter is the same as above.

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted using Pierce Agarose ChIP kit (Pierce) according to the manufacturer's instructions. In brief, BMSCs were transfected with sh-NC or sh-PDX1. Cells were crosslinked with 1% formaldehyde and lysed to prepare nuclei. Chromatin was sheared using micronuclease digestion. Sheared DNA was then incubated with anti-PDX1 antibody. Normal IgG was served as a negative control. DNA was purified and analyzed by qRT-PCR.

2.10. Hematoxylin and eosin (H&E) staining

Bone tissues were fixed with 4% PFA for 24 h and then sectioned into 30 μ m-thick slices. Sections were stained with hematoxylin for 5 min, washed with dH₂O, immersed in 1% hydrogen chloride–ethanol solution for 3 s and incubated in eosin for 20 s. Subsequently, the sections were dehydrated in a graded series of ethanol and xylene. Finally, the slices were mounted and photographed.

2.11. Statistical analyses

All data are presented as mean \pm SD. Data were analyzed by ANOVA, followed by Turkey post hoc test. A *p* value less than 0.05 were considered statistically significant.

3. Results

3.1. Circ_0134944, SPHK1 and PDX1 were upregulated while miR-127-5p was downregulated in PMOP patients and OVX mice

To explore whether circ_0134944 and miR-127-5p play a role in PMOP, we examined the expression of circ_0134944 and miR-127-5p in the BMCs of PMOP patients. Consistent with the findings of Yu et al. [13], the expression of circ_0134944 was significantly increased in the BMCs of PMOP patients (Fig. 1A). And miR-127-5p was downregulated in the BMCs of PMOP patients (Fig. 1B). Moreover, PDX1 and SPHK1 were upregulated in PMOP patients (Fig. 1C and D).

To verify the results from PMOP patients, the OVX model was established with mice. H&E staining was conducted to validate the model. As shown in Fig. 1E, the trabecular bone of the OVX group was small, thin and sparse and the cortical gap of the OVX group was much bigger compared with the sham group. Subsequently, the levels of circ_0134944, miR-127-5p, PDX1 and SPHK1 were quantified by qRT-PCR and Western blotting analysis. The results demonstrated that, compared with sham group, circ_0134944, PDX1 and SPHK1 were upregulated in OVX mice while miR-127-5p was downregulated (Fig. 1F and G). Moreover, we determined the relative expression levels of osteogenesis-related markers, Runx2, OPN and OCN: all three were downregulated in OVX group (Fig. 1G).

3.2. The expression of circ_0134944, SPHK1, miR-127-5p, Runx2, OPN and OCN in osteogenic differentiation of BMSCs

Osteogenic induction of human BMSCs for 14 days was conducted and the expression of Runx2, OPN and OCN, as well as

