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LncRNA PCBP1-AS1 induces osteoporosis by sponging miR-126-5p/PAK2 axis

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Aims

Long non-coding RNAs (lncRNAs) act as crucial regulators in osteoporosis (OP). Nonetheless, the effects and potential molecular mechanism of lncRNA PCBP1 Antisense RNA 1 (PCBP1-AS1) on OP remain largely unclear. The aim of this study was to explore the role of lncRNA PCBP1-AS1 in the pathogenesis of OP.

Methods

Using quantitative real-time polymerase chain reaction (qRT-PCR), osteogenesis-related genes (alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), and Runt-related transcription factor 2 (RUNX2)), PCBP1-AS1, microRNA (miR)-126-5p, group I Pak family member p21-activated kinase 2 (PAK2), and their relative expression levels were determined. Western blotting was used to examine the expression of PAK2 protein. Cell Counting Kit-8 (CCK-8) assay was used to measure cell proliferation. To examine the osteogenic differentiation, Alizarin red along with ALP staining was used. RNA immunoprecipitation assay and bioinformatics analysis, as well as a dual-luciferase reporter, were used to study the association between PCBP1-AS1, PAK2, and miR-126-5p.

Results

The expression of PCBP1-AS1 was pre-eminent in OP tissues and decreased throughout the development of human bone marrow-derived mesenchymal stem cells (hBMSCs) into osteoblasts. PCBP1-AS1 knockdown and overexpression respectively promoted and suppressed hBMSC proliferation and osteogenic differentiation capacity. Mechanistically, PCBP1-AS1 sponged miR-126-5p and consequently targeted PAK2. Inhibiting miR-126-5p significantly counteracted the beneficial effects of PCBP1-AS1 or PAK2 knockdown on hBMSCs' ability to differentiate into osteoblasts.

Conclusion

PCBP1-AS1 is responsible for the development of OP and promotes its progression by inducing PAK2 expression via competitively binding to miR-126-5p. PCBP1-AS1 may therefore be a new therapeutic target for OP patients.

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Keywords: PCBP1-AS1, miR-126-5p, PAK2, Osteoporosis

Article focus

- This article focuses on the interplay between PCBP1 Antisense RNA 1 (PCBP1-AS1) and osteoporosis (OP), and details the potential molecular mechanism of PCBP1-AS1 in OP.

Key messages

- PCBP1-AS1 promotes the development of OP by inducing group I Pak family member p21-activated kinase 2 (PAK2) expression via competitively binding to microRNA (miR)-126-5p.

Strengths and limitations

- This study offers fresh perspectives on the pathophysiology and management of OP, and a potential target in OP therapy.
- Translation to human clinical trials presents substantial challenges.

Introduction

Osteoporosis (OP) is the most prevalent metabolic bone disorder disease that is characterized by decreased bone formation, increased skeletal fragility, and damaged bone tissue microstructure.[1] The morbidity

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and mortality of OP patients increases with ageing, which imposes considerable socioeconomic and health burdens globally.[2] OP is a complex disorder caused by multiple risk factors such as age, alcohol intake, menopause, medication use, cigarette smoking, and sedentary lifestyle.[3,4] OP patients are usually treated with bisphosphonates, oestrogen, teriparatide, tamoxifen, strontium ranelate, vitamin D, calcium, calcitonin, and denosumab.[5,6] However, OP treatment outcomes remain unsatisfactory due to adverse side effects. Novel diagnostic and more effective therapies therefore need to be developed.

Long non-coding RNAs (lncRNAs) are endogenous RNA molecules with a length of more than 200 nucleotides that lack protein-coding potential.[7] LncRNAs have a substantial role in the incidence and progression of OP.[8,9] For example, lncRNA AK045490 reportedly impairs bone formation in an ovariectomized mouse model of OP and, via the β-catenin/T cell factor 1 (TCF1)/Runt-related transcription factor 2 (RUNX2) signalling pathway, blocks osteoblast differentiation in vitro.[10] LncRNA AK077216 promotes osteoclast overactivation via nuclear factor of activated T cells 1 (NFATc1) by inhibiting NFAT-interacting protein 45, and may be an indicator of postmenopausal OP.[11] In addition, Chen et al[12] demonstrated that the X-inactive specific transcript lncRNA facilitates OP and inhibits osteoblast differentiation by regulating nuclear factor erythroid 2-related factor 2 (Nrf2) expression via targeting Cullin 3. LncRNA PCBP1 Antisense RNA 1 (PCBP1-AS1) is an antisense RNA of PCBP1. This lncRNA slows the progression of several cancers that include lung adenocarcinoma, prostate cancer, hepatocellular carcinoma, and thyroid cancer.[13] PCBP1-AS1 inhibits metastasis of lung adenocarcinoma.[13] However, PCBP1-AS1 aggravates progression of hepatocellular carcinoma.[14] These findings suggest that PCBP1-AS1 could have different roles in different cancers. However, the biological function and molecular mechanism of PCBP1-AS1 in OP have not yet been explored.

LncRNAs act as competing endogenous RNAs (ceRNAs) that sponge microRNAs (miRNAs) to control gene expression.[15] Human cancers that are suppressed by miR-126-5p are glioblastomas, non-small cell lung cancer, and colorectal cancer.[16-18] For instance, lncRNA recombinant Thymopoietin (TMPO-AS-1) causes stomach cancer to grow more quickly by upregulating BRCA1-BRCA2-containing complex (BRCC3) and sponging miR-126-5p.[19] Wang and Liu[20] demonstrated the relationship of the miR-126-5p/transcriptional repressor GATA binding 1 (TRPS1) axis regulation by lncRNA KCNQ1OT1 to the development of osteoarthritis. Other evidence demonstrated that miR-126-5p sponged by PCBP1-AS1 can target group I Pak family member p21-activated kinase 2 (PAK2). PAK2 controls a variety of cellular processes including proliferation, cytoskeletal remodelling, mitosis, chromatin modification, apoptosis, and cell motility.[21] In cervical cancer, lncRNA LINC01006 increases PAK2 expression by sponging miR-28-5p and can promote tumours.[22] In addition, lncRNA ZEB1-AS1 accelerates the initiation of colon adenocarcinoma via the miR-455-3p/PAK2 axis.[23] However, so far no studies have addressed the biological functions of PAK2 and miR-126-5p in OP.

In this research, we investigated the role and potential mechanism of PCBP1-AS1 in OP. Mechanistically, we found that this lncRNA influences the miR-126-5p/PAK2 axis. The finding may offer a fresh perspective for investigations of potential OP treatment targets.

Methods

Clinical samples. Bone tissue samples were collected from 26 OP patients (55 to 75 years of age) and 26 age-matched healthy controls (56 to 77 years of age). All participants provided voluntary signed informed consent. OP was diagnosed by dual-energy X-ray absorptiometry, based on a T score < -2.5 standard deviation (SD) and the absence of other diseases. The healthy participants who needed surgery after the accident had no bone disorders and were able to move about freely. All clinical procedures were approved by the Ethical Committee of Chengdu First People's Hospital.

