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CircPSM3 inhibits the proliferation and differentiation of OA chondrocytes by targeting miRNA-296-5p

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Abstract. – OBJECTIVE: Osteoarthritis (OA) is a common chronic bone and joint disease. Circular RNA is a type of non-coding RNA that forms a circular structure with covalent bonds. There is growing evidence that circRNA can function as a functional RNA and play an important role in the occurrence and development of osteoarthritis chondrocytes. However, the exact role of circRNA on OA remains to be studied.

PATIENTS AND METHODS: Quantificational real-time polymerase chain reaction (qRT-PCR) was used to determine the expression levels of CircPSM3 and miRNA-296-5p in OA chondrocytes. Cell proliferation was detected by the Cell Counting Kit (CCK8), and BMP2, BMP4, BMP6 and RUN2 molecular levels in OA chondrocytes were detected by qRT-PCR and Western Blot (WB). Direct targets of CircPSM3 and miRNA-296-5p in OA chondrocytes were measured by Luciferase reporter assay.

RESULTS: CircPSM3 expression was upregulated in OA cartilage tissue and cells. Low expression of CircPSM3 promoted the proliferation and cell differentiation of OA chondrocytes. Meanwhile, miRNA-296-5p was down-regulated in OA cartilage tissue and cells. The Luciferase reporter gene showed that CircPSM3 could target miRNA-296-5p. The expression level of CircPSM3 and miRNA-296-5p showed a negative correlation. Further research found that a high expression of miRNA-296-5p could effectively promote the proliferation and cell differentiation of OA chondrocytes. Furthermore, miRNA-296-5p inhibitors reversed the effect of si-CircPSM3 on the proliferation and differentiation of OA chondrocytes, while miRNA-296-5p inhibitors enhanced the effect of si-CircPSM3 on the proliferation and differentiation of OA chondrocytes.

CONCLUSIONS: CircPSM3 was upregulated in OA chondrocytes. CircPSM3 participated in the proliferation and differentiation of OA chondrocytes through targeted binding to miRNA-296-5p. CircPSM3 may become a potential therapeutic target for osteoarthritis treatment.

Key Words:
Osteoarthritis, CircPSM3, MiRNA-296-5p, Proliferation, Differentiation.

Introduction

Osteoarthritis (OA) is a joint disease characterized by cartilage degeneration and secondary subchondral bone thickening, osteophyte formation[1-3]. It is widely spread all over the world. Its pathogenesis is still not clear because it involves genetic biology and biomechanics. Hence, the mechanism of formation and regulation of OA has become a research hotspot in the field of biomedicine. Circular RNA is a class of endogenous RNA molecules with a stable closed loop structure. Circular RNA plays important regulatory roles in cell biological functions such as protein synthesis, gene expression and post-transcriptional modification[4-8]. The abnormal expression of CircRNAs was closely related to the occurrence and development of OA[9]. CircRNAs mainly played a potential role in ECM degradation, chondrocyte proliferation, apoptosis and inflammation in OA tissues and cells[10]. Day et al[11] found that hundreds of miRNAs and circRNAs have significant differences in osteoclast gene expression profiles at different stages of differentiation. Li et al[12] explored the potential role of circular RNA in osteoarthritis and pointed out that circRNAs/miRNAs/mRNAs axis played an important role in the pathogenesis of OA. MiRNA is a type of endogenous non-coding single-stranded small-molecule RNA of approximately 22 nucleotides in length that interacts with homologous mRNA[13]. CircRNA can act as a sponge of miRNA to inhibit the translation of mRNA, thus participating in the occurrence and development of OA chondrocytes[14]. Lü et al[15] proved that CircRNA-Atp9b is related to the expression of multiple inflammatory factors and could target the binding of miR-138-5p to promote ECM degradation and chondrocyte inflammatory response. Hsa-circ-0404754 could up-regulate the expression of proteoglycan and type II collagen and promote cell proliferation

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while miR-193b can promote apoptosis[16]. Evidence demonstrated that hsacirc-0005105 increased its expression in chondrocytes after being stimulated by IL-1β, and it could up-regulate the target gene NAMPT of miR-26a[17]. Rong et al[18] found that CircPSMC3 inhibits the proliferation and metastasis of gastric cancer by directly targeting miR-296-5p. In the present study, we found that CircPSM3 could serve as a sponge of miR-296-5p to inhibit the proliferation and differentiation of OA chondrocytes, thus involving in regulating the occurrence and development of OA chondrocytes. Our research provides a theoretical basis for CircPSM3 as a target for OA treatment.