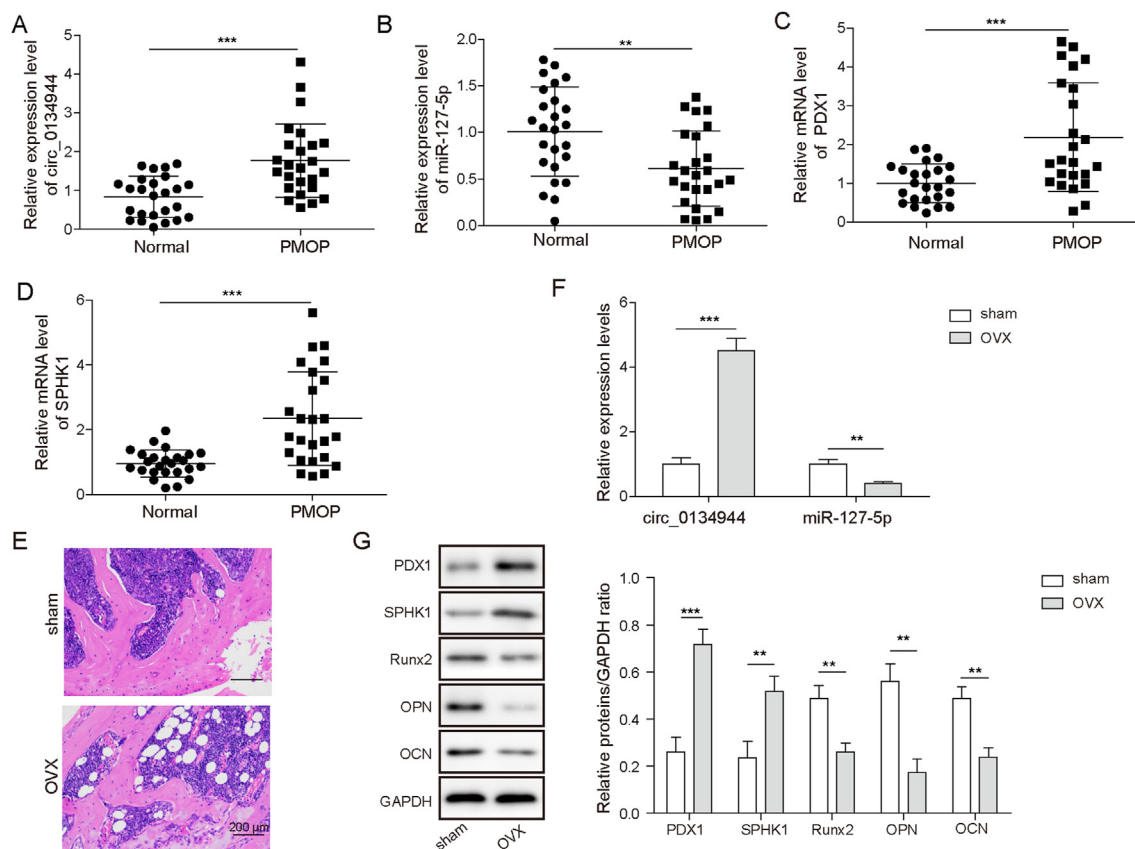


Fig. 1. Circ_0134944, PDX1 and SPHK1 were upregulated and miR-127-5p was downregulated in PMOP patients and OVX mice. (A–C) The expression of circ_0134944 (A), miR-127-5p (B), PDX1 (C) and SPHK1 (D) in the BMCs of PMOP patients were determined by qRT-PCR. N = 25. (E) H&E staining was performed with the bone samples from sham mice and OVX mice (N = 6). (F) The expression of circ_0134944 and miR-127-5p were determined by qRT-PCR (N = 6). (G) The protein levels of PDX1, SPHK1, Runx2, OPN and OCN were determined by Western blotting (N = 6). GAPDH was used as an internal control. ** $P < 0.01$; *** $P < 0.001$.

circ_0134944, miR-127-5p, PDX1 and SPHK1 on day 0, 7 and 14 were determined. As shown in Fig. 2A, the mRNA levels of Runx2, OPN, OCN and miR-127-5p were significantly increased during osteogenic differentiation, while the levels of circ_0134944, PDX1 and SPHK1 were significantly decreased. The protein expression was consistent with these results, confirming that Runx2, OPN and OCN were upregulated while PDX1 and SPHK1 were downregulated during osteogenic differentiation (Fig. 2B). Meanwhile, to validate the osteogenic differentiation of BMSCs, ALP activity and Alizarin red S staining were performed. As shown in Fig. 2C and D, enhanced ALP staining and Alizarin red S accumulation were observed over time. Taken together, these data suggest that, circ_0134944, PDX1, SPHK1 and miR-127-5p may be involved in the osteogenesis of BMSCs.

3.3. Circ_0134944 overexpression suppressed osteogenic differentiation of BMSCs

Circ_0134944 overexpression was established to investigate its implications in osteogenesis. A significant increase of circ_0134944 level was confirmed by qRT-PCR (Fig. 3A). qRT-PCR results also showed that, in circ_0134944-overexpressed BMSCs, miR-127-5p was downregulated while PDX1 and SPHK1 were upregulated (Fig. 3B–D). Meanwhile, the mRNA levels of osteogenic markers, Runx2, OPN and OCN were downregulated (Fig. 3E). Western blotting results further validated the upregulation of SPHK1 and PDX1, and the downregulation of OPN, OCN and Runx2 in circ_0134944-overexpressed BMSCs (Fig. 3F). Moreover, circ_0134944 overexpression decreased ALP activity and Alizarin

red S accumulation (Fig. 3G and H). These results suggest that circ_0134944 suppresses osteogenic differentiation of BMSCs.

3.4. Circ_0134944 bound with miR-127-5p to modulate osteogenesis of BMSCs

According to our bioinformatics analysis, circ_0134944 has binding sites with miR-127-5p (Fig. 4A). Together with the results that circ_0134944 negatively regulated the expression of miR-127-5p (Fig. 3B), we hypothesized that circ_0134944 might interact with miR-127-5p to regulate osteogenic differentiation in BMSCs.