Cell culture and osteogenic differentiation. Human bone marrow-derived mesenchymal stem cells (hBMSCs) purchased from the American Type Culture Collection (ATCC; Manassas, USA; Cat#: PCS-500-012) were cultured in MSC basal medium in a MSC growth kit (both ATCC) containing 7% fetal bovine serum, 15 ng/ml recombinant human insulin-like growth factor 1 (rhIGF-1), 125 pg/ml recombinant human fibroblast growth factor-basic (rhFGF-b), and 2.4 nM L-alanyl-L-glutamine under humid conditions of 95% O_2 and 5% CO_2 at 37°C. For osteoblast differentiation, an osteogenic differentiation medium containing 10 mM β-glycerophosphate (MilliporeSigma, USA), 200 μM ascorbic acid (Beyotime, China), and 100 nM dexamethasone sodium phosphate (MilliporeSigma) was used. The hBMSCs were cultured for 14 days, with the medium replenished every three days.

Quantitative real-time polymerase chain reaction. Total RNA was extracted from tissues or cells using TRIzol reagent (Thermo Fisher Scientific, USA), and then reverse-transcribed into complementary DNA (cDNA) using the Primescript RT Reagent Kit (RR047A; Takara Bio, China). The SYBR Green Kit (Takara Bio) was used in a quantitative real-time polymerase chain reaction (qRT-PCR) experiment using an ABI7500 PCR System (Applied Biosystems, USA). The miRNA internal control was U6. To evaluate the relative expression of lncRNAs and messenger RNAs (mRNAs), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The $2^{-\Delta\Delta Ct}$ procedure was used to calculate the relative expression levels.[24] The primer sequences are provided in Supplementary Table i.

Alkaline phosphatase assay. Cells grown in the osteoblastic induction medium for 14 days were washed three times with phosphate-buffered saline (PBS) before being lysed for 30 minutes in pre-chilled, 1% Triton X-100 on ice. After repeated freeze-thaw cycles, the samples were centrifuged at 600 ×g for ten minutes at 4°C. Each cell

lysate was added to the alkaline phosphatase (ALP) assay kit (Nanjing Jiancheng Bioengineering Institute, China). Measurements and calculations were performed using a 450 nm optical microscope reader (Promega, USA).

Alizarin red staining. Alizarin red was used to analyze osteogenesis in hBMSCs. According to the manufacturer's instructions (Cyagen Biosciences, USA), 4% neutral formaldehyde was used to fix the induced cells at room temperature for 30 minutes. The stain solution (Cyagen Biosciences) was applied after three PBS washes and left for five minutes. Stained cells were selected randomly for examination using a light microscope (Nikon, Japan).

Nuclear/cytoplasmic fractionation assay. The nuclear and cytoplasmic fractions of hBMSCs were prepared using the Nuclear and Cytoplasmic RNA Purification Kit (Norgen Biotek, Canada). The distribution of the PCBP1-AS1 was determined by RT-qPCR using the RNA from both fractions. The internal references were GAPDH for cytoplasm and U6 for nuclei.

Cell transfection. PCBP1-AS1 small interfering RNA (siRNA) (si-lnc), PCBP1-AS1 overexpression plasmids pcDNA3.1-PCBP1-AS1 (OE-lnc) and pcDNA3.1 (empty vector, OE-NC), miR-126-5p mimic, PAK2 siRNA (si-PAK2), mimic-NC, miR-126-5p inhibitor (inhibitor), inhibitor-NC, and negative control (si-NC) were obtained from GenePharma (China). Lipofectamine 2000 (Invitrogen, USA) was used to transfect hBMSCs with the vectors for 48 hours. The effectiveness of the transfection was assessed by RT-qPCR. The transfected cells were subsequently used in several investigations.

Western blotting analysis. Radioimmunoprecipitation assay (RIPA) lysis buffer was used to separate the cells (Beyotime). Protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Beyotime). The proteins in each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were electrotransferred to polyvinylidene fluoride membranes (MilliporeSigma). The membranes were blocked using a solution of 5% non-fat dried milk in Tris buffered saline-Tween (TBST; Thermo Fisher Scientific) applied for one hour at room temperature. Primary antibodies to PAK2 (Cat.#: 2615, 1:1,000; Cell Signaling Technology, USA) and GAPDH (Cat.#: 5174, 1:1,000; Cell Signaling Technology) were incubated overnight at 4°C. Following washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cat#: 14708, 1:2,000; Cell Signaling Technology) at room temperature for two hours. Target protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Aspen Biopharma Labs, India). ImageJ software (National Institutes of Health (NIH), USA) was used to quantitatively examine the protein bands.

Cell Counting Kit-8 assay. Transfected cells were added to wells of a 96-well plate at a density of 3×10^3 cells per well. A total of 10 µl of CCK-8 reagent (Dojindo Laboratories, Japan) was added to each well and incubated for a further four hours after 24, 48, and 72 hours. The optical density (OD) at 450 nm was measured with a Synergy H1 microplate reader (BioTek Instruments, USA).

Bioinformatic analysis. The GSE91033 miRNA microarray and GSE35958 mRNA microarray (Gene Expression Omnibus (GEO) DataSets) were used to screen the downregulated miRNAs and upregulated mRNAs in OP. Starbase 2.0 (http://starbase.sysu.edu.cn)[25] and TargetscanHuman 7.2[26] were used to predict the binding of miRNAs to lncRNA and mRNA. STRING[27] was used for protein-protein interaction analysis.

Luciferase reporter assay. The recombinant dual-luciferase reporter vectors synthesized by GenePharma included PCBP1-AS1-wild-type (WT), PCBP1-AS1-mutant (MUT), PAK2-WT, and PAK2-MUT. MUT or WT PCBP1-AS1 or PAK2 binding miR-126-5p was subcloned into a pGL3 basic vector obtained from Promega. Lipofectamine 2000 was used to co-transfect PCBP1-AS1 WT/MUT and PAK2-3' untranslated region (UTR) WT/MUT with mimic-NC and miR-126-5p mimics. An applied system (Luciferase Assay System; Promega) was applied to analyze the luciferase activity.

RNA immunoprecipitation assay. RNA immunoprecipitation (RIP) assay was performed using the EZMagna RIP-Kit (Millipore). Target cells were lysed in RIP lysis buffer and then conjugated to anti-Argonaute 2 (AGO2) antibody (Millipore) or control anti-immunoglobulin G (IgG) antibody (Millipore) for two hours at 4°C. Purified RNA was analyzed using qRT-PCR after beads were washed with RIP buffer.

RNA pull-down assay. Biotin-labelled miR-126-5p mimic (Bio-miR-126-5p mimic) and Bio-NC purchased from GenePharma were transfected into hBMSCs. The hBMSCs were lysed after 48 hours of transfection and the supernatant was collected after centrifugation. The supernatant was incubated with Streptavidin-Dyna beads (Thermo Fisher Scientific) at 4°C for two hours in a rotator. After repeatedly washing the beads with a lysis buffer, enrichment of PAK2, mitogen-activated protein 3 kinase 2 (MAP3K2), glycogen synthase kinase 3 beta (GSK3B), RUNX2, collagen, type I, alpha 1 (COL1A1), and lysyl oxidase (LOX) genes was detected via qRT-PCR.

