

# Circ\_0019693 promotes osteogenic differentiation of bone marrow mesenchymal stem cell and enhances osteogenesis-coupled angiogenesis via regulating microRNA-942-5p-targeted purkinje cell protein 4 in the development of osteoporosis

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## ABSTRACT

Circular RNA (circRNA) is a crucial regulator in multiple human diseases, including osteoporosis (OP). However, the function of numerous circRNAs remains unclear. This study aimed to explore the role and mechanism of circ\_0019693 in bone marrow mesenchymal stem cell (BMSC) osteogenic differentiation and osteogenesis-coupled angiogenesis. The expression of circ\_0019693, miR-942-5p and purkinje cell protein 4 (PCP4) was measured using quantitative real-time PCR (qPCR) or Western blot. Osteogenic differentiation was monitored according to the protein levels of RUNX family transcription factor 2 (RUNX2), osteopontin (OPN) and osteocalcin (OCN) by Western blot analysis, and the activity of alkaline phosphatase (ALP). Angiogenesis was evaluated by tube formation assay. The targeting relationship between miR-942-5p and circ\_0019693 or PCP4 was identified using pull-down, dual-luciferase reporter, and RNA immunoprecipitation assays. Circ\_0019693 was downregulated in serum samples and bone tissues from OP patients relative to normal subjects. Circ\_0019693 expression was enhanced in the stages of BMSC osteogenic differentiation. Circ\_0019693 overexpression enhanced the activity of ALP and the expression of RUNX2, OPN and OCN, and its overexpression also promoted angiogenesis. However, circ\_0019693 knockdown played the opposite effects. MiR-942-5p was ensured to be a target of circ\_0019693, and miR-942-5p enrichment reversed the effects of circ\_0019693. In addition, PCP4 was a target of miR-942-5p, and miR-942-5p inhibitor-promoted BMSC osteogenic differentiation and angiogenesis were partly repressed by PCP4 knockdown. In conclusion, circ\_0019693 promotes BMSC osteogenic differentiation osteogenesis-coupled angiogenesis via regulating miR-942-5p-targeted PCP4, thus blocking the development of OP.

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PCP4; osteoporosis;  
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## Introduction

Osteoporosis (OP) is a highly prevalent systemic bone disease, characterized by bone loss and deterioration of bone microstructure [1]. The prevalence of OP is increasing due to the aging of the population [2]. The progression of OP may be attributed to the imbalance between osteoblast bone formation and osteoclast bone resorption [3,4], but its pathogenesis still needs further elucidation. It is considered that bone formation and fracture are linked to moderate osteogenesis and angiogenesis that are often impaired by reduced recruitment of osteoblasts to the damaged area in osteoporosis conditions, leading to nonunion of the fracture [2,5,6]. Nowadays, the promotion of bone marrow mesenchymal stem cell (BMSC) osteogenic

differentiation is regarded as a therapeutic strategy for OP [7,8]. Therefore, it is essential to uncover the molecular mechanisms of BMSC osteogenic differentiation and osteogenesis-coupled angiogenesis.

Non-coding RNAs (ncRNAs) play considerable roles in epigenetics regulation of gene expression in multiple biological processes [9]. Accumulating reports record the pivotal roles of ncRNAs in the progression of OP [10,11]. Circular RNAs (circRNAs) are a class of newly identified ncRNAs, well-known for unique loop-closed structures. Although the role of circRNA is initially underestimated, the function of circRNA in biological processes, especially disease progression, has attracted more and more attention [12]. In

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view of the high stability of circRNAs in tissues, cells and body liquids, circRNAs are believed to be more promising biomarkers compared to other linear RNA molecules [12,13]. The application of circRNA sequencing and bioinformatics proves that certain circRNAs are differently expressed in OP groups compared with that in non-OP groups, hinting that several circRNAs are implicated with the development of OP [14,15]. Progressive researches illustrate that circRNAs regulate BMSC osteogenic differentiation. For example, circ\_0024097 contributed to the osteogenic differentiation of BMSCs via regulating its parental gene and Wnt/ $\beta$ -catenin pathway [16]. Whereas, the functions of multiple OP-related circRNAs on osteogenic differentiation remain unclear. A previous study performed circRNA microarray analysis and identified that circ\_0019693 was one of the upregulated circRNAs in OP serum samples [14]. Given that circ\_0019693 was rarely explored in any diseases, we aimed to investigate the role of circ\_0019693 in the development of OP.

CircRNAs have been widely studied for their molecular sponge effect on target microRNAs (miRNAs). With the prediction of bioinformatics tools [17,18], miRNAs targeted by circ\_0019693 are easily acquired. MiR-942-5p was shown to be involved in BMSC osteogenic differentiation by a recent study [19], and it was also predicted as one of the targets of circ\_0019693. We speculated that circ\_0019693 might regulate BMSC biological phenotypes by targeting miR-942-5p. It is canonical that miRNAs sequester gene expression via combining with gene 3' UTR. Likewise, it is easy to predict the target genes of miRNA by bioinformatics [20]. Purkinje cell protein 4 (PCP4) was predicted as a target of miR-942-5p, and it was declared to be downregulated in rat models of OP [21]. It should be addressed whether the involvement of PCP4 in OP was associated with circ\_0019693-mediated miR-942-5p.

This study for the first time focused on circ\_0019693 and hypothesized that circ\_0019693 played considerable roles in BMSC osteogenic differentiation and osteogenesis-coupled angiogenesis. Besides, we confirmed the interplays between miR-942-5p and circ\_0019693 or PCP4, which provided a new mechanism to explain the role of circ\_0019693. Our study aimed to disclose

the role and regulatory mechanism of circ\_0019693 in OP, further providing the potential management strategy for OP.

## Materials and methods

### Clinical specimen collection

A total of 43 patients with OP (including 27 females and 16 males, age range: 50–78 ages) and 17 normal controls (including 11 females and 6 males, age range: 53–80 ages) from the Second Hospital of Shanxi Medical University were enrolled in this study. Normal controls suffered from external traumatic fracture, without OP symptoms. These patients had no complications or diseases related to bone metabolisms, such as nephropathy and liver disease. Besides, before treatment, none of the patients have experienced any medical therapy. Blood samples and bone tissues were collected from these subjects, with the written informed consent from all subjects. Blood samples were left standing and centrifuged to obtain serum. This study obtained the permission of the Second Hospital of Shanxi Medical University.

### Cell treatment and cell culture

Human BMSCs purchased from Cyagen (Suzhou, China) were cultured in OriCell human BMSC complete medium (No. HUXMA-90011; Cyagen) at 37°C conditions with 5% CO<sub>2</sub>. BMSCs were plated in 6-well plates, and BMSC differentiation was induced by supplying osteogenic differentiation medium (No. HUXMA-90021) in cell culture medium. Osteogenic differentiation medium was renewed every 3 days, and BMSCs were induced for 3 weeks for use.

### Cell transfection

Circ\_0019693 overexpression vector (circ\_0019693) and empty vector control (pCD5-ciR), small interference RNA targeting circ\_0019693 (si-circ\_0019693) and negative control (si-con) were constructed by Genesee (Guangzhou, China). The mimic and inhibitor of miR-942-5p (miR-942-5p and in-miR-942-5p), and their matched negative controls (miR-con and in-miR-con) were purchased

from Ribobio (Guangzhou, China). PCP4 siRNA (si-PCP4) and si-con were also provided by Geneseeed. Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was utilized to perform cell transfection with the guideline of protocol.