Patients and Methods

Cell Cultures and Patient Tissues

Thirty-five OA patients and thirty-five meniscal injury patients who were admitted to our hospital from June 2017 to May 2018 were selected. The OA diagnostic criteria were based on the American College of Rheumatology. The average age of patients was 41.5 ± 10.2 years old, including 21 males and 14 females. The average age of meniscus injury patients was 40.7 ± 9.6 years old, including 22 males and 13 females. None of the patients had rheumatoid arthritis, gouty arthritis, rheumatoid arthritis, and secondary osteoarthritis. The cartilage tissue was removed during the operation, and all tissue samples were divided into the same size and stored in liquid nitrogen. All patients signed informed consent before surgery.

qRT-PCR

Total RNA was extracted from OA tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription kit (TaKaRa, Otsu, Shiga, Japan) was used to synthesize cDNA for circRNA and mRNA. RiboBio reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to reverse total RNAs for miRNA. SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan) and SYBR Green PCR Kit (RiboBio, Guangzhou, China) were used to perform the quantification of mRNA/circular RNA and miRNA. All primer sequences were designed and synthesized by Genery (Nanjing, China). The expression level of CircPSM3 was measured by the following primer pair: 5'-GTTTAGGGTCCCTGCCCTTTG-3' (F) and 5'-GTGTTGGGCTGGAAGCCATC-3' (R). The primer pair of miRNA-296-5p was 5'-TGCCTAATTCAGAGGGTTGG-3' (F) and 5'-CTCCACTCCTGGCACACAG-3' (R). The primer pair of PCNA was 5'-GACACATACCGCTGCGATCG-3' (F) and 5'-TCACCACAGCATCTCCAATAT-3' (R). The primer pairs of BMP2, BMP4, BMP6 and RUN2 were as follows: BMP2, 5'-ATCACCTGAACTCCACGAA-3' (F) and 5'-TACCACCTTCTCATTCTCAT-3'(R); BMP4, 5'-GGGGAAGAAAAAAGTCGCCGAGATT-3' (F) and 5'-CTCAGGATACTCCAGACCGATGCCC-3' (R); BMP6, 5'-GGTGGCAGGACTGGATCATT(F) and 5'-CACCGAGATGGCGTTCAGTT-3' (R); Run2, 5'-CACTGGCGCTGCAACAAGA-3'(F) and 5'-CATTCCGGAGCTCAGCAGAATAA-3' (R). The primer set for GAPDH was 5'-ACCACAGTCCATGCCATCAC-3' (F) and 5'-TCCACCACCCTGTTGCTGTA-3' (R). The primer set for U6 was 5'-CGCTTCGGCAGCACATATAC-3' (F) and 5'-AAATATGGAACGCTTCACGA-3' (R).

The results were measured using the $2^{-\Delta\Delta Ct}$ methods. GAPDH and U-6 were used to normalize the level of mRNA and CircRNA expression.

Luciferase Reporter Assay

The 3'-UTR WT sequence of circPSMC3 predicted to interact with miRNA-296-5p or a MUT sequence within the predicted target sites were inserted into pGL3-basic vectors (Realgene, Nanjing, China). Next, cells were co-transfected with the miRNA-296-5p mimic and circPSMC3 WT or MUT. After 48 h, the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) was used to collect and detect Luciferase activity in co-transfected cells.

CCK-8 Assay

OA cells were seeded in 96 wells plate with 4000 cells/well. The cells were maintained at 37°C with 10 μl of CCK-8 solution incubator overnight. Cell proliferation was assessed by a CCK-8 (CCK-8, CK04, Dojindo Molecular Technologies, Kumamoto, Japan). Then, the CCK-8 solution was added into each well after the cells were treated as indicated. OA cells were cultured for 24 h and microplate reader (Synergy4, BioTek, Winooski, VT, USA) was used to analyze the absorbance of cells at each time.

Western Blot

OA cells from each group were collected 24 hours after transfection, and the total protein was routinely extracted. The protein concentration was measured by bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Block the 5% skimmed milk powder at 37°C for 2 h. BMP2, BMP4, BMP6,

RUN2 and β-actin (Abcam, Cambridge, UK) primary antibodies were added and incubated overnight at 4°C, Tris-Buffered Saline and Tween-20 (TBST) was washed for 3 × 10 min, secondary antibodies were incubated for 1 h at 37°C, TBST was washed for 3 × 30 min, and enhanced chemiluminescence (ECL) was developed. Image J software was used to analyze the gray value of protein bands, and β-actin was used as an internal reference to calculate the relative expression.

Statistical Analysis

Data are expressed as mean ± standard deviation and analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Spearman correlation analysis was used to evaluate the correlation of circPSMC3 and miRNA-296-5p expression and $p < 0.05$ was considered statistically significant.