Statistical analysis. All data from this work, including at least three replicates for each experiment were analyzed using GraphPad Prism version 7 (GraphPad Software, USA). The results are expressed as mean (SD). Unpaired Student's *t*-tests or one-way analysis of variance was used to analyze differences between groups. Pearson correlation coefficient was used to determine correlation. Statistical significance was defined at $p < 0.05$.

Results

Downregulation of PCBP1-AS1 in osteoblasts and overexpression in differentiated hBMSCs from OP tissues. Our study explored the potential function of PCBP1-AS1 in OP by detecting its expression level in bone tissues from OP patients or healthy donors. PCBP1-AS1 levels were considerably higher in OP patients than in control subjects (Figure 1a). PCBP1-AS1 expression gradually decreased

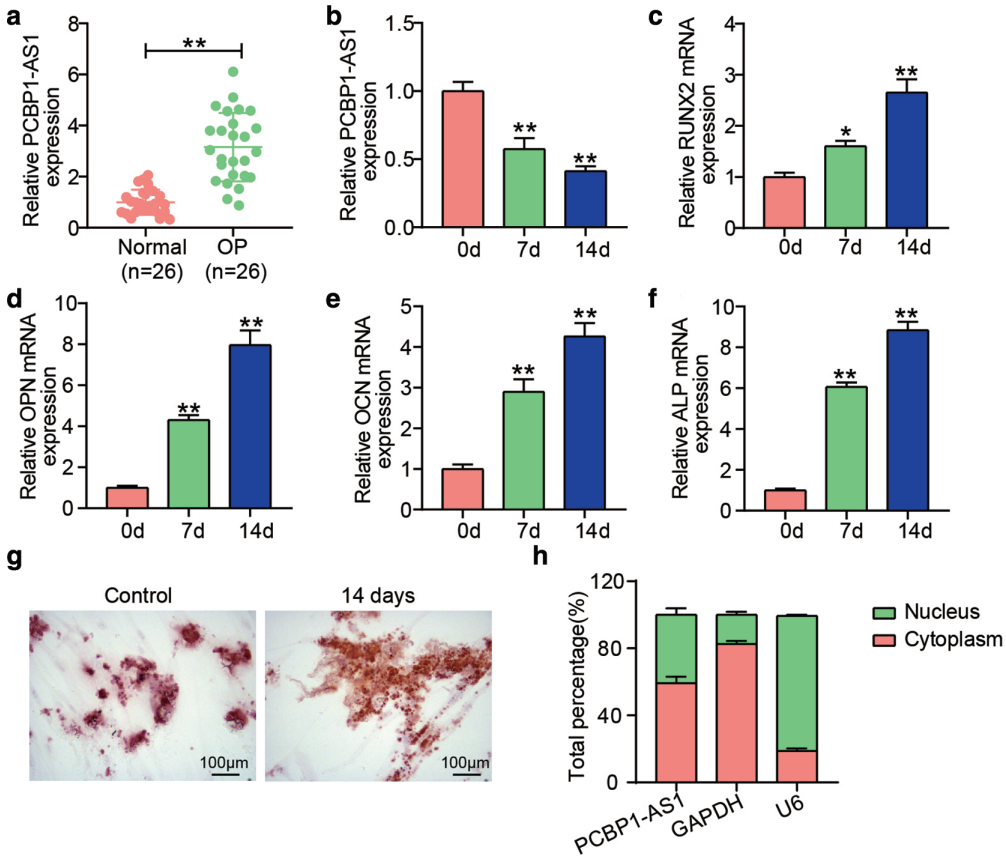

Fig. 1

The expression of PCBP1 Antisense RNA 1 (PCBP1-AS1) was elevated in osteoporosis (OP) tissues and reduced with human bone marrow-derived mesenchymal stem cell (hBMSC) osteoblast development. a) and b) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the amount of PCBP1-AS1 expression in OP tissues and hBMSCs. c) to f) The expression levels of osteogenesis-related genes (Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP)) were detected by qRT-PCR. g) Calcium deposits in hBMSCs were detected by Alizarin red staining. h) By subcellular fractionation and qRT-PCR analysis, the amount of PCBP1-AS1 expression in the nuclear and cytoplasmic fractions of hBMSCs was determined. The data represent the mean (standard deviation). *p < 0.05, **p < 0.001 compared with normal or zero days, calculated with one-way analysis of variance. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA.

during osteoblast differentiation of hBMSCs (Figure 1b). Subsequently, we established an osteoblastic differentiation cell model of hBMSCs. RT-qPCR revealed the gradually increased expressions of osteogenesis-related genes (RUNX2, osteopontin (OPN), osteocalcin (OCN), and ALP) (Figures 1c to 1f). Alizarin red staining showed that calcium deposits of hBMSCs were markedly enhanced at 14 days (Figure 1g). In addition, we detected the subcellular localization of PCBP1-AS1 by qRT-PCR, mainly in the cytoplasm (Figure 1h). These outcomes confirmed that PCBP1-AS1 was overexpressed in OP and downregulated upon the osteoblast differentiation of hBMSCs.

PCBP1-AS1 inhibits osteoblastic differentiation of hBMSCs. To reveal the function of PCBP1-AS1 in the osteoblast differentiation of hBMSCs, PCBP1-AS1 expression was knocked down or overexpressed in hBMSCs using plasmids. The transfection efficiencies were determined by qRT-PCR (Figure 2a). CCK-8 assay data demonstrated that PCBP1-AS1 knockdown and overexpression obviously promoted and inhibited proliferation of hBMSCs, respectively (Figure 2b). Knockdown of PCBP1-AS1 also augmented the expressions of osteogenesis-related genes (OCN, OPN, RUNX2, and ALP). The expressions of these genes were markedly reduced by PCBP1-AS1 overexpression (Figures 2c to 2f). ALP quantification and Alizarin red staining showed that PCBP1-AS1 knockdown and overexpression apparently increased and decreased the abilities of hBMSC osteoblast differentiation, respectively (Figures 2g and 2h). The collective data indicate that PCBP1-AS1 suppresses the osteoblast differentiation of hBMSCs.

miR-126-5p is a direct target of PCBP1-AS1 in OP. To additionally explore the mechanism of PCBP1-AS1 regulating osteoblast differentiation, we investigated the miRNA targets of PCBP1-AS1 using StarBase 2.0. The GSE91033 miRNA microarray was used to screen downregulated miRNAs in OP samples with adjusted $p < 0.05$ and logFC < -1. Three miRNAs (miR-378g, miR-126-5p, and miR-424-5p) overlapped from StarBase and GSE91033 (Figure 3a). After qRT-PCR analysis, miR-378g and miR-126-5p were downregulated in the OP samples (Figure 3b). However, only miR-126-5p expression was negatively correlated