### **Quantitative real-time PCR (qPCR)**

Total RNA was isolated by Trizol method (Invitrogen) and quantified by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, HiScript 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) and miRNA cDNA Synthesis Kit (Cwbio, Beijing, China) were applied for reverse transfection. Next, the UltraSYBR Mixture (Cwbio) was used to perform qPCR reaction. Here, GAPDH was used as an internal reference for circRNA and mRNA, and U6 was used as an internal reference for miRNA. Relative expression was obtained with the application of the  $2^{-\Delta\Delta C_t}$  method. Primer sequences were shown as follows:

circ\_0019693, F: 5'-AGTTGCGGACAACCTCTTG-3' and R: 5'-TGCCTTCTCCTCCTCTGAGT-3'; nucleolar and coiled-body phosphoprotein 1 (NOLC1), F: 5'-TTCCTGCGCGATAACCAACT-3' and R: 5'-TGGCAGACTTGAGCCAGAAG-3'; miR-942-5p, F: 5'-GTATGATCTTCTCTGTTTTGGC-3' and R: 5'-CTCAACTGGTGTCGTGGAG-3'; PCP4, F: 5'-TGGGTCTCAGTCTAGTGGG-3' and R: 5'-TGGAGGTTTGCTATGCGTGT-3'; U6, F: 5'-CTCGCTTCGGCAGCACA-3' and R: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH, F: 5'-ACAGCCTCAAGATCATCAGC-3' and R: 5'-GGTCATGAGTCCTTCCACGAT-3'.

### **CircRNA subcellular location**

RNA from cytoplasmic fraction or nuclear fraction was isolated using the PARTIS kit (Invitrogen). The expression of circ\_0019693 in each fraction was measured by qPCR, with  $\beta$ -actin as a control in cytoplasm and U6 as a control in nucleus.

### **CircRNA identification**

CircRNA was resistant to RNase R digestion due to the lack of 3' and 5' tails. Total RNA was

treated with RNase R (BioVision, Milpitas, CA, USA) and then used for qPCR to detect the expression of circ\_0019693 and linear NOLC1.

Oligo(dT)<sub>18</sub> primers (amplifying mRNAs with poly(A) tail) and random primers were purchased from Solarbio (Beijing, China) and used as reverse transcription primers. The synthesized cDNA was used for qPCR to examine the expression of circ\_0019693 and linear NOLC1.

### **Western blot assay**

Western blot assay was carried out to examine the expression of RUNX family transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN) and PCP4 proteins. According to the previous description [22], total protein was extracted using the RIPA method (Solarbio) and then quantified by the BCA method (Solarbio). After separating by 12% SDS-PAGE, proteins were transferred onto PVDF membranes, following by blocking with 5% skim milk. Next, the membranes were cultured with the primary antibodies (Abcam, Cambridge, MA, USA), such as anti-RUNX2 (ab236639; dilution: 1/1000), anti-OPN (ab214050; dilution: 1/1000), anti-OCN (ab133612; dilution: 1/3000), anti-PCP4 (ab197377; dilution: 1/1000) and anti- $\beta$ -actin (ab8227; dilution: 1/3000). Subsequently, the membranes were cultured with HRP-conjugated secondary antibody (ab205718; dilution: 1/5000). The signaling of proteins was observed using the ECL reagent (Solarbio).

### **Alkaline phosphatase (ALP) staining assay**

BMSCs in 96-well plates were cultured in osteogenic differentiation medium for 21 days. Then, cells were washed with PBS and fixed with 4% paraformaldehyde. Next, cells were stained using a BCIP/NBT ALP Color Development Kit (Beyotime, Shanghai, China) according to the protocol. The differentiated cells were stained with blue violet.

### **Tube formation assay**

According to the previous method [23], Matrigel (BD Biosciences, San Jose, CA, USA) was plated into 96-well plates for 1 h at 37°C. The supernatant of BMSCs with various transfections was collected, and HUVECs cultured in the

supernatant of BMSCs were added into the Matrigel-coated 96-well plates. After incubation at 37°C for 4 h, the efficiency of tube formation was visualized under a microscope (Nikon, Tokyo, Japan).

### **Bioinformatics tools**

A total of 3 databases were employed in this study, including starbase (<http://starbase.sysu.edu.cn/>), circular RNA interactome (<https://circinteractome.nia.nih.gov/>), and circbank (<http://www.circbank.cn/>).

### **RNA pull-down assay**

Biotin probes of circ\_0019693 (circ\_0019693 probe) and miR-942-5p (bio-miR-942-5p-WT, containing wild-type miR-942-5p sequence; bio-miR-942-5p-MUT, containing mutant-type miR-942-5p sequence) were provided by Ribobio. The probes were incubated with streptavidin magnetic beads (Thermo Fisher Scientific) for 2 h. BMSCs were lysed and then cultured with probe-coated beads overnight at 4°C. RNA compounds bound to beads were eluted and isolated for qPCR analysis.

### **Dual-luciferase reporter assay**

Luciferase reporter plasmids of circ\_0019693-WT (containing miR-942-5p binding site), circ\_0019693-MUT (containing mutant miR-942-5p binding site), PCP4-3 UTR-WT (containing miR-942-5p binding site), and PCP4-3 UTR-MUT (containing mutant miR-942-5p binding site) were constructed using pmirGLO plasmid (Promega, Madison, WI, USA). BMSCs were cotransfected with circ\_0019693-WT, circ\_0019693-MUT, PCP4-3 UTR-WT or PCP4-3 UTR-MUT and miR-942-5p or miR-con. After culturing for 48 h, luciferase activity was monitored using a Dual-Luciferase Reporter Assay System (Promega).

### **RNA immunoprecipitation (RIP) assay**

RIP assay was conducted in accordance with the protocol from the Magna RIP kit (Millipore, Bedford, MA, USA). BMSCs were lysed and then

incubated with magnetic beads pre-conjugated with the antibody of anti-Ago2 (Millipore) or anti-IgG (control; Millipore) to capture RNA compounds. RNA compounds bound to beads were eluted and isolated for qPCR analysis.

### **Statistical analysis**

Three independent biological experiments were performed to obtain the experimental data, followed by data processing using GraphPad Prism 7.0 (GraphPad, La Jolla, CA, USA). Statistical difference in different groups was analyzed using Student's *t*-test or analysis of variance. The correlation between two groups was analyzed using Pearson's correlation analysis. Data were finally exhibited as the mean  $\pm$  standard deviation. A *P*-value less than 0.05 was defined to be statistical difference.

## **Results**

We reported a circRNA, circ\_0019693, whose expression was aberrantly declined in OP patients, and its expression was gradually increased during BMSC osteogenic differentiation. We conducted gain- and loss-of-function assays to explore the function of circ\_0019693 and monitored that circ\_0019693 overexpression enhanced BMSC osteogenic differentiation and angiogenesis. We further summarized that circ\_0019693 regulated miR-942-5p-targeted PCP4 to promote osteogenesis and angiogenesis, thus affecting the progression of OP.

### **Circ\_0019693 expression was reduced in serum and bone tissues from OP patients**

Expression analysis showed that circ\_0019693 expression was strikingly decreased in serum and bone tissues from OP patients compared with that from normal subjects (Figure 1(a and b)). Circ\_0019693 was derived from the exon6-exon12 of NOLC1 gene by back-splicing, with 1334 bp in length (Figure 1(c)). Subcellular location analysis presented that circ\_0019693 was mainly located in the cytoplasm relative to nucleus (Figure 1(d)). After RNase R digestion, circ\_0019693 expression was rarely degraded by



RNase R, while NOLC1 expression was largely degraded by RNase R, verifying the existence of circ\_0019693 (Figure 1(e)). In addition, circ\_0019693 was hardly amplified by oligo(dT)<sub>18</sub> primers, while NOLC1 could be amplified by random primers and oligo(dT)<sub>18</sub> primers, which verified the circular structure of circ\_0019693 (Figure 1(f)). The data indicated that circ\_0019693 was upregulated in OP.