Dual-luciferase assay was carried out to examine the potential binding between circ_0134944 and miR-127-5p. We observed that overexpression of miR-127-5p suppressed the luciferase activity, while mutated circ_0134944 prevented this repression (Fig. 4B), indicating that circ_0134944 could bind with miR-127-5p in this putative binding site. Furthermore, we investigated whether miR-127-5p mediated the regulatory role of circ_0134944 in osteogenic differentiation of BMSCs. qRT-PCR results showed that circ_0134944 overexpression suppressed the expression of miR-127-5p which was restored by miR-127-5p overexpression (Fig. 4C). MiR-127-5p overexpression also reduced PDX1 and SPHK1 expression which were induced in circ_0134944-overexpressed BMSCs (Fig. 4D). Moreover, the expression of osteogenic markers (Runx2, OCN and OPN) was restored by miR-127-5p mimics in circ_0134944-overexpressed BMSCs (Fig. 4E). Besides, miR-127-5p overexpression abolished the suppression of circ_0134944 on both ALP activity and mineralization (Fig. 4F and G). Western blotting assay also showed that miR-127-5p

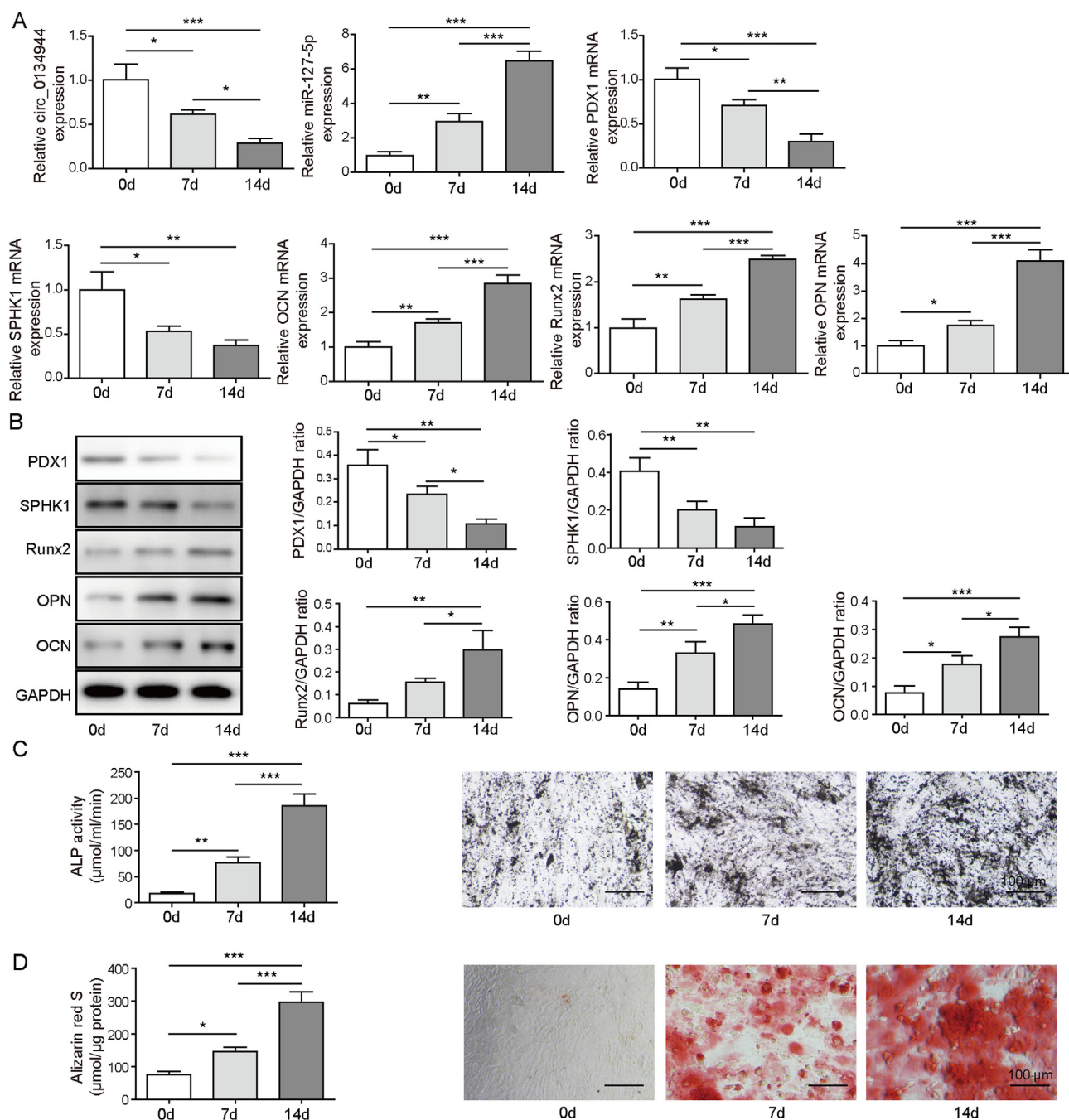


Fig. 2. The expression of circ_0134944, PDX1, SPHK1, miR-127-5p, Runx2, OPN and OCN in osteogenic differentiation of BMSCs. To induce osteogenic differentiation, BMSCs were incubated in osteogenic differentiation medium for 0, 7 and 14 days. (A) The levels of circ_0134944, miR-127-5p, PDX1, SPHK1, OCN, Runx2, and OPN in BMSCs were determined by qRT-PCR. (B) The protein levels of PDX1, SPHK1, OCN, Runx2, and OPN in BMSCs were determined by Western blotting. GAPDH was used as an internal control. (C) ALP activity was both assessed by quantitative assay (left) and qualitative staining assay (right). (D) Mineralization was both assessed by quantitative ARS assay (left) and qualitative ARS staining assay (right). N = 6. *P < 0.05; **P < 0.01; *** P < 0.001.