Results

CircPSM3 Was Upregulated In Both OA Tissues and OA Chondrocytes

We investigated the expression levels of CircPSM3 in OA Tissues and OA Chondrocytes. As a result, the relative expression of CircPSM3 was significantly increased in cartilage tissue of knee joint as compared with that in patients with meniscus injury (Figure 1A). Similarly, a high-regulation of CircPSM3 was also found in OA chondrocytes as compared with normal cells (Figure 1B). Meanwhile, the cells were transfected with si-NC and si-CircPSM3, respectively. The results showed that the expression level of CircPSM3 in cells after transfection of si-CircPSM3 was significantly lower than that of si-NC (Figure 1C). On the contrary, the expression

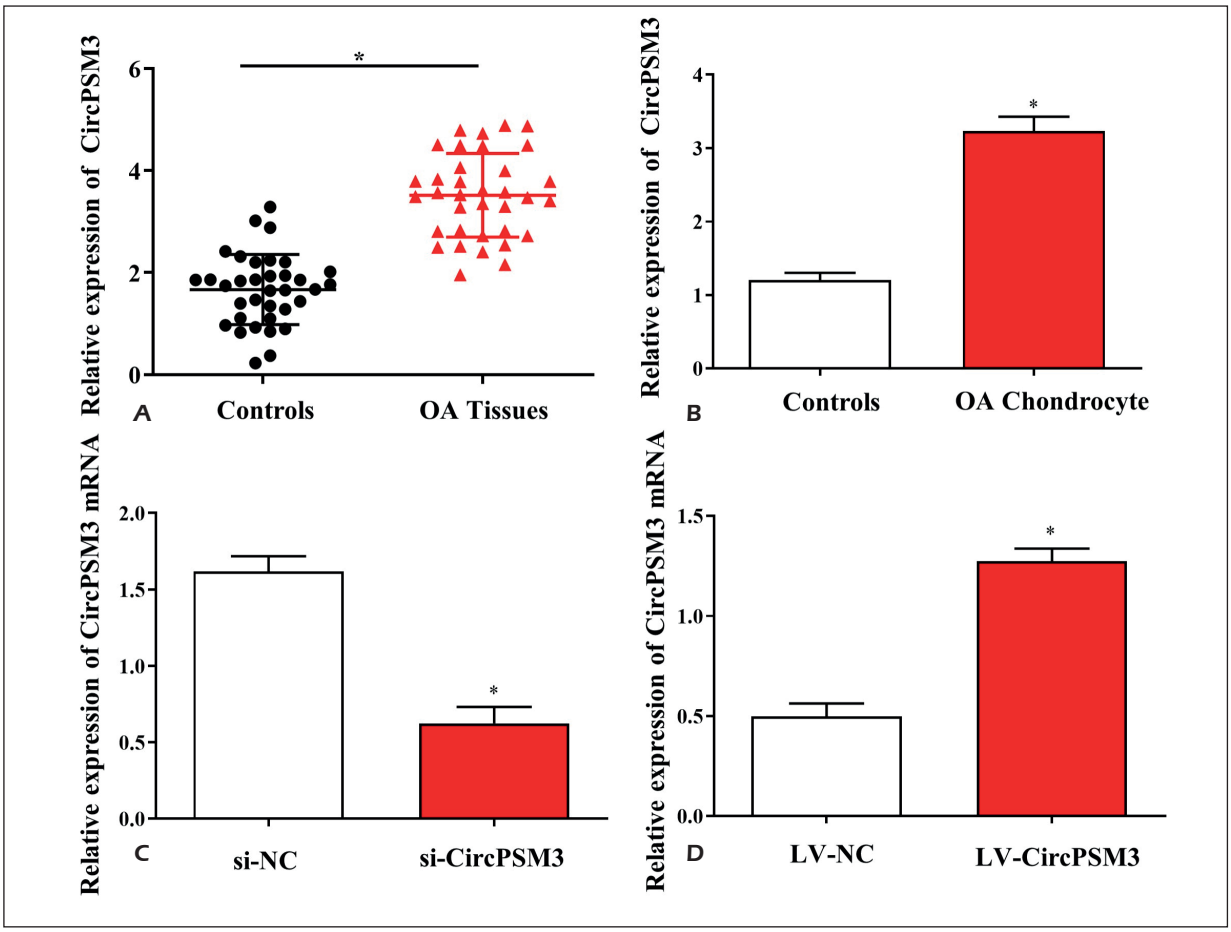

Figure 1. CircPSM3 was upregulated in OA tissues and OA chondrocytes. **A**, CircPSM3 expression was measured by qRT-PCR in OA tissues and meniscus damaged tissues. **B**, The expression of CircPSM3 was detected by qRT-PCR in OA chondrocytes and normal chondrocytes. **C**, si-CircPSM3 mRNA expression levels were measured by qRT-PCR after transfection of si-CircPSM3. **D**, The expression of CircPSM3 mRNA was detected by qRT-PCR after transfection of LV-CircPSM3. The data were expressed as mean ± SD. *$p<0.05$.