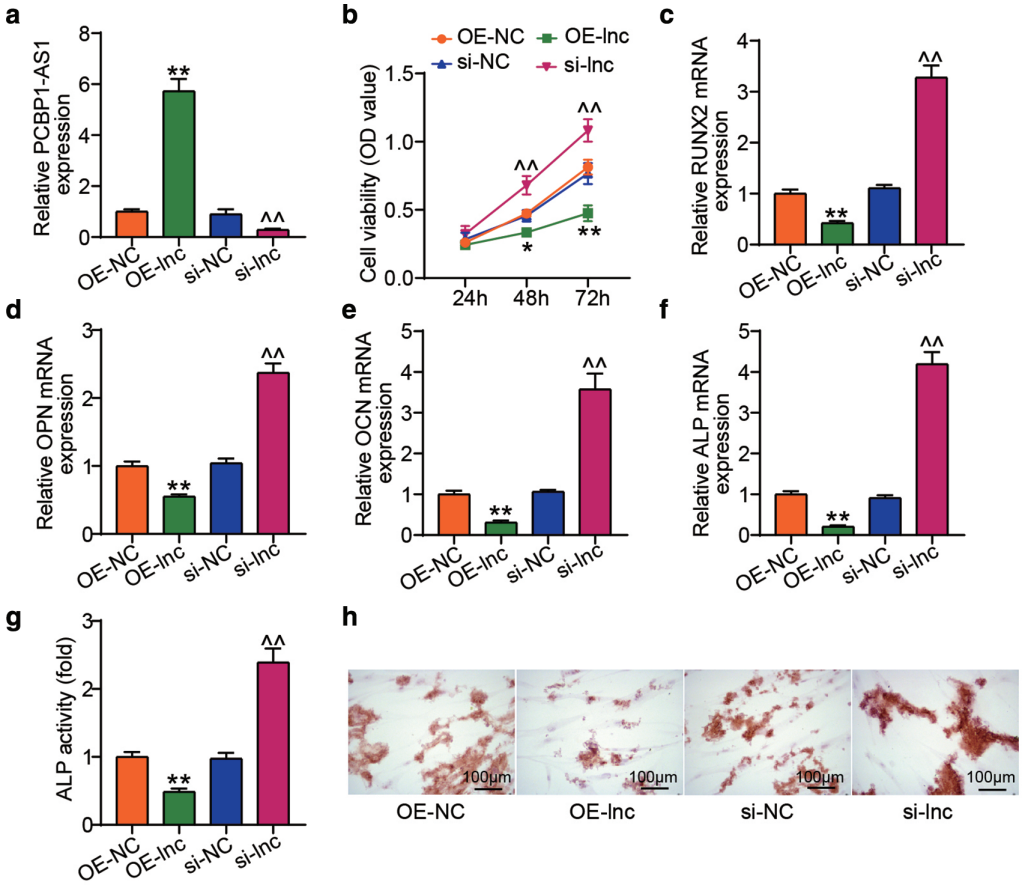

Fig. 2

PCBP1 Antisense RNA 1 (PCBP1-AS1) inhibited human bone marrow-derived mesenchymal stem cell (hBMSC) osteoblast differentiation. a) Transfection efficiency of PCBP1-AS1 overexpression vectors (OE-lnc) or si-PCBP1-AS1 (si-lnc) was recognized by quantitative real-time polymerase chain reaction (qRT-PCR). b) Cell proliferation of hBMSCs was measured by Cell Counting Kit-8 (CCK-8) assay. c) to f) qRT-PCR was used to determine the degree of osteogenesis-related gene expression for the genes Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP). g) ALP quantification identified ALP activity. h) Calcium deposits in hBMSCs were detected by Alizarin red staining. The data represent the mean (standard deviation). *p < 0.05, **p < 0.001 compared with empty vector (OE-NC), ^^p < 0.001 compared with negative control (si-NC); calculated using one-way analysis of variance. mRNA, messenger RNA; OD, optical density.

to PCBP1-AS1 expression in OP samples (Figure 3c). Therefore, miR-126-5p was identified as a miRNA of interest. The two binding sites between miR-126-5p and PCBP1-AS1 are shown in Figure 3d. The experiment revealed that the miR-126-5p mimic and PCBP1-AS1-WT group had the lowest luciferase activity in comparison with other groups (Figure 3e). The RIP test revealed that Ago2 pellets enhanced PCBP1-AS1 and miR-126-5p in contrast to IgG (Figure 3f). PCBP1-AS1 overexpression could inhibit miR-126-5p expression, whereas PCBP1-AS1 knockdown enhanced miR-126-5p expression in hBMSCs (Figure 3g). The expression of miR-126-5p was gradually increased with hBMSC osteoblast differentiation (Figure 3h). In this study, miR-126-5p directly interacted with PCBP1-AS1.

Inhibition of miR-126-5p restores osteoblast differentiation of hBMSCs after PCBP1-AS1 knockdown. To reveal the association of miR-126-5p and PCBP1-AS1, we co-transfected hBMSCs with inhibitor-NC, si-NC, si-PCBP1-AS1 (si-lnc), miR-126-5p inhibitor (inhibitor), or si-PCBP1-AS1 + miR-126-5p inhibitor (si-lnc + inhibitor). The miR-126-5p expression was dramatically boosted by PCBP1-AS1 knockdown but decreased by miR-126-5p inhibitor (Figure 4a). The CCK-8 assay confirmed that miR-126-5p inhibitor clearly inhibited hBMSC proliferation and inverted the impact of PCBP1-AS1 knockdown on the ability of hBMSCs to proliferate (Figure 4b). In addition, miR-126-5p inhibitor reduced the levels of osteogenesis-related genes (OPN, OCN, RUNX2, and ALP), and the up-regulation of osteogenesis-related genes (OPN, RUNX2, OCN, and ALP) by si-PCBP1-AS1 was reversed after miR-126-5p inhibitor transfection (Figures 4c to 4f). Alizarin red staining and ALP measurement showed similar results (Figures 4g and 4h). The findings indicate that miR-126-5p inhibitor impaired the ability of hBMSC osteoblast differentiation, and counteracted the impact of PCBP1-AS1 knockdown on differentiation of hBMSCs to osteoblasts.

miR-126-5p directly targets PAK2 gene. To clarify the possible molecular processes of miR-126-5p in the differentiation of hBMSCs to osteoblasts, the miR-126-5p target