overexpression prevented the upregulations of PDX1 and SPHK1 and repression of Runx2, OPN and OCN in circ_013499-overexpressed cells (Fig. 4H). Taken together, these data indicated that miR-127-5p reverses the suppression of circ_0134944 on osteogenic differentiation in BMSCs.

3.5. MiR-127-5p interacted with PDX1 to modulate SPHK1-mediated osteogenic differentiation

It has been demonstrated that circRNAs can compete for the same pool of miRNA with specific mRNAs, thereby sponging the

effects of the miRNAs on the target mRNAs [19,20]. According to our bioinformation analysis, PDX1 has one binding site with miR-127-5p (Fig. 5A), which can compete for the same pool of miR-127-5p with circ_0134944. Together with the results that miR-127-5p counteracted the suppression of circ_0134944 on osteogenesis in BMSCs, we hypothesized that PDX1 might be a downstream molecule of circ_0134944/miR-127-5p axis in osteogenic differentiation.

Dual-luciferase assay was carried out to examine the potential binding between miR-127-5p and PDX1. Overexpression of miR-127-5p suppressed the luciferase activity, while mutated PDX1

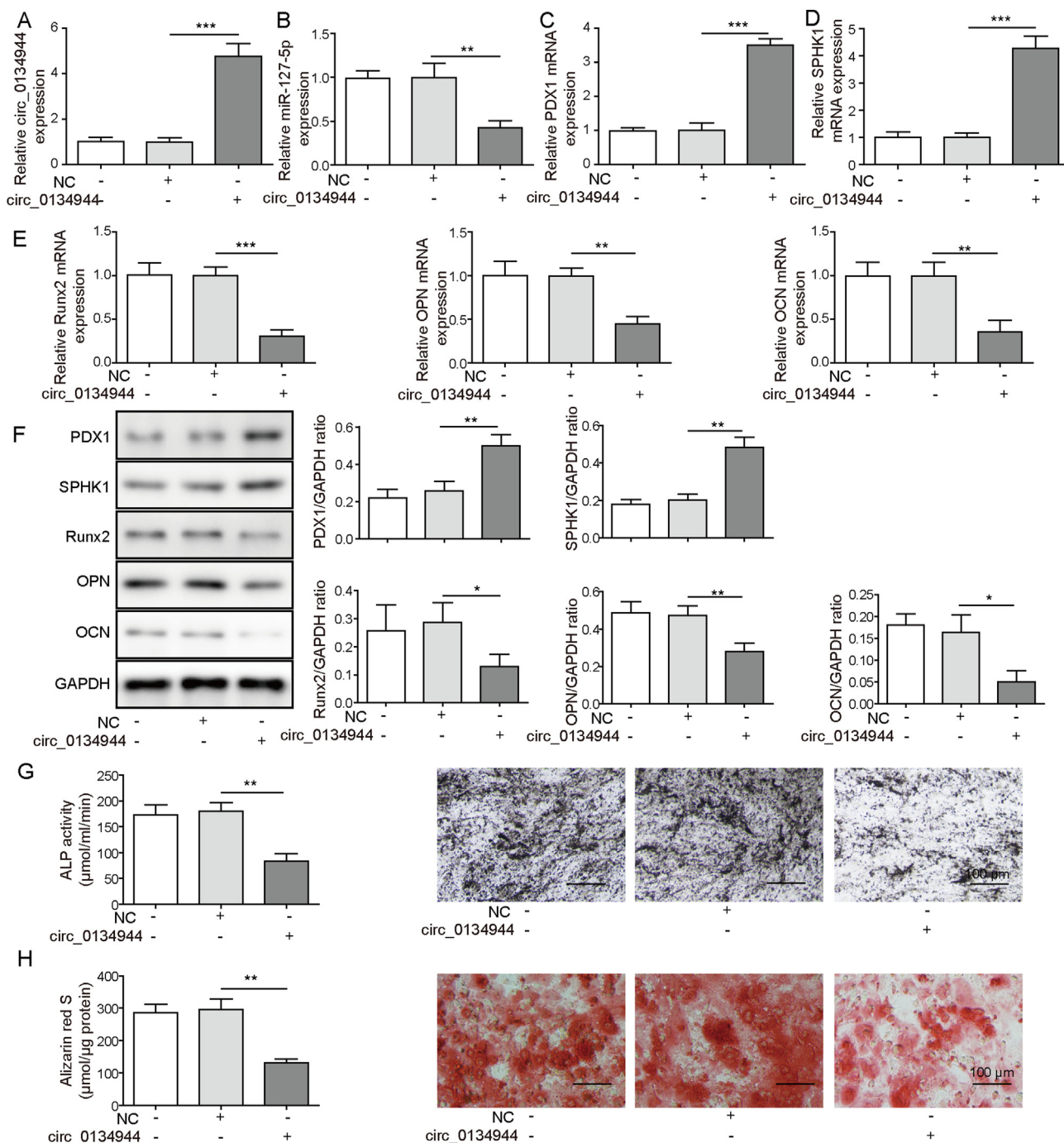


Fig. 3. Circ_0134944 overexpression suppressed osteogenic differentiation of BMSCs. Circ_0134944 was overexpressed by transfecting circ_0134944 plasmids. BMSCs were incubated with osteogenic differentiation medium for 14 days. (A–D) The expression of circ_0134944, miR-127-5p, PDX1 and SPHK1 were determined