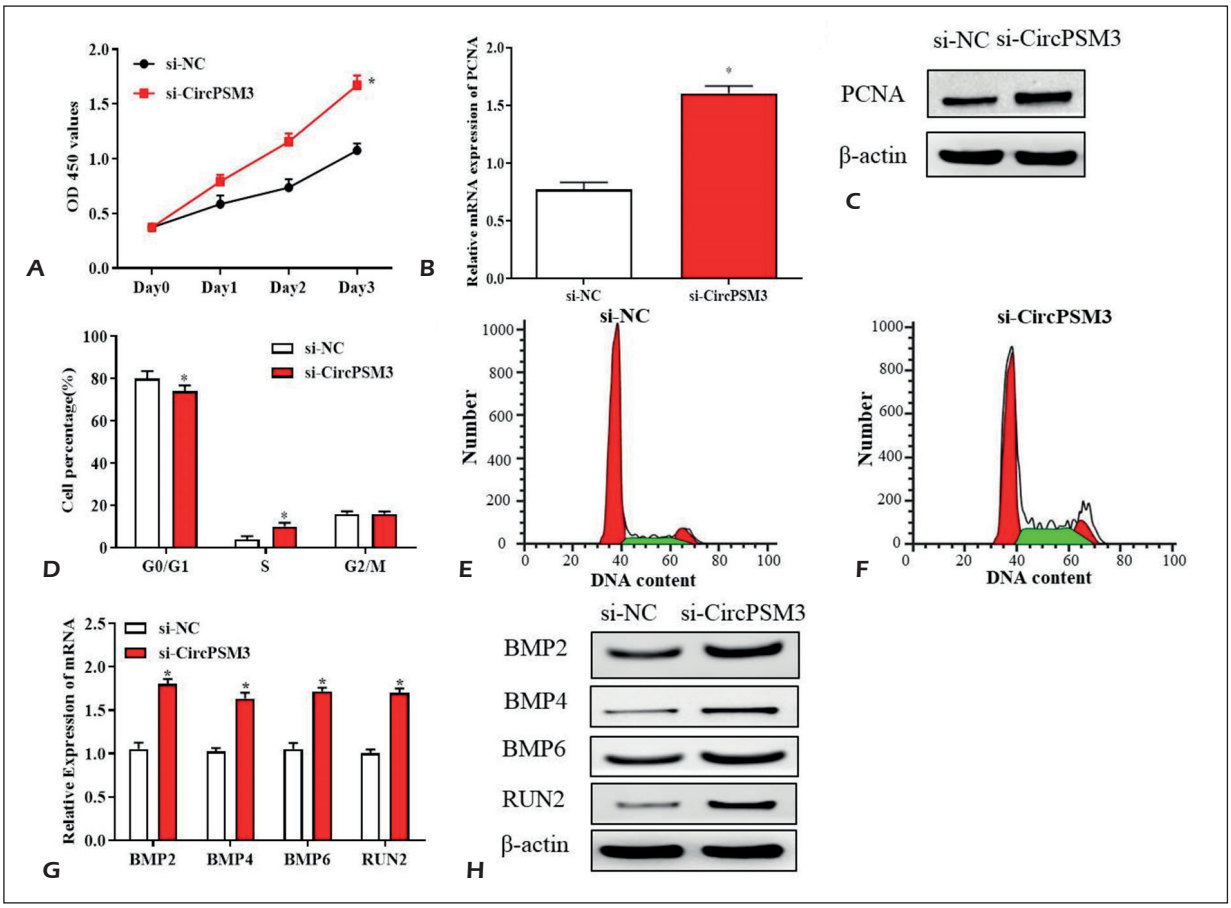

Figure 2. Low expression of CircPSM3 promoted proliferation and differentiation of OA chondrocytes. **A-B**, The proliferation of OA chondrocytes was measured by CCK-8 assay after transfection of si-CircPSM3. **C**, Proliferation-related molecular expression levels were measured by Western blotting. **D-F**, Cell cycle of OA chondrocytes was obtained by flow cytometry. **G-H**, Cell differentiation related molecules expression levels were measured by Western blotting and mRNA expression levels were detected by qRT-PCR. The data were expressed as mean ± SD. *$p<0.05$.

level of CircPSM3 was significantly higher than that of transfected LV-NC after further transfection of LV-CircPSM3 in cells (Figure 1D).

Low Expression of CircPSM3 Promotes Proliferation and Differentiation of OA Chondrocytes

To further analyze the effect of CircPSM3 on the proliferation and differentiation of OA chondrocytes, OA chondrocytes were transfected with si-CircPSM3 for 3 days. The results revealed that the cell proliferation ability was significantly higher than transfection with si-NC (Figure 2A), and the expression levels of proliferation-related molecules PCNA mRNA and protein were also increased (Figures 2B and 2C). Flow cytometry showed that downregulated CircPSM3 significantly decreased cell ratio at G0/G1 phase, and increased cell ratio at S phase of OA chondrocytes cells, and there was no statistical difference between the two groups in the proportion of M phase of OA chondrocytes cells (Figures 2D-2F). At the same time, the expression levels of mRNA and protein of cell differentiation related molecules BMP2, BMP4, BMP6 and RUN2 increased significantly (Figures 2G-2H).