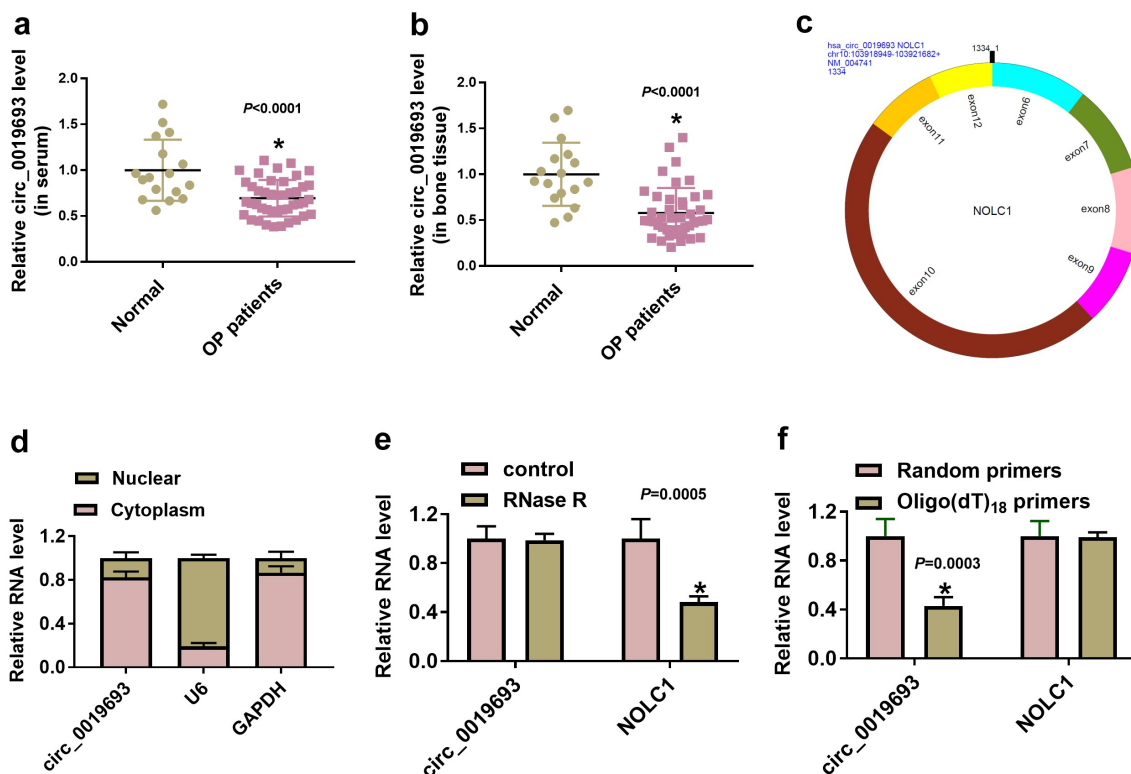
### Circ\_0019693 overexpression promoted BMSC osteogenic differentiation and angiogenesis

The expression of circ\_0019693 was gradually upregulated during the osteogenic differentiation of BMSCs (Figure 2(a)). Gain- or loss-function assays were performed to determine the functional role of circ\_0019693. The expression of circ\_0019693 was markedly strengthened in BMSCs transfected with circ\_0019693, while its expression was markedly reduced in BMSCs transfected with si-circ\_0019693 (Figure 2(b)). Several osteogenic differentiation-related markers were

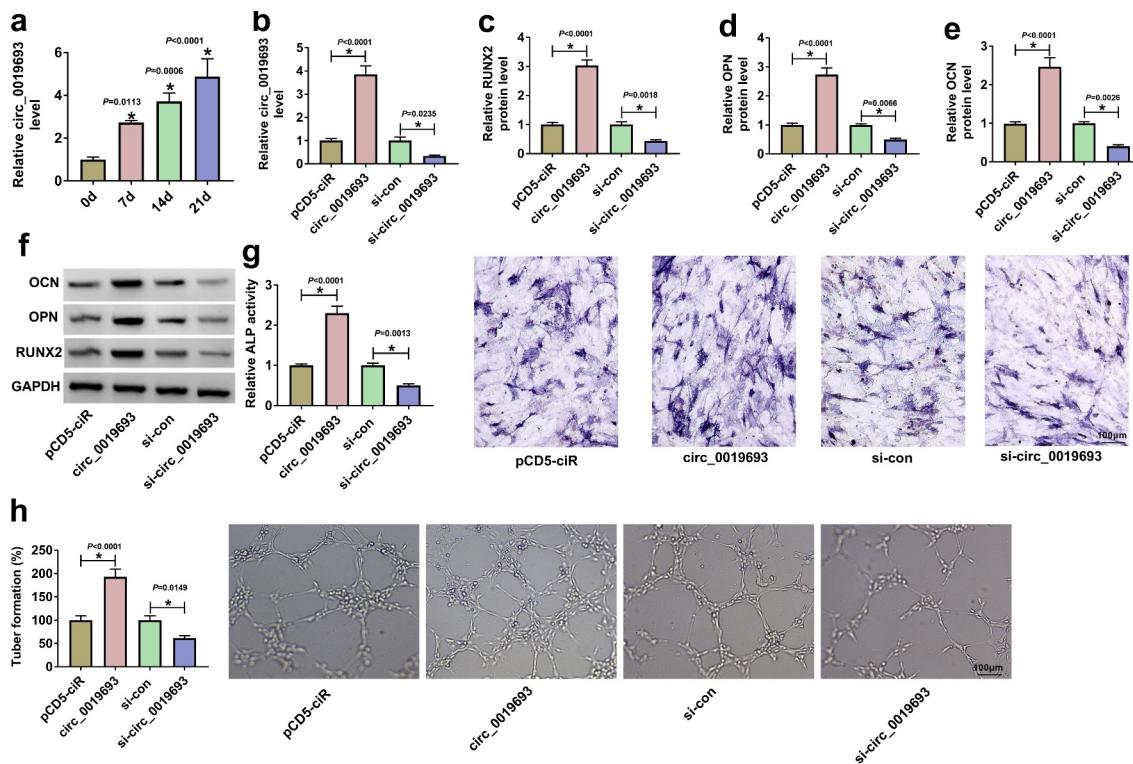
quantified by Western blot. The results presented that the expression of RUNX2, OPN and OCN was notably enhanced in BMSCs with circ\_0019693 overexpression but largely decreased in BMSCs with circ\_0019693 knockdown (Figure 2(c-f)). Besides, ALP activity was shown to be markedly increased in BMSCs with circ\_0019693 overexpression but largely decreased in BMSCs with circ\_0019693 knockdown (Figure 2(g)). Tube formation assay uncovered that circ\_0019693 overexpression promoted tube formation of HUVECs, while circ\_0019693 knockdown blocked tube formation (Figure 2(h)). These data summarized that circ\_0019693 promoted BMSC osteogenic differentiation and angiogenesis.

### Circ\_0019693 targeted and negatively regulated miR-942-5p

Starbase, circular RNA interactome and circbank were utilized to predicted miRNAs targeted by circ\_0019693, and miR-942-5p and miR-432-5p



**Figure 1. Circ\_0019693 expression was reduced in OP serum and bone tissues.** (a and b) Circ\_0019693 expression was detected using qPCR in serum samples and bone tissues. (c) The formation and structure of circ\_0019693. (d) The location of circ\_0019693 in cytoplasm or in nucleus. (e) The stability of circ\_0019693 was checked using RNase R. (f) The existence of circ\_0019693 was ensured using oligo(dT)<sub>18</sub> primers. \* $P < 0.05$ .