by qRT-PCR. (E) The expression of Runx2, OPN and OCN were determined by qRT-PCR. (F) The protein levels of PDX1, SPHK1, Runx2, OPN and OCN in BMSCs were determined by Western blotting. GAPDH was used as an internal control. (G) Quantitative ALP activity (left) and ALP staining (right) of BMSCs on day 14. (H) Quantitative Alizarin red S level (left) and ARS staining (right) of BMSCs on day 14. N = 6. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

prevented this repression (Fig. 5B). Moreover, PDX1 overexpression significantly increased the expression of SPHK1, which was blocked by miR-127-5p mimics in BMSCs (Fig. 5C). These results highlighted the regulation of miR-127-5p on SPHK1 via PDX1. PDX1, as a transcription factor, has been known to regulate the activity of multiple genes by binding to their promoter regions [21,22]. Here, we explored whether PDX1 regulated the expression of SPHK1. PDX1 was knocked down using shRNA, and the expression of PDX1 and

SPHK1 were determined. Western blotting results showed that PDX1 shRNA effectively decreased the expression of PDX1 and SPHK1 (Fig. 5D). We further validated the binding between PDX1 and SPHK1 promoter region with ChIP assay. The results showed that, PDX1 was enriched in SPHK1 promoter region while PDX1 knockdown blocked the enrichment (Fig. 5E). The binding between PDX1 and SPHK1 promoter region was further supported by the result that PDX1 was only recruited in wild type SPHK1 promoter