CircPSM3 Acted as a Sponge of MiR-296-5p In OA Chondrocytes

To determine the interaction between CircPSM3 and miR-296-5p, the target binding between CircPSM3 and miR-296-5p is shown in Figure 3A. The Dual-Luciferase results showed that the overexpression of miR-296-5p significantly attenuated the Luciferase activity of CircPSM3-WT but not CircPSM3-WT -MUT reporter (Fig-

ure 3B). Transfection of miR-296-5p mimics in OA chondrocytes could significantly promote the expression of miR-296-5p, and miR-296-5p inhibitors could significantly inhibit the expression of miR-296-5p (Figure 3C). The expression level of miR-296-5p in OA tissues was significantly lower than that of meniscus injury tissues (Figure 3D). Further, the transfection of si-CircPSM3 in OA chondrocytes could significantly promote the expression level of miR-296-5p (Figure 3E), whereas transfection of LV-CircPSM3 significantly inhibited the expression level of miR-296-5p (Figure 3F). Spearman's correlation analysis revealed a significantly negative correlation between CircPSM3 and miR-296-5p in OA chondrocytes (Figure 3G).

Overexpression of MiRNA-296-5p Facilitates Proliferation and Differentiation of OA Chondrocytes

To further analyze the effect of miRNA-296-5p on the proliferation and differentiation of OA chondrocytes, OA chondrocytes were transfected with miRNA-296-5p mimics for 3 days. The results showed that the cell proliferation ability was significantly higher than transfection with miR-NC (Figure 4A), and the expression levels of proliferation-related molecules PCNA mRNA and protein were also increased (Figures 4B and 4C). Flow cytometry suggested that the percentage of G0/G1 cells in OA chondrocytes after transfection with miRNA-296-5p mimics was significantly lower than transfection with miR-NC group. However, the percentage of S phase cells in OA chondrocytes was significantly higher than transfection with miR-NC group, and there was no statistical difference between the two groups in the proportion of M phase of OA chondrocytes cells (Figures 4D-4F). Additionally, the expression levels of mRNA and protein of cell differentiation related molecules BMP2, BMP4, BMP6 and RUN2 increased significantly (Figures 4F-4H).

CircPSM3 Regulates Proliferation and Differentiation of OA Chondrocytes Through Targeting MiR-296-5p

To investigate whether CircPSM3 affects the proliferation and differentiation by targeting miRNA-296-5p, OA chondrocytes were transfected with si-NC and si-CircPSM3, or si-CircPSM3 and miR-296-5p inhibitors, respectively. The results revealed that the low expression of miR-296-

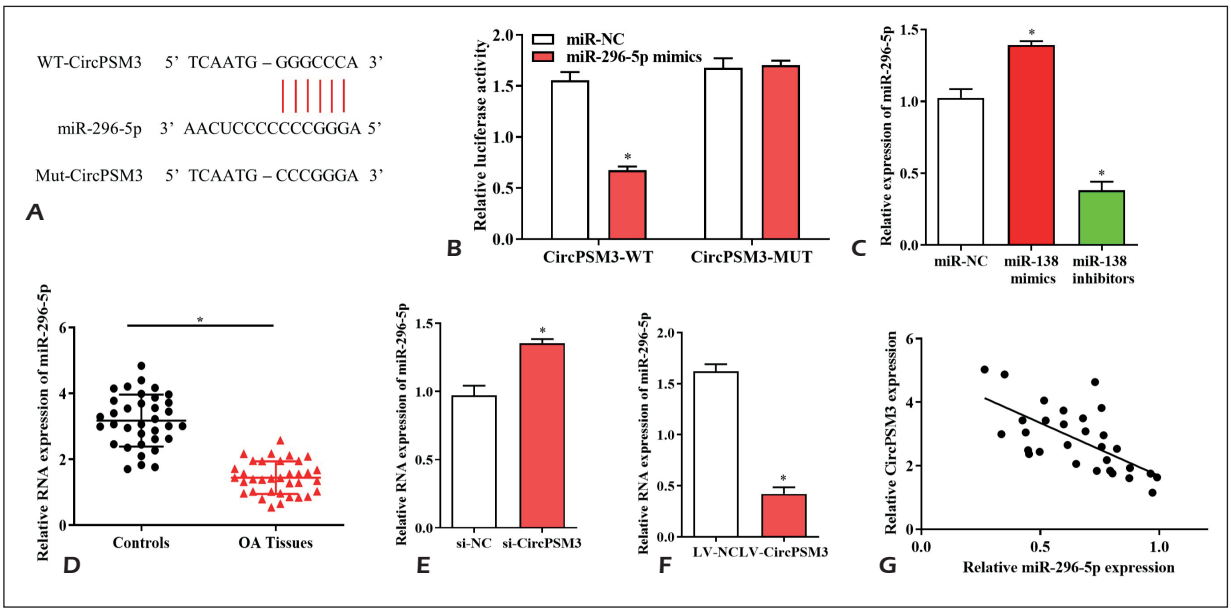

Figure 3. CircPSM3 acted as a sponge of miR-296-5p in OA chondrocytes. **A**, The predicted miR-296-5p binging sites in CircPSM3 mRNA 3'-UTR. **B**, The Luciferase activity of CircPSM3-WT and CircPSM3-MUT reporter was measured by luciferase assays in OA chondrocytes. **C**, The expression levels of miR-296-5p were measured by qRT-PCR after transfection of miR-296-5p mimics and miR-296-5p inhibitors. **D**, The expression of CircPSM3 was detected by qRT-PCR in OA tissue and meniscus damaged tissues. **E**, The expression levels of miR-296-5p were measured by qRT-PCR after transfection of si-CircPSM3 and LV-CircPSM3. **F**, The correlation between CircPSM3 and miR-296-5p was measured by Spear¬man's correlation analysis. The data were expressed as mean ± SD. *p<0.05.