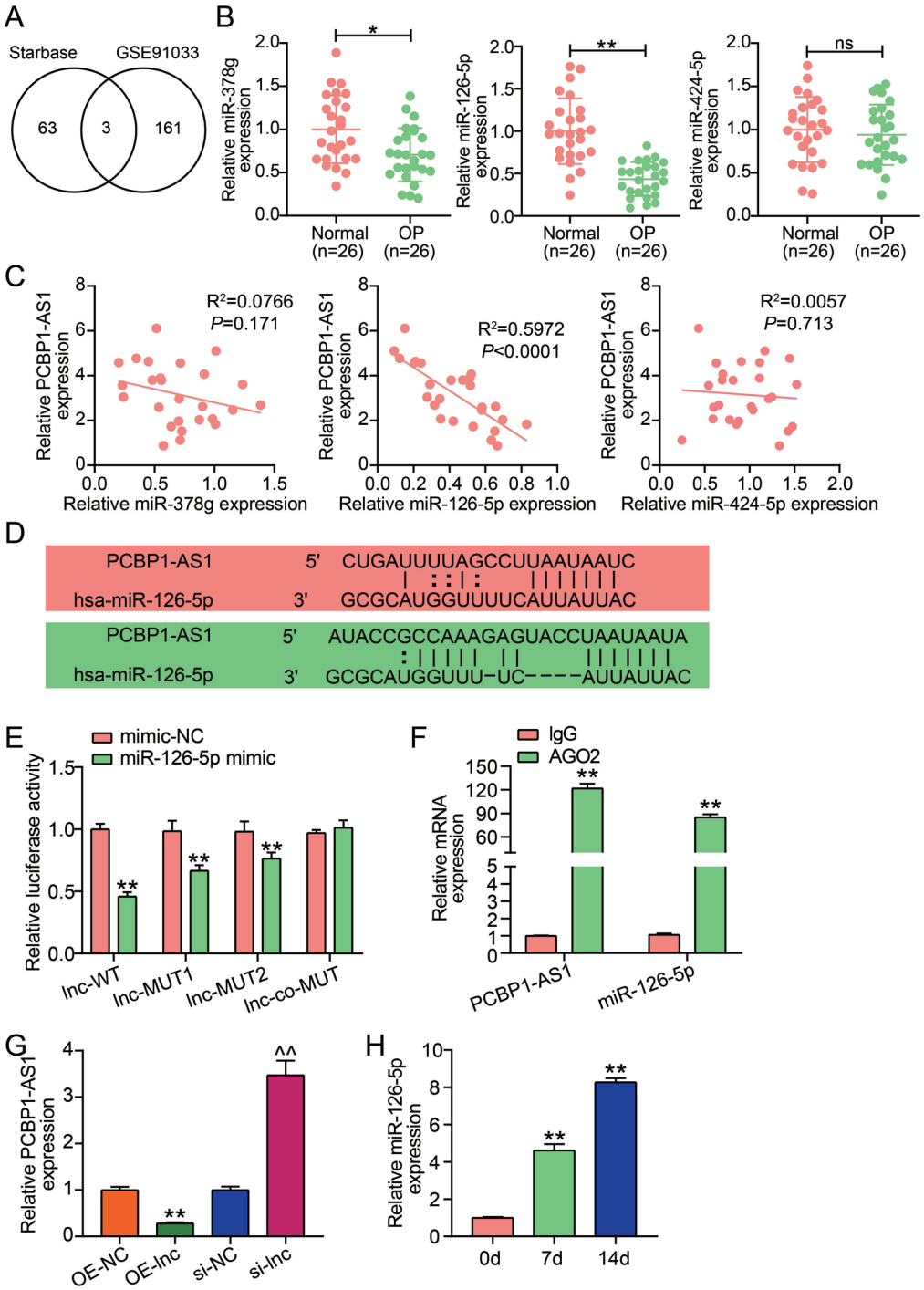

Fig. 3

PCBP1 Antisense RNA 1 (PCBP1-AS1) acted as a sponge for microRNA (miR)-126-5p in human bone marrow-derived mesenchymal stem cells (hBMSCs). a) Three miRNAs were overlapped from Starbase and GSE91033. b) Quantitative real-time polymerase chain reaction (qRT-PCR) detected the levels of miR-378g, miR-126-5p, and miR-424-5p in our osteoporosis (OP) samples. c) The correlation between miR-378g/miR-126-5p/miR-424-5p expression and PCBP1-AS1 expression in OP samples. d) The predicted binding sites of miR-126-5p with PCBP1-AS1 through Starbase. e) In hBMSCs co-transfected with mimic-NC or miR-126-5p mimic and PCBP1-AS1-wild type (WT) or PCBP1-AS1-mutant (MUT), the relative luciferase activity was assessed. f) Binding of miR-126-5p to PCBP1-AS1 was determined by RNA immunoprecipitation (RIP) assay. g) The expression level of miR-126-5p in transfected hBMSCs was detected by qRT-PCR. h) qRT-PCR was applied to determine the miR-126-5p expression level in hBMSCs with osteoblast differentiation. The data signify the mean (standard deviation). **p < 0.001 compared with mimic-NC, immunoglobulin G (IgG), or zero days. ^^p < 0.001 compared with negative control (si-NC). All p-values calculated using one-way analysis of variance. Ago2, Argonaute 2; hsa, Homo sapiens; IgG, immunoglobulin G; lnc, long non-coding; ns, not significant.