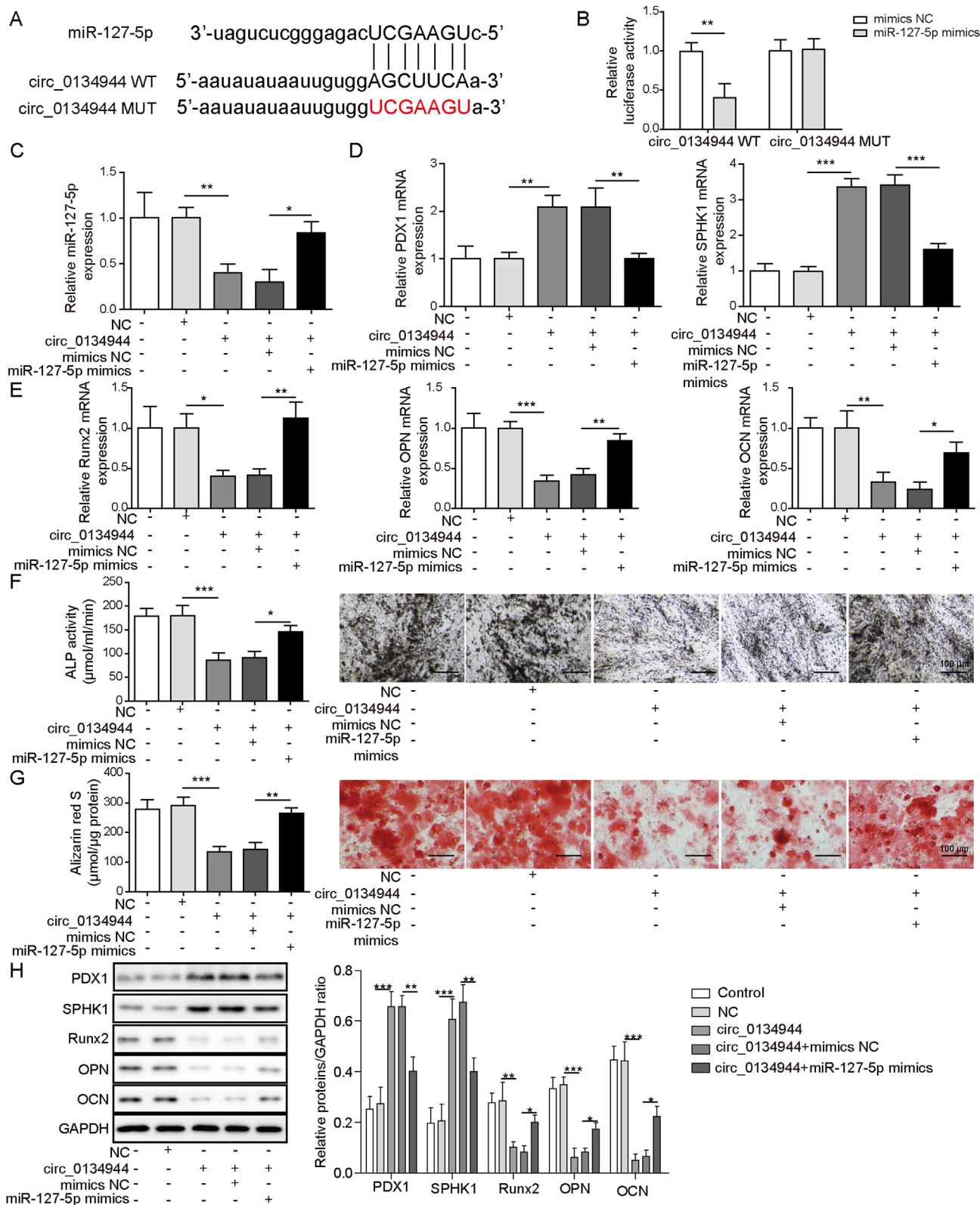


Fig. 4. Circ_0134944 bound with miR-127-5p to modulate osteogenesis of BMSCs. (A) Binding sites between circ_0134944 and miR-127-5p were predicted by circinteractome. (B) The association between miR-127-5p and circ_0134944 was validated by dual-luciferase reporter assay. The levels of miR-127-5p (C), PDX1 and SPHK1 (D) in BMSCs transfected with circ_0134944 and/or miR-127-5p mimics were determined by qRT-PCR. (E) The expression of osteogenesis related markers Runx2, OPN and OCN was determined by qRT-PCR. (F) ALP expression was both examined by quantitative ALP activity assay (left) and qualitative ALP staining (right). (G) Mineralization was confirmed both by quantitative ARS activity assay (left) and qualitative ARS staining (right). (H) The protein levels of PDX1, SPHK1, Runx2, OCN and OPN were determined by Western blotting. GAPDH was used as an internal control. For C–H, BMSCs were transfected with circ_0134944 plasmids and/or miR-127-5p plasmids for 2 days, and then incubated with osteogenic differentiation medium for 14 days. Wt, wild type; MUT, mutated type. N = 6. *P < 0.05; **P < 0.01; ***P < 0.001.