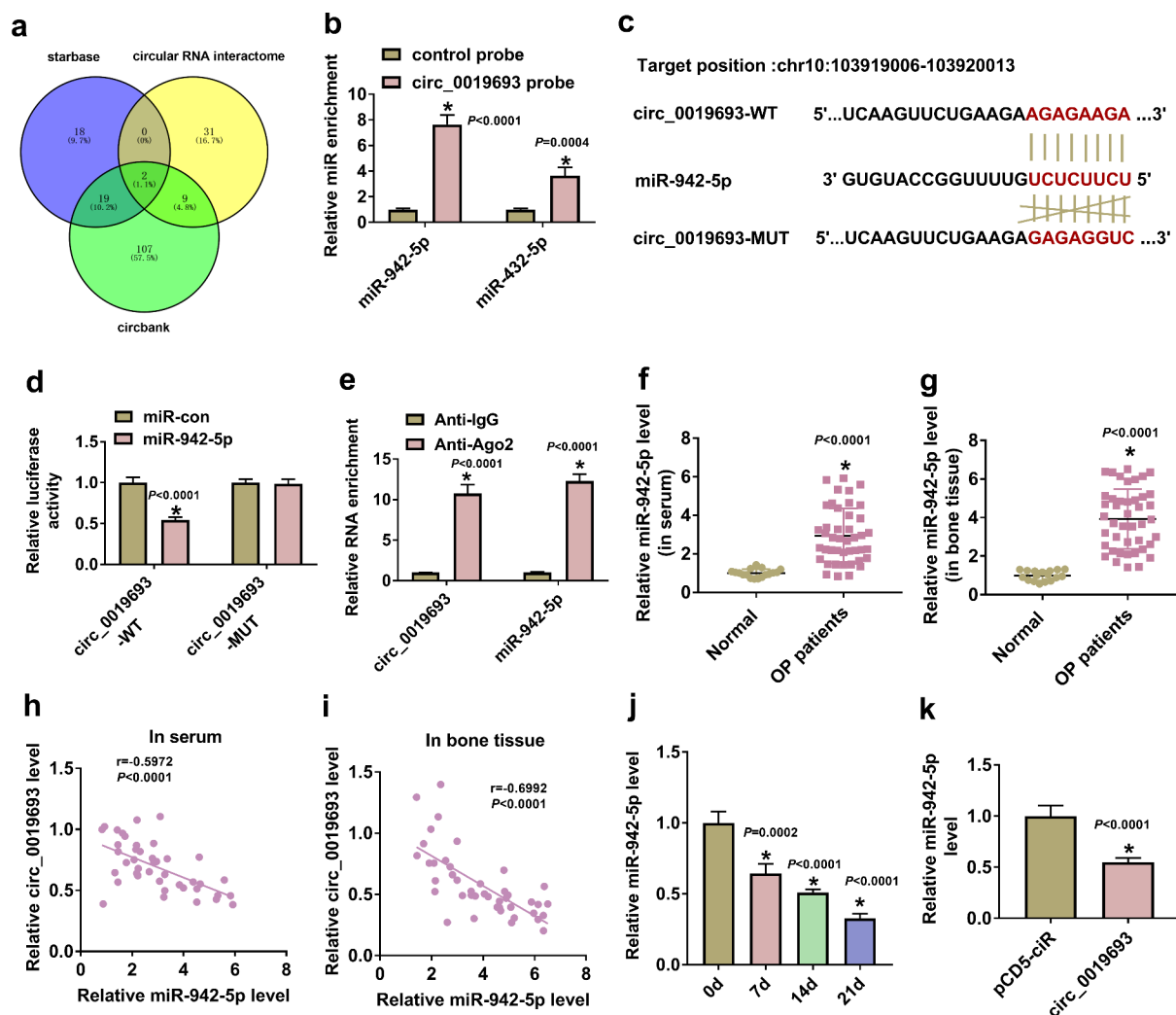
**Figure 2. Circ\_0019693 overexpression promoted BMSC osteogenic differentiation and angiogenesis.** (a) The expression of circ\_0019693 during the osteogenic differentiation of BMSCs was measured by qPCR. (b) The efficiency of circ\_0019693 overexpression vector and si-circ\_0019693 was checked by qPCR. (c-f) The protein levels of RUNX2, OPN and OCN affected by circ\_0019693 overexpression or knockdown were measured by Western blot. (g) ALP activity was examined to determine osteogenic differentiation. (h) Cell angiogenesis was assessed by tube formation assay. \* $P < 0.05$ .

were overlapped by three databases (Figure 3(a)). We used circ\_0019693 probe by pull-down assay to determine whether miR-942-5p and miR-432-5p could be enriched by circ\_0019693. The data showed that miR-942-5p, compared to miR-432-5p, with more abundance, was enriched by circ\_0019693 probe (Figure 3(b)). Then, we designed the mutant sequence fragment of circ\_0019693 and constructed circ\_0019693-WT and circ\_0019693-MUT luciferase reporter plasmids (Figure 3(c)). The data revealed that miR-942-5p overexpression significantly reduced luciferase activity in BMSCs transfected with circ\_0019693-WT (Figure 3(d)). In addition, circ\_0019693 and miR-942-5p were both enriched in the anti-Ago2-mediated RIP group relative to anti-IgG (Figure 3(e)). The expression of miR-942-5p was notably higher in serum and bone tissues from OP patients compared with that from normal subjects (Figure 3(f-g)). Circ\_0019693 expression in serum samples and bone tissues showed negative correlation with miR-942-5p expression

(Figure 3(h-i)). Moreover, miR-942-5p expression was gradually declined during the osteogenic differentiation of BMSCs (Figure 3(j)). MiR-942-5p expression was markedly impaired by circ\_0019693 overexpression in BMSCs (Figure 3(k)). Overall, miR-942-5p was a target of circ\_0019693, and circ\_0019693 negatively modulated miR-942-5p expression.

### **MiR-942-5p upregulation reversed the role of circ\_0019693 overexpression**

Rescue experiments were performed to determine whether circ\_0019693 promoted BMSC osteogenic differentiation and phenotype changes by targeting miR-942-5p. MiR-942-5p mimic significantly enhanced the expression of miR-942-5p in BMSCs (Figure 4(a)). Then, miR-942-5p expression decreased in circ\_0019693-transfected BMSCs but partially enhanced in circ\_0019693+ miR-942-5p-transfected BMSCs (Figure 4(b)). The protein levels of RUNX2, OPN and OCN were