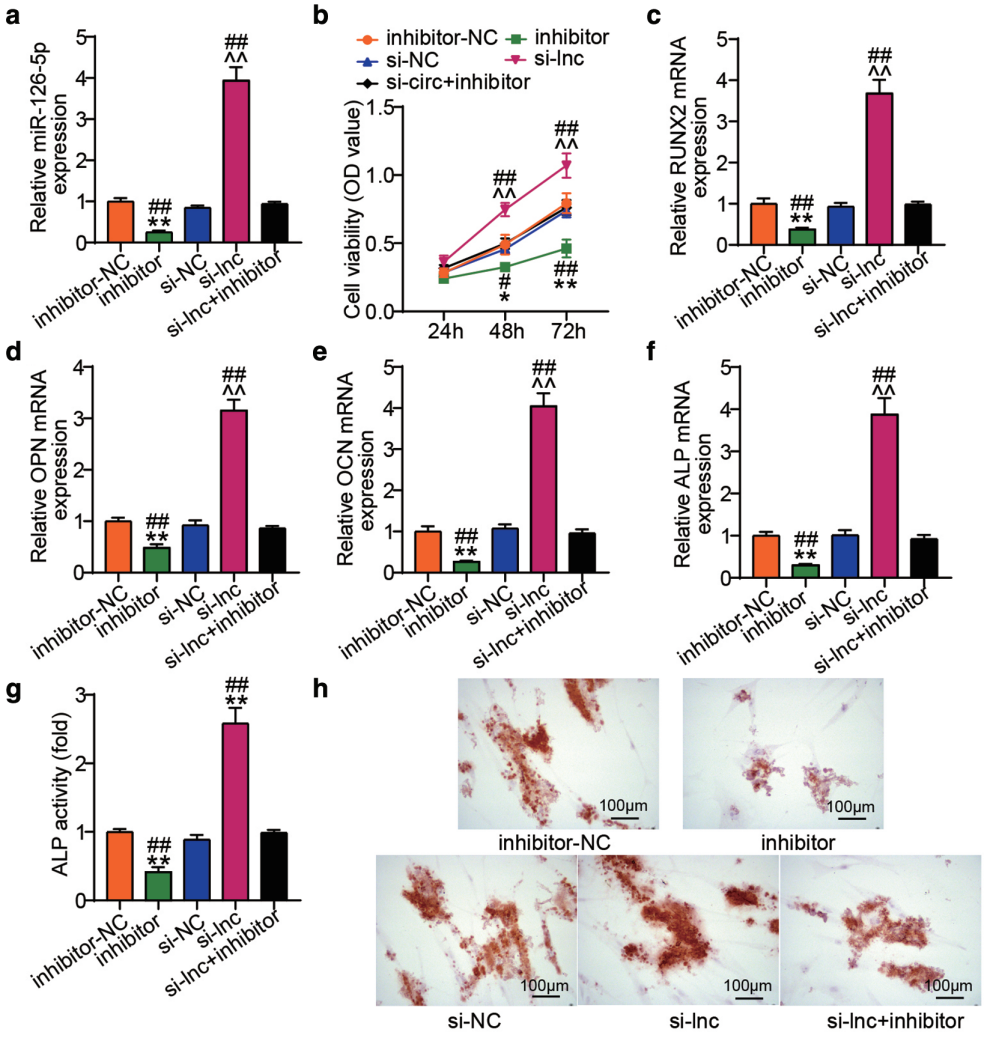

Fig. 4

PCBP1 Antisense RNA 1 (PCBP1-AS1) affected human bone marrow-derived mesenchymal stem cell (hBMSC) osteoblast differentiation by sponging microRNA (miR)-126-5p. hBMSCs were co-transfected hBMSCs with inhibitor-NC, miR-126-5p inhibitor (inhibitor), negative control (si-NC), si-PCBP1-AS1 (si-lnc), or si-PCBP1-AS1 + miR-126-5p inhibitor (si-lnc + inhibitor). a) The expression level of miR-126-5p in hBMSCs was detected by quantitative real-time polymerase chain reaction (qRT-PCR). b) Cell proliferation of hBMSCs was measured by Cell Counting Kit-8 (CCK-8) assay. c) to f) The expression level of osteogenesis-related genes (Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP)) were determined with the help of qRT-PCR. g) ALP activity was determined by ALP quantification. h) Calcium deposits in hBMSCs were detected by Alizarin red staining. The data represent the mean (standard deviation). *p < 0.05, **p < 0.001 compared with inhibitor-NC, ^^p < 0.001 compared with si-NC, #p < 0.05, ##p < 0.001 compared with si-lnc+ inhibitor; calculated using one-way analysis of variance. mRNA, messenger RNA; OD, optical density; si-circ, silence circle RNA.

gene was searched for using the TargetscanHuman 7.2 and StarBase tools. The GSE35958 mRNA microarray was used to identify the upregulated genes in OP samples with adjusted p < 0.05 and logFC > 2. A total of 25 genes overlapped in TargetScan, GSE35958, and StarBase (Figure 5a). The 25 genes were uploaded to STRING for protein-protein interaction analysis. Only six genes were correlated (Supplementary Figure a). RNA pull-down assay data revealed that only PAK2 was enriched in the miR-126-5p mimic group (Figure 5b). Therefore, PAK2 was confirmed as the gene of interest. The two binding sites in the 3′-UTR of PAK2 are shown in Figure 5c. The association between miR-126-5p and PAK2 was further demonstrated by the luciferase reporter assay (Figure 5d). Subsequently, we detected PAK2 expression in OP tissues by qRT-PCR. PAK2 was upregulated in OP tissues (Figure 5e). Expressions of miR-126-5p and PAK2 were negatively correlated in OP tissues (Figure 5f). Additionally, following transfection with inhibitor-NC, mimic-NC, miR-126-5p inhibitor, and miR-126-5p mimic, PAK2 expression level was ascertained by qRT-PCR and western blot. The miR-126-5p mimic suppressed PAK2 expression, whereas miR-126-5p inhibitor promoted PAK2 expression (Figures 5g and 5h). The data implicate the involvement of miR-126-5p with PAK2 via the 3′-UTR site.

miR-126-5p functions by interacting with PAK2. To further confirm the effect of PAK2 on osteoblast differentiation of hBMSCs, a series of cell experiments were performed by transfection with inhibitor-NC, si-NC, miR-126-5p