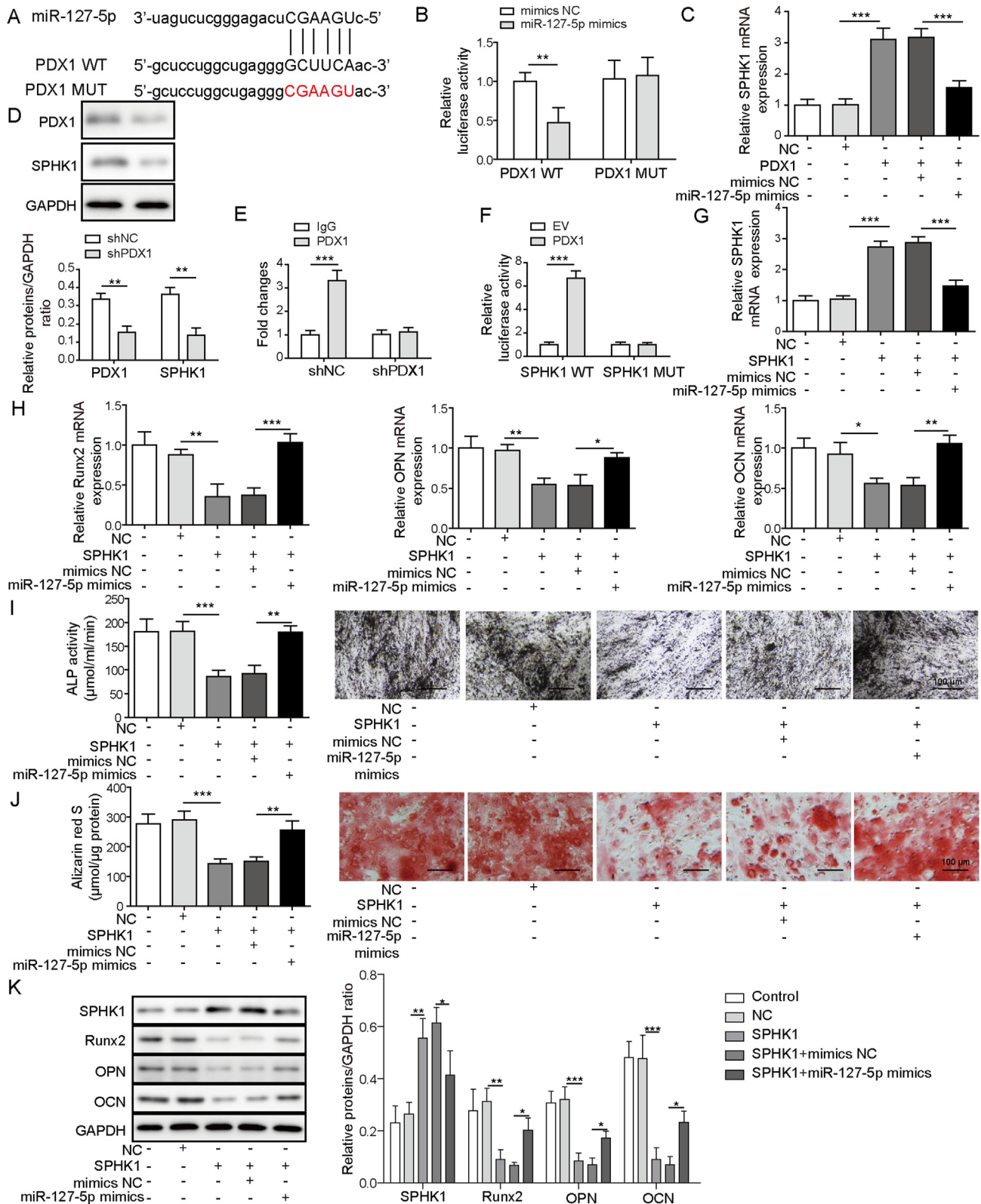


Fig. 5. MiR-127-5p bound with and suppressed SPHK1 to modulate osteogenic differentiation. (A) Binding sites between PDX1 and miR-127-5p were predicted by StarBase. (B) The association between miR-127-5p and 3'UTR of PDX1 was validated by dual-luciferase reporter assay. (C) The levels of SPHK1 in BMSCs transfected with PDX1 and/or miR-127-5p mimics were determined by qRT-PCR. (D) The protein levels of PDX1 and SPHK1 in cells transfected with shRNA against PDX1 were determined by Western blotting. GAPDH was used as an internal control. (E, F) The binding between the promoter region of SPHK1 and PDX1 was confirmed with ChIP assay and dual-luciferase reporter assay. For E, cells were transfected with negative control shRNAs or shRNAs against PDX1. For F, cells were transfected with wild-type SPHK1 or mutated-SPHK1. (G) The mRNA levels of SPHK1 in BMSCs transfected with SPHK1 and/or miR-127-5p mimics were determined by qRT-PCR. (H) The expression of osteogenesis related markers Runx2, OPN and OCN were determined by qRT-PCR. (I) ALP expression was both examined by quantitative ALP activity assay (left) and qualitative ALP staining (right). (J) Mineralization was confirmed both by quantitative ARS activity assay (left) and qualitative ARS staining (right). (K) The protein levels of SPHK1, Runx2, OCN and OPN were determined by Western blotting. GAPDH was used as an internal control. For G-K, BMSCs were transfected with SPHK1 and/or miR-127-5p plasmids for 2 days, and then incubated with osteogenic differentiation medium for 14 days. shNC, negative control shRNA; Wt, wild type; MUT, mutated type. N = 6. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

region; in mutated SPHK1 promoter, PDX1 was not recruited (Fig. 5F). Next, the regulatory role of SPHK1 on osteogenic differentiation was investigated with SPHK1 overexpression. As shown in Fig. 5G and H, SPHK1 overexpression increased the expression of SPHK1, while decreased the expression of Runx2, OPN and OCN, which were all reversed by adding miR-127-5p mimics. ALP staining and activity assay, and Alizarin red S staining also confirmed that miR-127-5p overexpression counteracted the suppression effect of SPHK1 on osteogenesis (Fig. 5I and J). Besides, Western blotting assay showed that miR-127-5p overexpression restored the expression of Runx2, OPN and OCN in SPHK1-overexpressed cells (Fig. 5K). Thus, these data demonstrate that miR-127-5p maintains osteogenic differentiation of BMSCs by counteracting PDX1/SPHK1 pathway.