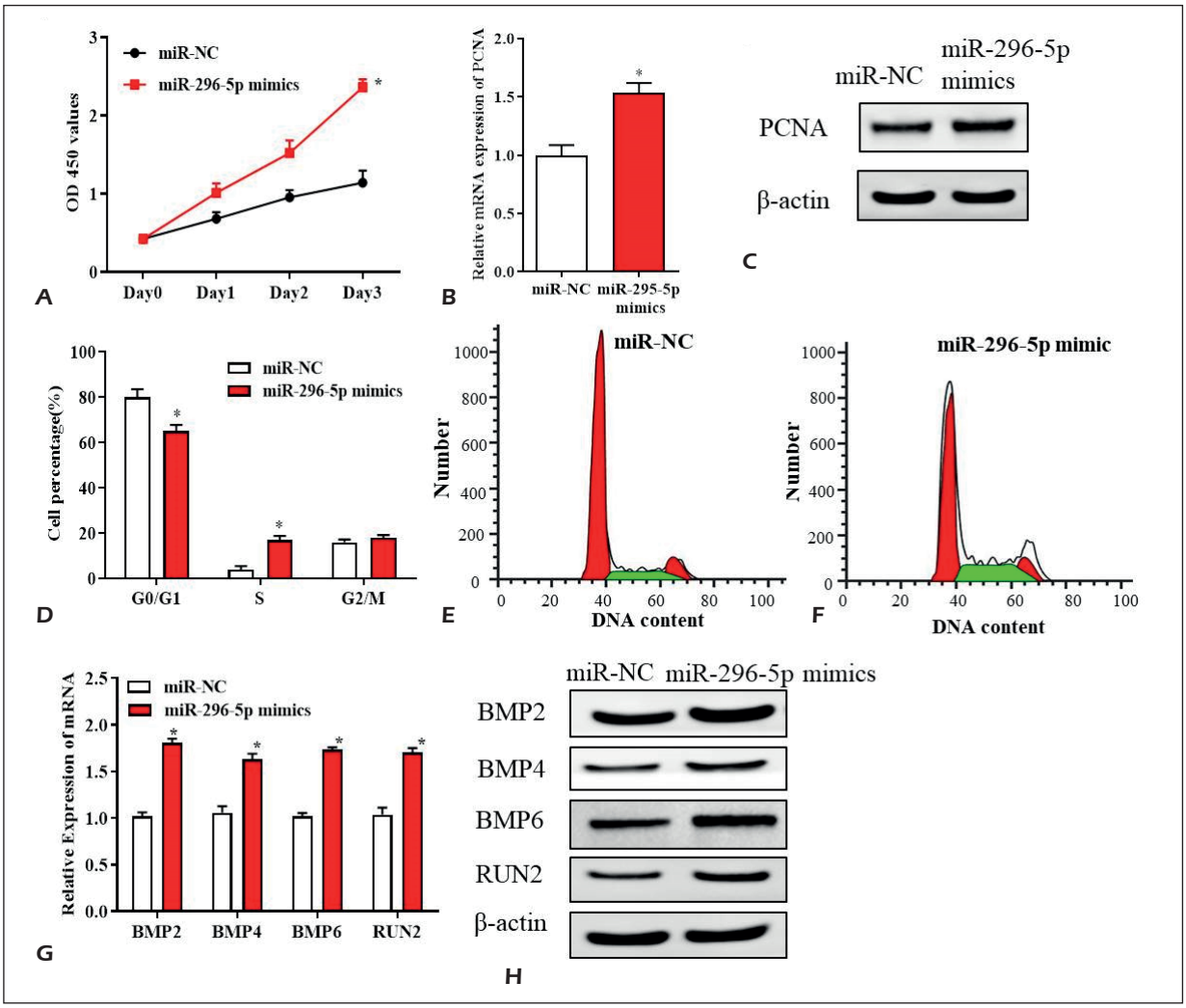

Figure 4. Overexpression of miRNA-296-5p facilitated proliferation and differentiation of OA chondrocytes. **A-B**, The proliferation of OA chondrocytes was measured by CCK-8 assay after transfection of miRNA-296-5p mimics. **C**, Proliferation-related molecular expression levels were measured by Western blotting. **D-F**, Cell cycle of OA chondrocytes was obtained by flow cytometry. **G-H**, Cell differentiation related molecules expression levels were measured by Western blotting and mRNA expression levels were detected by qRT-PCR. The data were expressed as mean ± SD. *$p<0.05$.