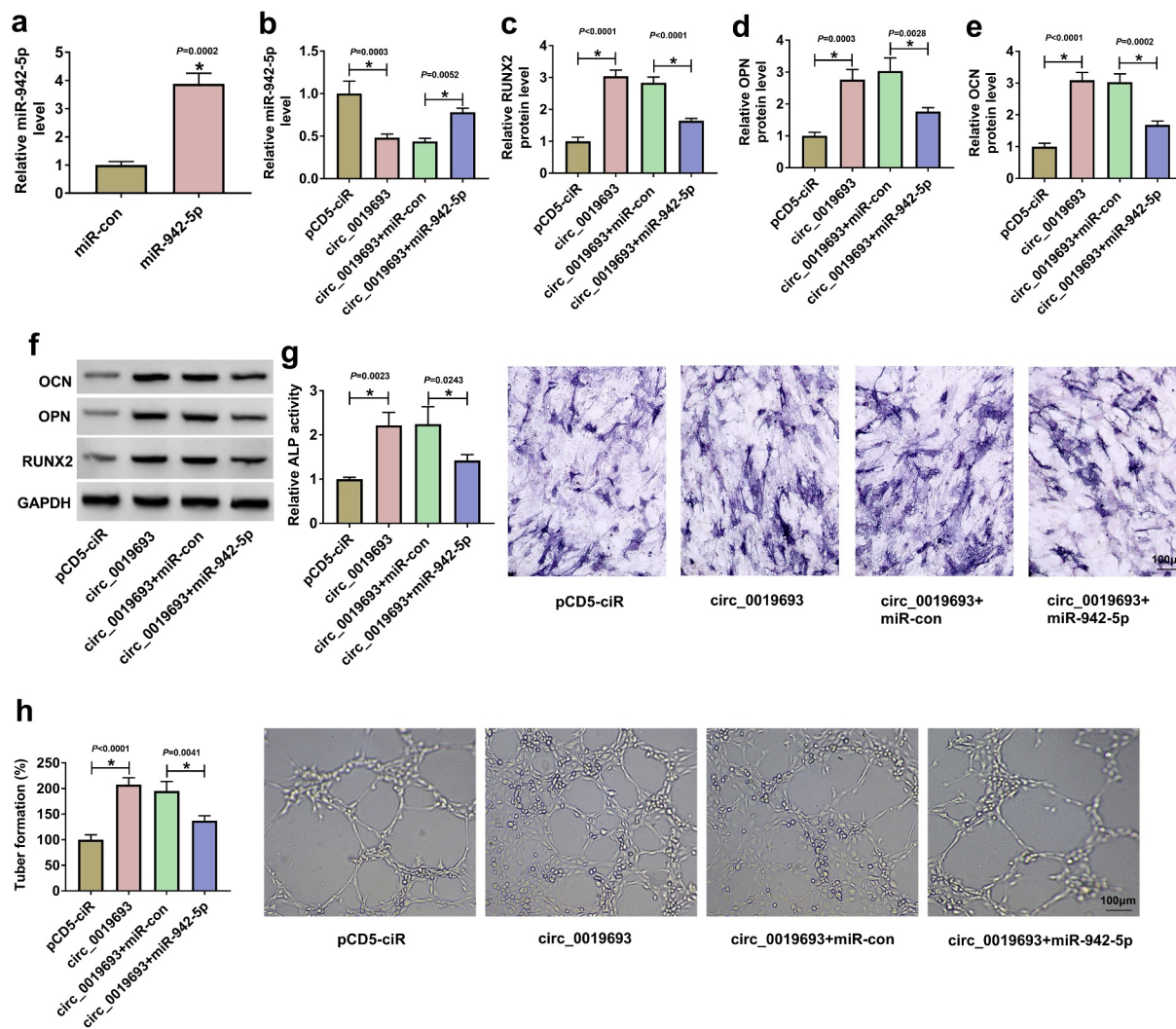
**Figure 3. MiR-942-5p was targeted by circ\_0019693.** (a) MiRNAs potentially targeted by circ\_0019693 were predicted by starbase, circular RNA interactome, and circbank. (b) Pull-down assay was performed to analyze miRNAs enriched by circ\_0019693 probe. (c) The binding site between circ\_0019693 and miR-942-5p. (d and e) The binding between circ\_0019693 and miR-942-5p was verified by dual-luciferase reporter assay and RIP assay. (f and g) MiR-942-5p expression in serum and bone tissues was measured by qPCR. (h and i) The correlation between circ\_0019693 expression and miR-942-5p expression in serum samples and bone tissues. (j) The expression of miR-942-5p during the osteogenic differentiation of BMSCs was measured by qPCR. (k) The expression of miR-942-5p during the osteogenic differentiation of BMSCs with circ\_0019693 overexpression was measured by qPCR. \* $P < 0.05$ .

strengthened by circ\_0019693 overexpression, while further miR-942-5p upregulation repressed their expression levels (Figure 4(c-f)). The increased ALP activity caused by circ\_0019693 overexpression was also reversed by the reintroduction of miR-942-5p (Figure 4(g)). Moreover, we found that the overexpression of circ\_0019693 and miR-942-5p markedly reversed the enhanced angiogenesis of BMSCs relative to alone circ\_0019693 upregulation (Figure 4(h)). These data proved that circ\_0019693 overexpression promoted BMSC osteogenic differentiation and angiogenesis via targeting miR-942-5p.

### MiR-942-5p bound to PCP4 and negatively regulated PCP4 expression

The potential binding relationship between miR-942-5p and PCP4 was predicted by starbase, and then PCP4-3'UTR-WT and PCP4-3'UTR-MUT reporter plasmids were constructed (Figure 5(a)). The data from pull-down assay exhibited that high abundance of PCP4 was enriched by bio-miR-942-5p-WT probe (Figure 5(b)). Luciferase reporter assay pointed out that miR-942-5p enrichment markedly weakened luciferase activity in BMSCs transfected with PCP4-3'UTR-WT (Figure 5(c)).





**Figure 4. Circ\_0019693 played functions by targeting miR-942-5p.** (a) The efficiency of miR-942-5p mimic was checked by qPCR. (b) The expression of miR-942-5p in BMSCs transfected with circ\_0019693 or circ\_0019693+ miR-942-5p was checked by qPCR. (c-f) The protein levels of RUNX2, OPN and OCN in these cells were detected by Western blot. (g) ALP activity was measured to assess osteogenic differentiation of BMSCs in different groups. (h) Angiogenesis was evaluated by tube formation assay. \* $P < 0.05$ .

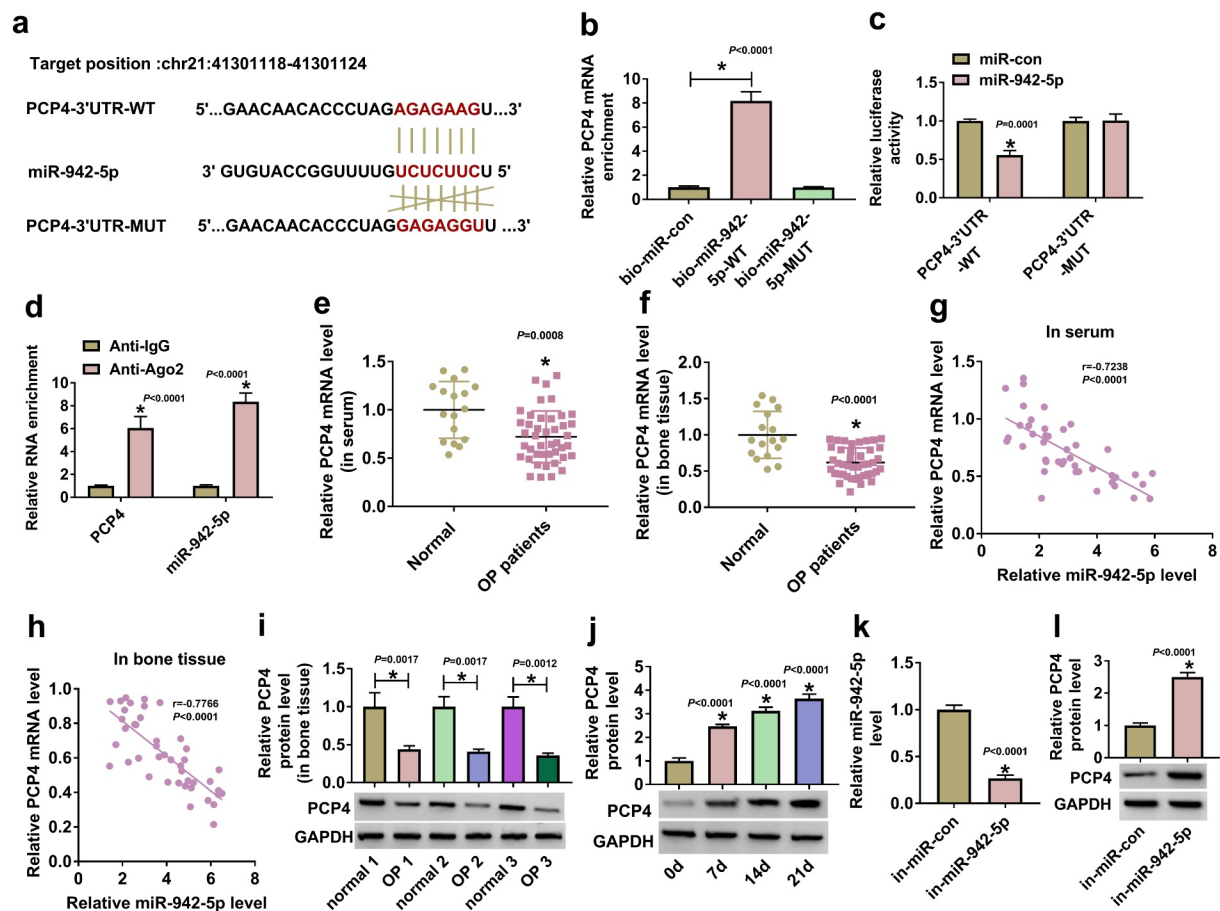
RIP assay presented that PCP4 and miR-942-5p were both enriched in the anti-Ago2-mediated RIP group relative to anti-IgG (Figure 5(d)). The expression of PCP4 mRNA was strikingly decreased in serum samples and bone tissues from OP patients compared with that from normal subjects (Figure 5(e-f)). In addition, PCP4 mRNA expression was negatively correlated with miR-942-5p expression in serum samples and bone tissues (Figure 5(g-h)). The expression of PCP4 protein in bone tissues from OP patients was also markedly declined (Figure 5(i)). However, PCP4 expression was gradually increased during the osteogenic differentiation of BMSCs (Figure 5(j)). The transfection of in-miR-942-5p markedly reduced the expression of miR-

942-5p but strengthened the expression of PCP4 in BMSCs (Figure 5(k-l)). The data indicated that miR-942-5p negatively regulated the expression of PCP4.