4. Discussion

Insufficient osteogenesis of BMSCs to counteract bone resorption is a vital cause of PMOP [23]. Hence, it's of significant importance to reveal underlying mechanisms regulating the osteogenic differentiation of BMSCs. In the present study, we investigated the role of circ_0134944 in this process. We found that circ_0134944 suppressed the osteogenic differentiation of BMSCs. Moreover, circ_0134944 acted as a ceRNA of miR-127-5p to regulate SPHK1, which generated S1P. The miR-127-5p/PDX1/SPHK1 signaling was confirmed to regulate osteogenesis. Taken together, our study has identified a novel role of circ_0134944 in osteogenic differentiation of BMSCs and the underlying mechanism.

Circ_0134944 is a rarely explored circRNA which has recently been reported to be upregulated in osteoporosis [13]. However, its biological functions in osteogenesis remain largely unknown. Here, we also observed the aberrant expression of circ_0134944 in PMOP patients and OVX mice. Our results demonstrated that circ_0134944 was upregulated during osteoblast differentiation, indicating that circ_0134944 was possibly involved in regulating osteoblastogenesis. Subsequently, we overexpressed circ_0134944, and the results of ALP and Alizarin red S staining showed a considerable repression of osteogenic differentiation. These data suggest that circ_0134944 suppresses osteoblast formation. To our knowledge, this is the first research reporting the essential role of circ_0134944 in osteogenesis of BMSCs.

Recently, mounting evidences have shown that there is a novel regulatory mechanism between circRNAs and miRNAs. CircRNAs can indirectly regulate protein-coding RNA by binding miRNAs via shared binding sites [20]. To elucidate the molecular mechanism of circ_0134944 in osteoporosis, bioinformatics analysis was performed to explore the potential targeted gene of circ_0134944. Our results showed that miR-127-5p was a target of circ_0134944. Moreover, bioinformatic analysis, dual-luciferase assay results demonstrated that circ_0134944 and PDX1 share a same binding site on miR-127-5p. An earlier paper has reported the aberrant expression of miR-127-5p during osteogenic differentiation [24]. Kuang et al. also reported that miR-127-5p enhanced osteogenesis by interacting with PTEN/AKT axis [25]. Our findings also reveal the dominant role of miR-127-5p in PMOP: miR-127-5p was aberrantly expressed in PMOP patients and in BMSCs during osteogenic differentiation; restoring miR-127-5p to its normal level with miR-127-5p mimics could completely reverse the inhibition of osteogenesis induced by circ_0134944 overexpression; and miR-127-5p overexpression could only partially prevented the increase in SPHK1 level, but almost completely reversed the osteogenesis-suppressive effect of SPHK1 overexpression. These data showed that miR-127-5p might exert a regulative role on osteogenesis by modulating multiple pathways. In line with this hypothesis, several negative regulators of osteogenesis has been identified as the target

for miR-127-5p, like Gremlin2 [26], which is a bone morphogenetic protein antagonists and suppresses osteogenesis in BMSCs [27], and PTEN [25]. Further study is warranted to reveal other mechanisms underlying the regulation of miR-127-5p in osteogenesis.

PDX1 is a central transcription factor for pancreatogenesis [28]. Yang et al. firstly uncovered that PDX1 acted as a negative regulator of osteogenesis [18], but the molecular details remained unexplored. Herein, we found PDX1 was upregulated in osteoporosis and share the same binding pool on miR-127-5p with circ_0134944. Furthermore, our study demonstrates that circ_0134944 positively regulate the expression of PDX1 by counteracting miR-127-5p, and miR-127-5p overexpression restored osteogenesis in both circ_0134944-overexpressed cells. Hence, these findings indicate that circ_0134944 acts as a ceRNA of miR-127-5p to regulate the expression of PDX1, thus suppressing osteoblast formation. Moreover, we first revealed that PDX1 functioned as a regulator of SPHK1 by binding to its promoter region. We also found that SPHK1 overexpression significantly attenuated osteogenic differentiation, and this effect was abolished by miR-127-5p overexpression. Thus, we concluded that SPHK1 counteracted the promotive effective of miR-127-5p on osteogenesis by modulating the expression of osteogenic related genes. Thus, our data showed that by suppressing miR-127-5p, circ_0134944 increased the expression of PDX1 and upregulated the levels of SPHK1, finally suppressing osteogenesis.

In summary, we have established a novel role of circ_0134944 in PMOP. We have also identified the interaction between circ_0134944 and miR-127-5p as a crucial regulator of SPHK1/S1P pathway. Furthermore, we first identified the interaction between PDX1 and SPHK1. Moreover, we confirmed the vital role of PDX1 in regulating osteogenesis. These results have provided new insights into the molecular mechanism of osteogenesis, thus shedding new light on a new therapeutic strategy of PMOP through the suppression of circ_0134944 or the activation of miR-127-5p.

Ethics approval

This study was approved by Research Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University. Written informed consent was obtained from all subjects.

All experiments were approved by the Animal Care and Use Committee of the Fifth Affiliated Hospital of Sun Yat-sen University.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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