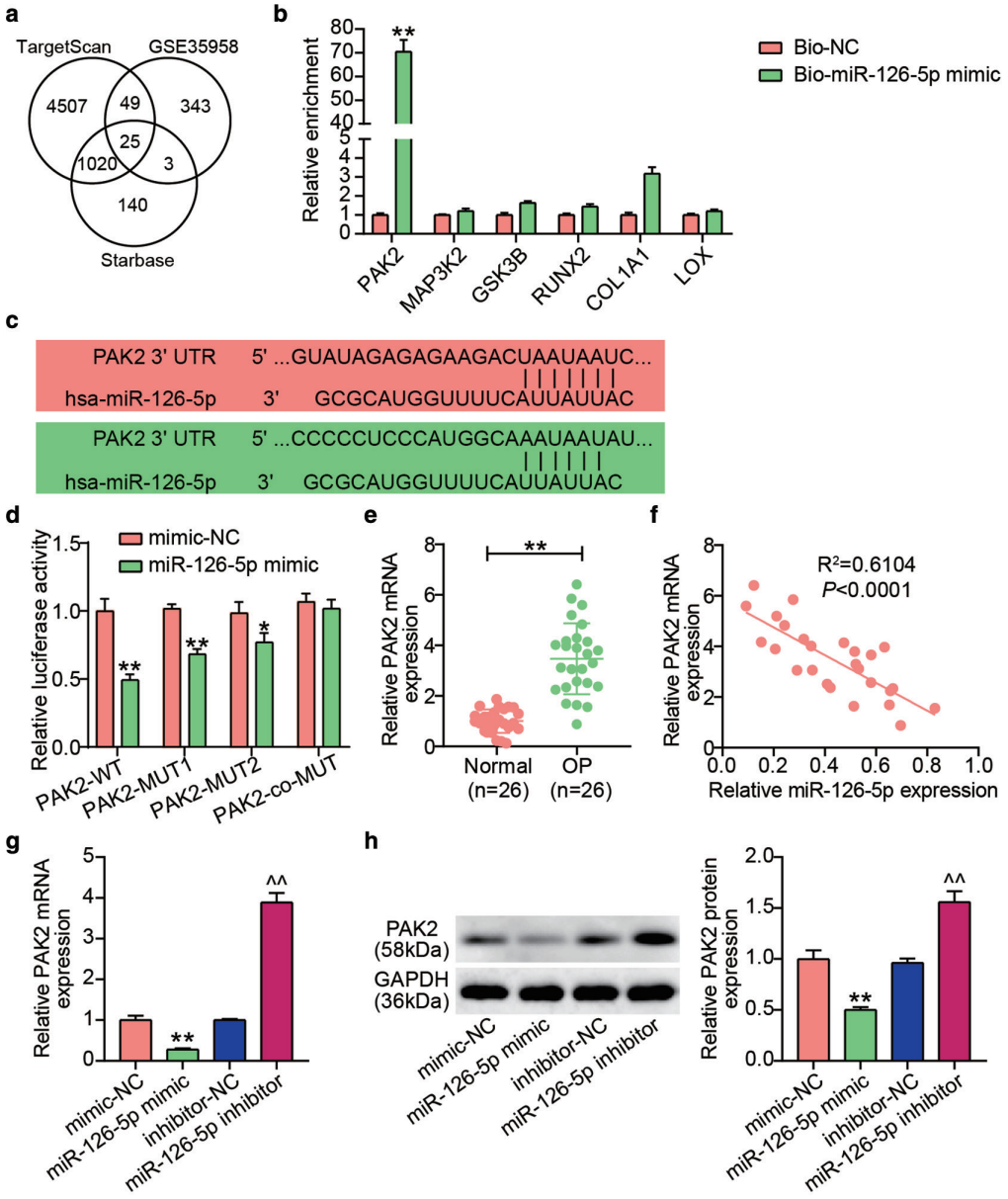

Fig. 5

Group I Pak family member p21-activated kinase 2 (PAK2) was a direct target of microRNA (miR)-126-5p in human bone marrow-derived mesenchymal stem cells (hBMSCs). a) A total of 25 genes were overlapped from TargetScan, GSE35958, and Starbase. b) The enrichments of PAK2, mitogen-activated protein 3 kinase 2 (MAP3K2), glycogen synthase kinase 3 beta (GSK3B), Runt-related transcription factor 2 (RUNX2), collagen, type I, alpha 1 (COL1A1), and lysyl oxidase (LOX) were detected in hBMSCs by RNA pull-down assay. c) Targetscan showed the predicted binding sequences of PAK2 for miR-126-5p. d) The relative luciferase activity was determined in hBMSCs co-transfected with PAK2-wild type (WT) or PAK2-mutant (MUT) and mimic-negative control (NC) or miR-126-5p mimic. e) PAK2 expression in osteoporosis (OP) tissues was determined with quantitative real-time polymerase chain reaction (qRT-PCR). f) A negative association between miR-126-5p and PAK2. g) and h) PAK expression in transfected hBMSCs was measured by g) qRT-PCR and h) western blot. The data represent the mean (standard deviation). *p < 0.05, **p < 0.001 compared with Bio-NC or mimic-NC. ^^p < 0.001 compared with inhibitor-NC. All p-values calculated using one-way analysis of variance. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hsa, Homo sapiens; mRNA, messenger RNA; UTR, untranslated region.

inhibitor, and si-PAK2 or si-PAK2 + miR-126-5p inhibitor. qRT-PCR and western blot analyses showed that PAK2 expression was markedly impaired by si-PAK2, with the impairment released by miR-126-5p inhibitor (Figures 6a and 6b). CCK-8 assay results showed that PAK2 silencing promoted cell proliferation of hBMSCs, although miR-126-5p inhibition may reverse this impact (Figure 6c). Notably, PAK2 suppression resulted in the upregulation of genes linked to osteogenesis (OPN, RUNX2, OCN, and ALP), which was reversed by the addition of the miR-126-5p inhibitor (Figures 6d to 6g). ALP quantification and Alizarin red staining showed that osteoblastic differentiation was enhanced by si-PAK2 and remained unchanged in miR-126-5p inhibitior/si-PAK2 co-transfection

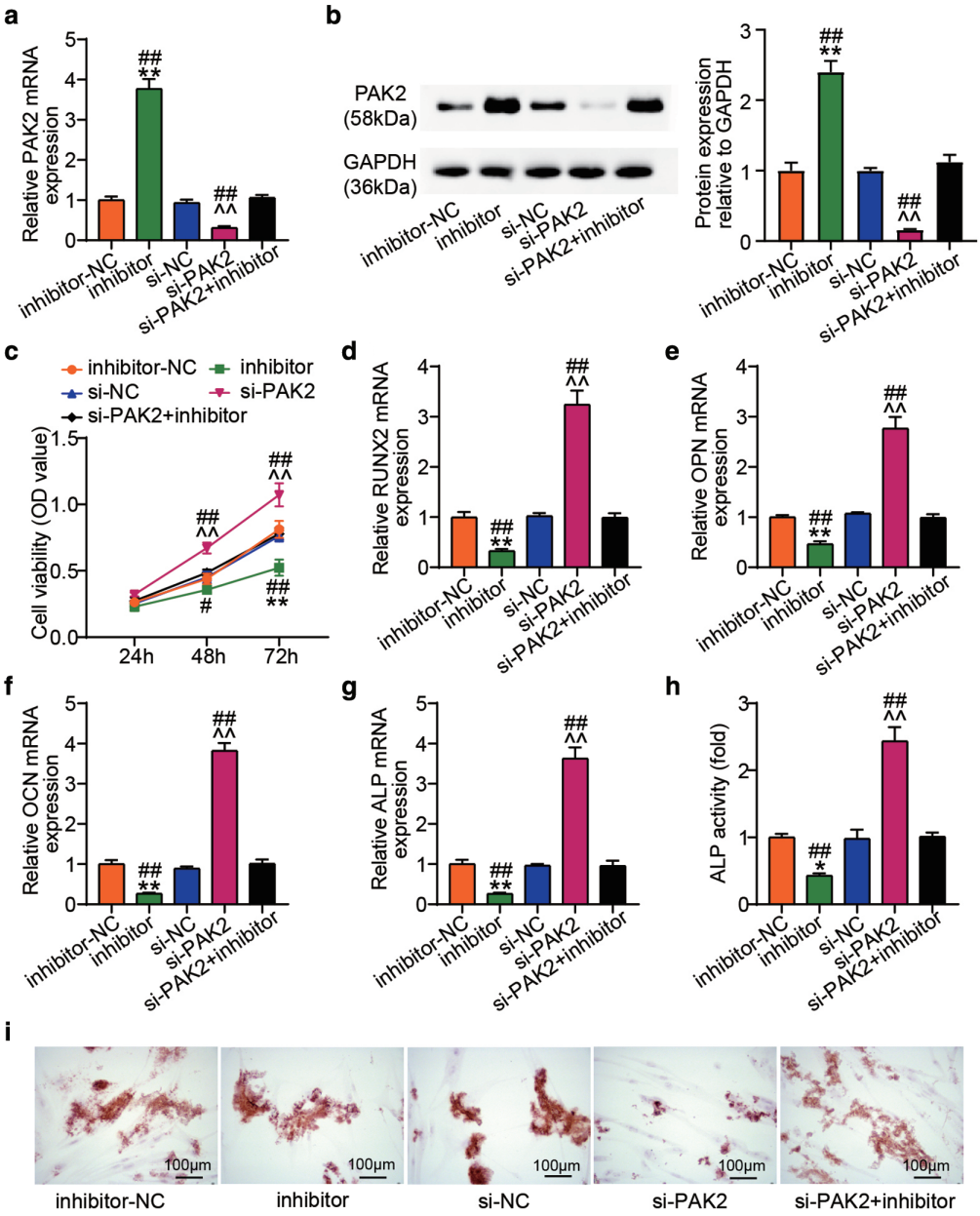

Fig. 6

MicroRNA (miR)-126-5p regulated osteoblast differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) by targeting group I Pak family member p21-activated kinase 2 (PAK2). hBMSCs were co-transfected hBMSCs with inhibitor-NC, negative control (si-NC), miR-126-5p inhibitor (inhibitor), and si-PAK2 or si-PAK2 + miR-126-5p inhibitor (si-PAK2 + inhibitor). a) and b) PAK expression was measured by a) quantitative real-time polymerase chain reaction (qRT-PCR) and b) western blot. c) Cell proliferation of hBMSCs was measured by Cell Counting Kit-8 (CCK-8) assay. d) to g) The expression level of osteogenesis-related genes (Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP)) detected by qRT-PCR. h) ALP activity was detected by ALP staining and quantification. i) Calcium deposits in hBMSCs were detected by Alizarin red staining. The data represent the mean (standard deviation). *p < 0.05, **p < 0.001 compared with inhibitor-NC, ^^p < 0.001 compared with si-NC, #p < 0.05, ##p < 0.001 compared with si-PAK2+ inhibitor; calculated using one-way analysis of variance. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; OD, optical density.

condition (Figures 6h and 6i). These findings indicate that miR-126-5p induces hBMSCs to differentiate to osteoblasts by suppressing PAK2 expression.