#### **MiR-942-5p repression promoted BMSC osteogenic differentiation and angiogenesis by increasing PCP4 expression**

The expression of PCP4 was remarkably weakened in BMSCs transfected with si-PCP4 (Figure 6(a)). In addition, PCP4 expression was markedly upregulated in BMSCs transfected with in-miR-942-5p but partly repressed in BMSCs transfected with in-miR-942-5p+si-PCP4 (Figure 6(b)). The protein levels of RUNX2, OPN and OCN were enhanced





**Figure 5. PCP4 was a target of miR-942-5p.** (a) The binding site between miR-942-5p and PCP4 3' UTR was provided by starbase. (b-d) The relationship between PCP4 and miR-942-5p was validated by pull-down, dual-luciferase reporter and RIP assays. (e and f) The expression of PCP4 mRNA in serum samples and bone tissues was measured by qPCR. (g and h) The correlation between PCP4 expression and miR-942-5p expression in serum samples and bone tissues. (i) The expression of PCP4 protein in bone tissues was measured by Western blot. (j) The expression of PCP4 protein during the osteogenic differentiation of BMSCs was checked by Western blot. (k) The efficiency of in-miR-942-5p was checked by qPCR. (l) The expression of PCP4 protein in BMSCs with miR-942-5p inhibition was measured by Western blot. \* $P < 0.05$ .

by miR-942-5p suppression, while further PCP4 downregulation repressed the protein levels of these markers (Figure 6(c-f)). The strengthened ALP activity caused by miR-942-5p inhibition was partially restrained by PCP4 knockdown (Figure 6(g)). Additionally, the simultaneous knockdown of miR-942-5p and PCP4 reversed the capacities of angiogenesis promoted by alone miR-942-5p knockdown (Figure 6(h)). These findings exposed that miR-942-5p functioned in osteoporosis by targeting PCP4.

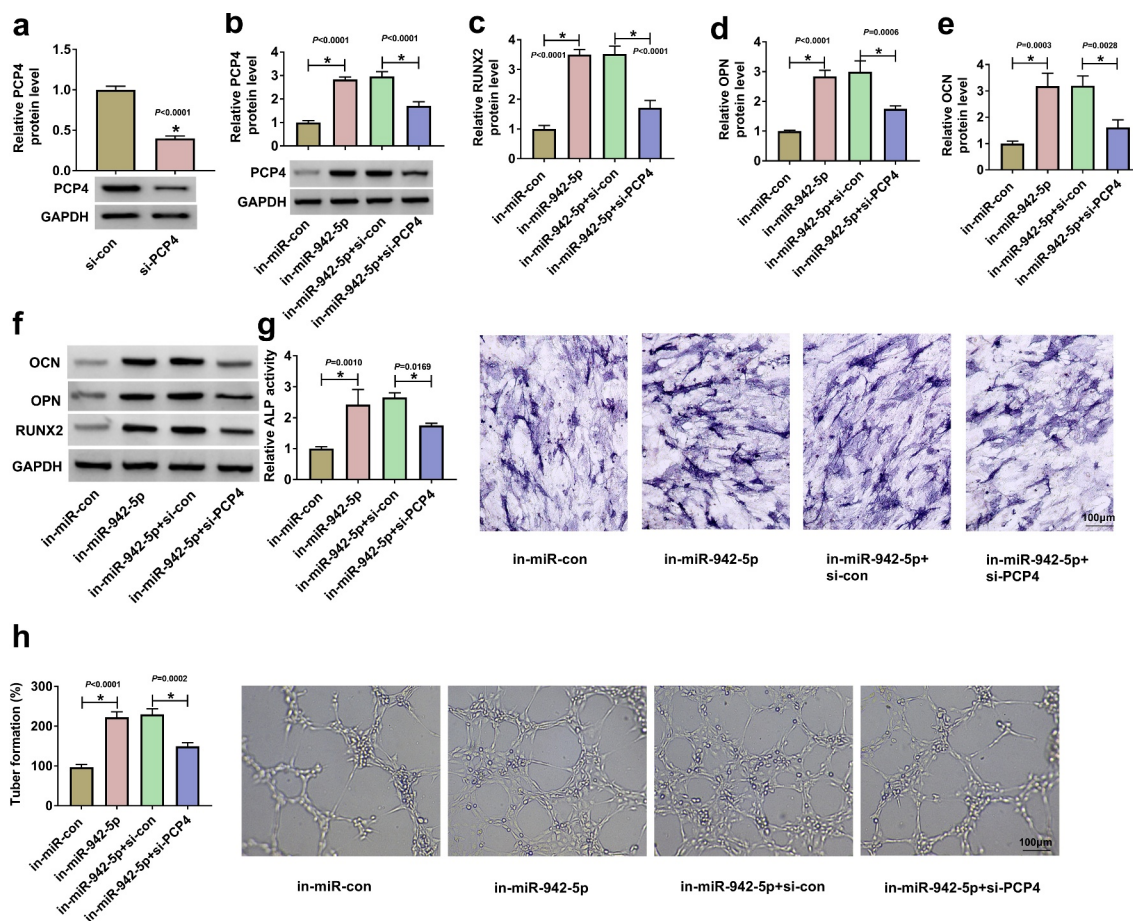
### Circ\_0019693 positively regulated PCP4 expression by decoying miR-942-5p

In serum samples and bone tissues from OP patients, we found that PCP4 mRNA expression was

markedly positively correlated with circ\_0019693 expression (Figure 7(a-b)). In addition, the expression of PCP4 protein was notably enhanced in BMSCs transfected with circ\_0019693 but partly impaired in BMSCs transfected with circ\_0019693 + miR-942-5p (Figure 7(c)). Unsurprisingly, the expression of PCP4 protein was significantly downregulated in BMSCs transfected with si-circ\_0019693 but partially restored in BMSCs transfected with si-circ\_0019693+ in-miR-942-5p (Figure 7(d)). The data suggested that circ\_0019693 regulated PCP4 expression by targeting miR-942-5p.

### Discussion

Patients with OP often suffer from the delayed union of bone fractures because of impaired