5p reversed the effects caused by downregulated CircPSM3. Molecular expression levels related to cell proliferation and differentiation in OA chondrocytes after transfection with si-CircPSM3 were much higher than transfection with si-CircPSM3 and miR-296-5p inhibitors (Figures 5A-5D). Flow cytometry suggested that the percentage of G0/G1 cells in OA chondrocytes after transfection with si-CircPSM3 was significantly lower than transfection with si-CircPSM3 and miR-296-5p inhibitors group. However, the percentage of S phase cells in OA chondrocytes was significantly higher than transfection with si-CircPSM3 and miR-296-5p inhibitors group (Figures 5E-5H). Overexpression of 296-5p promoted the effects caused by downregulated CircPSM3. Molecular expression levels related to cell proliferation and differentiation in OA chondrocytes after transfection with si-CircPSM3 and miR-296-5p mimics were much higher than transfection with si-CircPSM3 (Figures 5I-5L). Flow cytometry suggested that the percentage of G0/G1 cells in OA chondrocytes after transfection with si-CircPSM3 and miR-296-5p mimics was significantly lower than transfection with si-CircPSM3 group. However, the percentage of S phase cells in OA chondrocytes was significantly higher than transfection with si-CircPSM3 group (Figures 5M-5P). This evidence suggested that CircPSM3 may affect the proliferation and differentiation by targeting miRNA-296-5p.