Discussion

The findings clarify the expression levels of PCBP1-AS1 in OP tissues and health tissues. The overexpression of PCBP1-AS1 in OP samples affected the development and progression of OP by regulating osteoblast differentiation of hBMSCs. PCBP1-AS1 inhibited cell proliferation and expression of downregulated osteogenesis-related genes in osteoblast differentiation of hBMSCs. Mechanistically, PCBP1-AS1 impaired hBMSC osteoblast differentiation by regulating PAK2 expression due to the sponging of

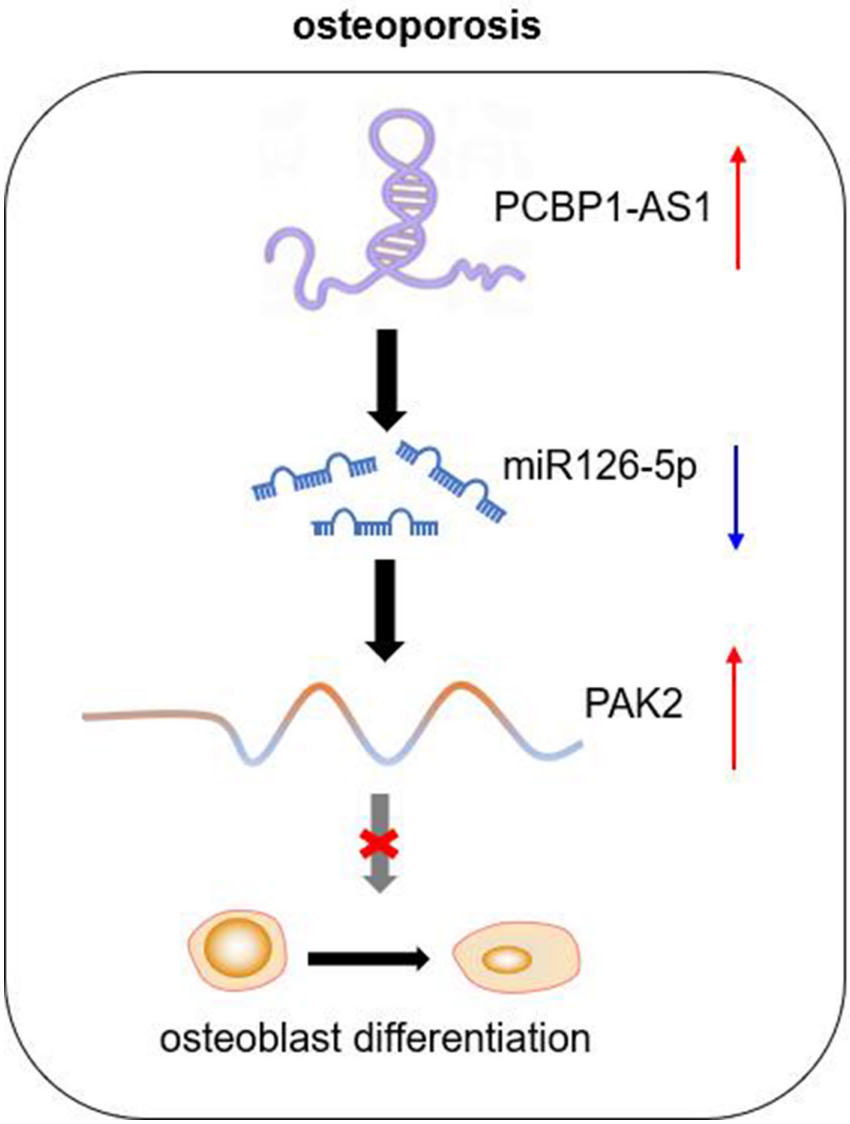

Fig. 7

The mechanism of PCBP1 Antisense RNA 1 (PCBP1-AS1)/microRNA (miR)-126-5p/group I Pak family member p21-activated kinase 2 (PAK2) axis in osteoporosis. The upregulation of PCBP1-AS1 could induce osteoporosis by downregulating miR-126-5p to enhance PAK2 expression.

miR126-5p (Figure 7). The finding implicates PCBP1-AS1 as a possible therapeutic target for OP patients.

OP is a common bone disease that detrimentally affects quality of life. Recent investigations have revealed the participation of lncRNAs in regulating different pathophysiological events in various human diseases, such as OP and cancer.[28] Microarray-based transcriptome profiling initially revealed PCBP1-AS1 in cervical cancer tissues.[29] Subsequently, functioning of this lncRNA has been identified in various cancers. These functions include promoting growth of hepatocellular carcinoma through controlling the PCBP1/PRL-3/AKT signalling pathway[14] and enhancing prostate cancer enzalutamide resistance.[30] The present data are the first evidence that PCBP1-AS1 affects the differentiation of hBMSCs to osteoblasts. We observed that PCBP1-AS1 was upregulated in OP tissues and gradually decreased during osteogenesis. In addition, PCBP1-AS1 knockdown and overexpression promoted and inhibited hBMSC osteoblast differentiation, respectively, implicating PCBP1-AS1 as a possible new diagnostic biomarker of OP.

The role of miRNAs in the regulation of biological events via regulation of functional mRNA expression has been described.[31] Differentiation and cell fate choices of hBMSCs are regulated by miRNAs. For example, miR-19a-3p encourages the osteogenic differentiation of hMSCs by decreasing the expression of histone deacetylase 4.[32] In addition, miR-205-5p inhibits osteogenic differentiation of hBMSCs by regulating RUNX2, facilitating the development of OP.[33] miR-126-5p, an intronic miRNA, has been identified as a tumour suppressor in a variety of human cancer types, including cervical, breast,

and liver cancers.[34–36] Recent research has shown that miR-126-5p can control bone resorption and osteoclast development in giant cell tumours by blocking matrix metalloproteinase-13.[37] Evidence from the present study indicates that miR-126-5p and PCBP1-AS1 might interact chemically. Additionally, miR-126-5p loss can prevent hBMSCs from differentiating into osteoblasts. Furthermore, miR-126-5p may target PAK2.

PAK2 is essential for an abundance of cellular processes.[38,39] Dynactin subunit contributes to osteoclast differentiation by activating the Cdc42/PAK2 signalling pathway.[40] Previous data suggest that PAK2 plays a vital role in bone metabolism. In the present study, PAK2 expression in OP was elevated and inversely linked with miR-126-5p expression. Following knockdown of PAK2, differentiation of hBMSCs was enhanced. This differentiation was stopped by inhibiting miR-126-5p. The findings are evidence of the significance of the miR-126-5p/PAK2 signal in the differentiation of hBMSCs to osteoclasts.

There are multiple limitations in the present study. First, the functions of the PCBP1-AS1/miR-126-5p/PAK2 axis in OP were not determined in vivo. Second, PCBP1-AS1 may influence OP by other channels, such as polyC-RNA-binding protein 1,[14] which needs to be further explored.

In conclusion, PCBP1-AS1 was upregulated in OP and hindered the differentiation of hBMSCs to osteoclasts. PCBP1-AS1 supported OP growth by mechanically functioning as a ceRNA to regulate the expression of PAK2 by sponging miR-126-5p. Our findings could offer fresh perspectives on the pathophysiology and management of OP.

Supplementary material

Table showing the polymerase chain reaction primer sequences used in this study, and figure showing the results of protein-protein interactions of 25 genes by STRING.

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