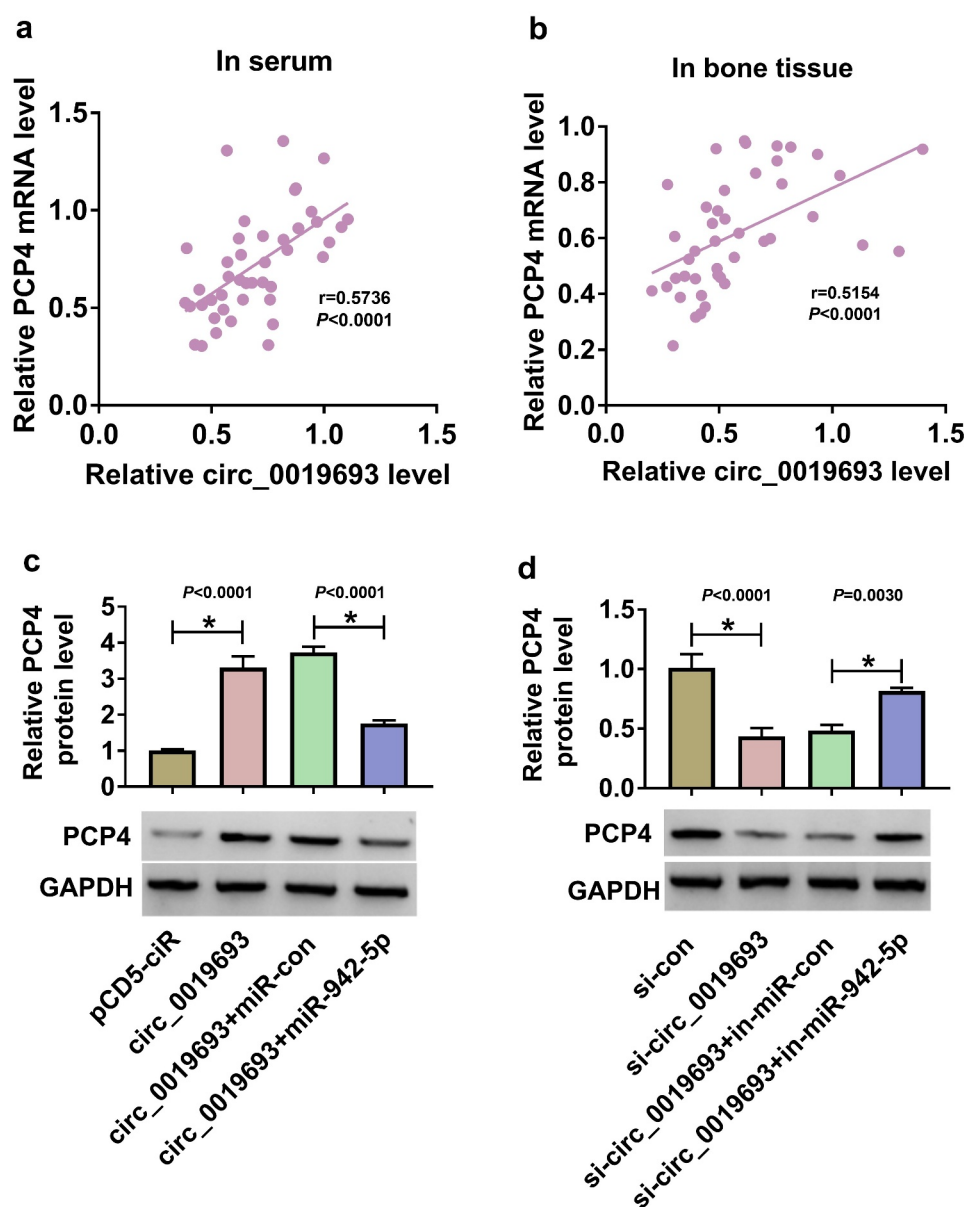


**Figure 6. MiR-942-5p played functions by targeting PCP4.** (a) The efficiency of si-PCP4 was checked by Western blot. (b) The expression of PCP4 protein in BMSCs transfected with in-miR-942-5p or in-miR-942-5p+si-PCP4 was checked by Western blot. (c-f) The protein levels of RUNX2, OPN and OCN in these cells were detected by qPCR. (g) ALP activity was measured to assess osteogenic differentiation of BMSCs in different groups. (h) Angiogenesis was evaluated by tube formation assay. \* $P < 0.05$ .

osteogenesis and inhibitory angiogenesis [24]. Therefore, it is necessary to exploit the factors involving in osteogenesis and angiogenesis to supply promising therapeutic strategies for OP. We mainly reported the function circ\_0019693 on BMSC osteogenic differentiation and angiogenesis, thus determining the underlying role of circ\_0019693 in the development of OP.

BMSCs are a kind of multifunctional, undifferentiated cells with regenerative capacity. When bone is injured, BMSCs spontaneously migrate to the bone lesion and differentiate into osteoblasts, and osteoblasts next secrete extracellular matrix (ECM) to achieve bone repair [25,26]. Blood vessels are essential to transfer oxygen, materials and nutrients, which is crucial for bone mass maintenance and bone regeneration [2,25]. Bone loss repair is a complex process with

complex signaling cascades, involving osteogenic differentiation of BMSCs and well-organized angiogenesis [26,27]. Therefore, the induction of BSMC osteogenic differentiation and the coupling of osteogenesis and angiogenesis are of great significance for the repair of OP-induced bone injury. Vascular endothelial growth factor (VEGF) was required for the coupling of bone formation and angiogenesis during bone development [28]. Emerging evidence revealed that certain circRNAs were implicated with these processes. For instance, circ\_0113689 was highly expressed in BMSCs during osteogenic differentiation, and its overexpression accelerated BMSC proliferation and osteogenic differentiation [29]. Besides, circ\_0006215 promoted BMSC osteogenic differentiation, and the medium of BMSCs overexpressing circ\_0006245 promoted HUVEC



**Figure 7. Circ\_0019693 positively regulated PCP4 expression by targeting miR-942-5p.** (a and b) The correlation between PCP4 mRNA expression and circ\_0019693 expression in serum samples and bone tissues. (c) The expression of PCP4 protein in BMSCs transfected with circ\_0019693 or circ\_0019693+ miR-942-5p was detected by Western blot. (d) The expression of PCP4 protein in BMSCs transfected with si-circ\_0019693 or si-circ\_0019693+ in-miR-942-5p was detected by Western blot. \* $P < 0.05$ .

angiogenesis [19]. Consistent with these studies, we discovered that circ0019693 expression was markedly increased during BMSC osteogenic differentiation, and circ\_0019693 upregulation enhanced the expression of RUNX2, OPN and OCN, and the activity of ALP. RUNX2, OPN and OCN are common markers of osteogenic differentiation [30], and ALP, produced by osteogenic cells (such as osteoblasts), is one of the most reliable markers for osteogenic differentiation [31]. The changes in the expression of RUNX2,

OPN and OCN and the activity of ALP in BMSCs overexpressing circ\_0019693 indicated that circ\_0019693 promoted BMSC osteogenic differentiation. Besides, the supernatant of BMSCs overexpressing circ\_0019693 strengthened HUVEC angiogenesis. Unsurprisingly, the knock-down of circ\_0019693 showed the opposite effects.

The results from pull-down, dual-luciferase reporter and RIP assays verified that miR-942-5p was targeted by circ\_0019693. Previous study reported

that miR-942-5p acted as a target of circ\_0006215, and its inhibition recovered the ability of BMSC osteogenic differentiation [19]. Coincidentally, circ\_0074834 overexpression promoted BMSC osteogenesis and angiogenesis by targeting miR-942-5p [32]. Largely in agreement with these data, our data defined miR-942-5p as a target of circ\_0019693. In function, miR-942-5p enrichment partly depleted the expression of RUNX2, OPN and OCN, the activity of ALP, and HUVEC angiogenesis in BMSCs overexpressing circ\_0019693, while miR-942-5p deficiency enhanced the expression of these markers and ALP activity. The data strongly supported that circ\_0019693 promoted BMSC osteogenic differentiation and angiogenesis by suppressing miR-942-5p.

To further exploit the functional mechanism of circ\_0019693, the downstream mRNA molecules of miR-942-5p were screened. PCP4 attracted our attention because its expression was shown to present an upward trend during BMSC osteogenic differentiation [33]. Bioinformatics study exposed that PCP4 was associated with calcium deposition and the regulation of CaM-dependent protein kinase, hinting that osteogenic differentiation was enhanced by PCP4 depending on PCP4-induced calcium deposition [33]. In addition, PCP4 expression was declined in rat models of OP, and PCP4 overexpression promoted the differentiation of osteoblasts obtained from OP rat models [21]. Consistently, our data presented that PCP4 expression was notably decreased in OP patients but gradually enhanced during osteogenic differentiation in BMSCs. PCP4 knockdown reversed the role of miR-942-5p inhibition, leading to the inhibition of BMSC osteogenic differentiation and angiogenesis.