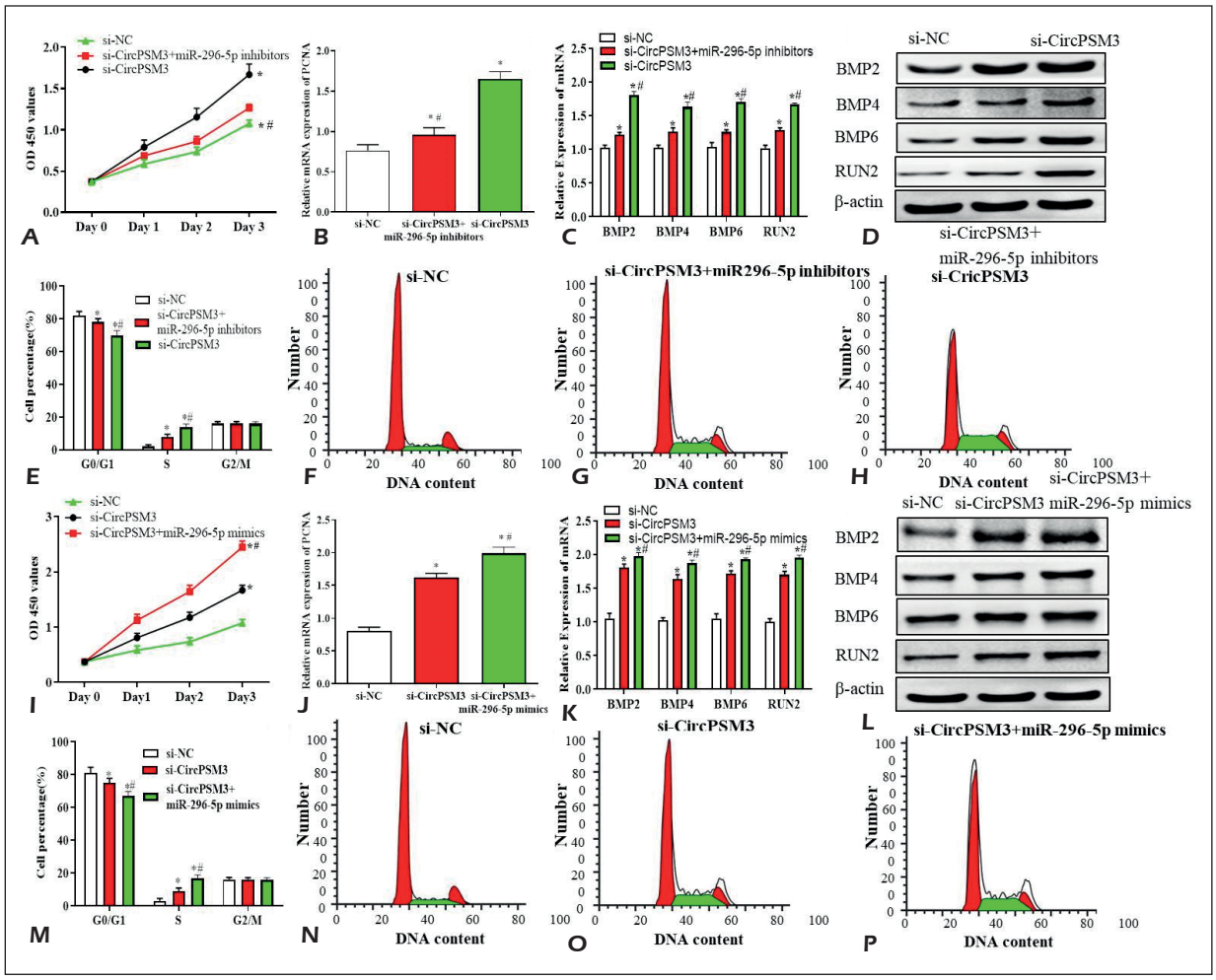

Figure 5. CircPSM3 regulated proliferation and differentiation of OA chondrocytes through targeting miR-296-5p. **A-B**, The proliferation of OA chondrocytes was measured by CCK-8 assay after transfection of si-NC, si-CircPSM3 and miR-296-5p inhibitors, si-CircPSM3. **C-D**, Cell differentiation related molecules expression levels were measured by Western blotting and mRNA expression levels were detected by qRT-PCR. **E-H**, Cell cycle of OA chondrocytes was obtained by flow cytometry. **I-J**, The proliferation of OA chondrocytes was measured by CCK-8 assay after transfection of si-NC, si-CircPSM3, si-CircPSM3 and miR-296-5p mimics. **K-L**, Cell differentiation related molecules expression levels were measured by Western blotting and mRNA expression levels were detected by qRT-PCR. **M-P**, Cell cycle of OA chondrocytes was obtained by flow cytometry.

Discussion

Osteoarthritis is one of the most common conditions in clinical orthopedic diseases in elderly group. However, osteoarthritis is a complex disease which is caused by multiple factors[1,4,18] such as obesity, aging, and genetic factors[12]. Patients with OA have to take longer treatment periods, as well as a heavy financial burden on individuals and families. The clinically effective intervention to improve osteoarthritis has not been put forward until now because the pathogenesis of osteoarthritis is still not clear.

At present, unusual expressions in osteoarthritis of CircRNA have a great effect on pathogenesis of osteoarthritis[5,14,17]. CircRNA is a type of RNA molecule that does not have a 5 'and 3' end-to-end structure and forms a cyclic structure with covalent bonds. CircRNA could serve as miRNA sponges, which means CircRNA could regulate osteoarthritis proliferation, differentiation, invasion and apoptosis by targeting miRNA[6,19]. The expression of CircRNA is closely related to the proliferation and apoptosis of OA chondrocytes[4,18,20]. Wu et al[17] also found that CircRNA hsa_circ_0005105 serves as miR-26 sponges to promotes chondrocyte extracellular matrix degradation. Xia et al[21] demonstrated that circular RNA circ-CBFB had a positive effect on proliferation and inhibited apoptosis in chronic lymphocytic leukemia by targeting miR-

607. In our study, we found that CircPSM3 was significantly upregulated in OA tissues, as well as OA chondrocytes. Meanwhile, low expression of CircPSM3 promoted proliferation and differentiation of OA chondrocytes. Collectively, these results suggested that CircPSM3 may be an important target molecule in OA chondrocytes and CircPSM3 could affect the progression of osteoarthritis.

MicroRNA belongs to a class of single-stranded non-coding small RNA molecules with a length of about 19-23 nt. MiRNAs play a vital role in a range of biological activities[22-24]. Besides, miRNAs could regulate cell proliferation, growth, differentiation, apoptosis, and metabolism in various tissue cells[25,26]. A large number of miRNAs are involved in the pathogenesis of osteoarthritis[2,20]. Of note, miR-136 participated in the process of chondrocyte ECM degradation by targeting MMP13[27]. Expression levels of miRNA-155, miRNA-181a and miRNA-223 were different in chondrocyte[28]. Rong et al[20] found that CircPSM3 could target miR-296-5p to repress the proliferation and metastasis of gastric cancer. We showed that CircPSM3 acted as a sponge of miR-296-5p in OA chondrocytes. To further determine the interaction between CircPSM3 and miR-296-5p, the expression level of miR-296-5p was upregulated in OA tissues transfected with si-CircPSM3 while downregulated when transfected with LV-CircPSM3. This indicated a negative correlation between CircPSM3 and miR-296-5p expression levels. When miR-296-5p was highly expressed, the expression levels of mRNA and protein of cell differentiation and proliferation were upregulated in OA tissues. These results of overexpression of miR-296-5p facilitated proliferation and differentiation of OA chondrocytes. In addition, low expression of 296-5p reversed the effects caused by downregulated CircPSM3, while overexpression of miR-296-5p promoted the effects caused by downregulated CircPSM3. All these data suggested that CircPSM3 inhibits the proliferation and differentiation of OA chondrocytes by targeting miR-296-5p.

Conclusions

Summarily, CircPSM3 was highly expressed in OA chondrocytes. CircPSM3 acts as a sponge of miR-296-5p to inhibit the proliferation and differentiation of OA chondrocytes. We provided evidence that CircPSM3/miR-296-5p pathway has a significant impact on OA chondrocyte proliferation and differentiation. It will provide a theoretical basis for the application of CircPSM3 in the treatment of osteoarthritis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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