## Conclusion

Taken together, the expression of circ\_0019693 was declined in serum samples and bone tissues from OP patients, and its expression was gradually increased in BMSCs during osteogenic differentiation. Circ\_0019693 overexpression promoted BMSC osteogenic differentiation and enhanced osteogenesis-coupled angiogenesis via regulating miR-942-5p-targeted PCP4, thus potentially preventing the progression of OP. This study for the

first time explores the potential role of circ\_0019693 in OP, which may help to develop new strategies for OP therapy.

## Highlight

- Circ\_0019693 is downregulated in osteoporosis.
- Circ\_0019693 overexpression promotes BMSC osteogenic differentiation and osteogenesis-coupled angiogenesis.
- Circ\_0019693 competitively binds to miR-942-5p to regulate PCP4 expression.
- Circ\_0019693 governs the miR-942-5p/PCP4 axis to suppress osteoporosis development.

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## Ethics approval and consent to participate

Written informed consents were obtained from all participants and this study was permitted by the Ethics Committee of the Second Hospital of Shanxi Medical University.

## References

- [1] Nuti R, Brandi ML, Checchia G, et al. Guidelines for the management of osteoporosis and fragility fractures. *Intern Emerg Med.* 2019;14(1):85–102.
- [2] Chen L, Zhang R-Y, Xie J, et al. STAT3 activation by catalpol promotes osteogenesis-angiogenesis coupling, thus accelerating osteoporotic bone repair. *Stem Cell Res Ther.* 2021;12(1):108.
- [3] Noh JY, Yang Y, Jung H. Molecular mechanisms and emerging therapeutics for osteoporosis. *Int J Mol Sci.* 2020;21(20):7623.
- [4] Tang P, Xiong Q, Ge W, et al. The role of microRNAs in osteoclasts and osteoporosis. *RNA Biol.* 2014;11(11):1355–1363.
- [5] Jing H, Liao L, Su X, et al. Declining histone acetyltransferase GCN5 represses BMSC-mediated angiogenesis during osteoporosis. *FASEB J.* 2017;31(10):4422–4433.
- [6] Grellier M, Granja PL, Fricain J-C, et al. The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on



- mineralization in a bone defect. *Biomaterials*. 2009;30(19):3271–3278.
- [7] Yang A, Yu C, You F, et al. Mechanisms of Zuogui Pill in treating osteoporosis: perspective from bone marrow mesenchymal stem cells. *Evid Based Complement Alternat Med*. 2018;2018:3717391.
- [8] Arthur A, Gronthos S. Clinical application of bone marrow mesenchymal stem/stromal cells to repair skeletal tissue. *Int J Mol Sci*. 2020;21(24):9759.
- [9] Wei JW, Huang K, Yang C, et al. Non-coding RNAs as regulators in epigenetics (Review). *Oncol Rep*. 2017;37(1):3–9.
- [10] Yang Y, Yujiao W, Fang W, et al. The roles of miRNA, lncRNA and circRNA in the development of osteoporosis. *Biol Res*. 2020;53(1):40.
- [11] Patil S, Dang K, Zhao X, et al. Role of lncRNAs and CircRNAs in bone metabolism and osteoporosis. *Front Genet*. 2020;11:584118.
- [12] Ojha R, Nandani R, Chatterjee N, et al. Emerging role of circular RNAs as potential biomarkers for the diagnosis of human diseases. *Adv Exp Med Biol*. 2018;1087:141–157.
- [13] Zhang Z, Yang T, Xiao J. Circular RNAs: promising biomarkers for human diseases. *EBioMedicine*. 2018;34:267–274.
- [14] Xu X, Chen Y, Tan B, et al. Circular RNA circ\_0011269 sponges miR-122 to regulate RUNX2 expression and promotes osteoporosis progression. *J Cell Biochem*. 2020;121:4819–4826.
- [15] Huang Y, Xie J, Li E. Comprehensive circular RNA profiling reveals circ\_0002060 as a potential diagnostic biomarkers for osteoporosis. *J Cell Biochem*. 2019;120(9):15688–15694.
- [16] Huang Y, Xiao D, Huang S, et al. Circular RNA YAP1 attenuates osteoporosis through up-regulation of YAP1 and activation of Wnt/ $\beta$ -catenin pathway. *Biomed Pharmacother*. 2020;129:110365.
- [17] Dudekula DB, Panda AC, Grammatikakis I, et al. CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs. *RNA Biol*. 2016;13(1):34–42.
- [18] Liu M, Wang Q, Shen J, et al. Circbank: a comprehensive database for circRNA with standard nomenclature. *RNA Biol*. 2019;16(7):899–905.
- [19] Ji H, Cui X, Yang Y, et al. CircRNA hsa\_circ\_0006215 promotes osteogenic differentiation of BMSCs and enhances osteogenesis-angiogenesis coupling by competitively binding to miR-942-5p and regulating RUNX2 and VEGF. *Aging (Albany NY)*. 2021;13(7):10275–10288.
- [20] Yang JH, Li J-H, Shao P, et al. starBase: a database for exploring microRNA-mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data. *Nucleic Acids Res*. 2011;39(Database issue):D202–9.
- [21] Meng YC, Lin T, Jiang H, et al. miR-122 exerts inhibitory effects on osteoblast proliferation/differentiation in osteoporosis by activating the PCP4-mediated JNK pathway. *Mol Ther Nucleic Acids*. 2020;20:345–358.
- [22] Liu B, Li X, Li C, et al. miR-25 mediates metastasis and epithelial-mesenchymal-transition in human esophageal squamous cell carcinoma via regulation of E-cadherin signaling. *Bioengineered*. 2019;10(1):679–688.
- [23] Hu X, Chen J, Huang H, et al. Syndecan-4 promotes vascular beds formation in tissue engineered liver via thrombospondin 1. *Bioengineered*. 2020;11(1):1313–1324.
- [24] Portal-Nunez S, Lozano D, Esbrit P. Role of angiogenesis on bone formation. *Histol Histopathol*. 2012;27(5):559–566.
- [25] Granero-Molto F, Weis JA, Miga MI, et al. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells*. 2009;27(8):1887–1898.
- [26] Mousaei Ghasroldasht M, Matin MM, Kazemi Mehrjerdi H, et al. Application of mesenchymal stem cells to enhance non-union bone fracture healing. *J Biomed Mater Res A*. 2019;107(2):301–311.
- [27] Dirckx N, Van Hul M, Maes C. Osteoblast recruitment to sites of bone formation in skeletal development, homeostasis, and regeneration. *Birth Defects Res C Embryo Today*. 2013;99(3):170–191.
- [28] Liu Y, Olsen BR. Distinct VEGF functions during bone development and homeostasis. *Arch Immunol Ther Exp (Warsz)*. 2014;62(5):363–368.
- [29] Chia W, Liu J, Huang Y-G, et al. A circular RNA derived from DAB1 promotes cell proliferation and osteogenic differentiation of BMSCs via RBPJ/DAB1 axis. *Cell Death Dis*. 2020;11(5):372.
- [30] Fu Y, Zhang P, Ge J, et al. Histone deacetylase 8 suppresses osteogenic differentiation of bone marrow stromal cells by inhibiting histone H3K9 acetylation and RUNX2 activity. *Int J Biochem Cell Biol*. 2014;54:68–77.
- [31] Westhauser F, Karadjian M, Essers C, et al. Osteogenic differentiation of mesenchymal stem cells is enhanced in a 45S5-supplemented beta-TCP composite scaffold: an in-vitro comparison of Vitoss and Vitoss BA. *PLoS One*. 2019;14(2):e0212799.
- [32] Ouyang Z, Tan T, Zhang X, et al. CircRNA hsa\_circ\_0074834 promotes the osteogenesis-angiogenesis coupling process in bone mesenchymal stem cells (BMSCs) by acting as a ceRNA for miR-942-5p. *Cell Death Dis*. 2019;10(12):932.
- [33] Xiao J, Wu Y, Chen R, et al. Expression of Pcp4 gene during osteogenic differentiation of bone marrow mesenchymal stem cells in vitro. *Mol Cell Biochem*. 2008;309(1–2):143–150.