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LncRNA CCAT1 promotes cell proliferation and differentiation via negative modulation of miRNA-218 in human DPSCs

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Abstract. – OBJECTIVE: To investigate the expression of Long non-coding RNA (LncRNA) CCAT1 and potential functions in promoting cell proliferation and differentiation, via miRNA-218 in human adult Dental Pulp Stem Cells (DPSCs).

PATIENTS AND METHODS: CCAT1 expressions in Periodontal Ligament Cells (PDLCs), DPSCs, differentiated main population (MP) cells and stem-cell-enriched Side Population (SP) cells in DPSCs were detected by qRT-PCR. MTT assay and ELISA assay were performed to evaluate the DPSCs cell proliferation and differentiation. The correlation between miR-218 and CCAT1 was detected by statistical analysis. The bioinformatics and luciferase assay were performed to explore the interaction and binding site of CCAT1 and miR-218.

RESULTS: Results showed the CCAT1 expression was up-regulated in DPSCs cells. And the expression level in MP cell was higher than SP cell. MTT assay and showed overexpression CCAT1 significantly increased cell proliferation of DPSCs. ELISA assay showed the expressions of collagen I, Osteopontin (OPN) and Osteocalcin (OCN) were significantly increased in DPSCs compared with control ($p<0.05$). The bioinformatics and luciferase assay showed that the CCAT1 directly interacted with miR-218. In addition, miR-218 expression was negatively correlated with CCAT1 expression in DPSCs

CONCLUSIONS: For the first time, we found that lncRNA-CCAT1 was upregulated in DPSCs, which could promote cell proliferation and differentiation by repressing the expression of miR-218.

Key Words:
LncRNA-CCAT1, miR-218, DPSCs, Proliferation, Differentiation.

Introduction

Stem cells are biological cells that have the potentials of differentiating into multiple types of cells and dividing to produce the same type of stem cells. There are two types of stem cells in mammals, the embryonic stem cells and the adult stem cells which are found in various tissues[1-3]. Dental Pulp Stem Cells (DPSCs) belong to the second one, which is derived from neural crest-external mesenchymal stem cells and can be isolated from adult dental pulp[4]. In adult organisms, DPSCs and progenitor cells are served as body's repair system, complementing the pulpal adult tissue. As a result, they are important in tissue engineering and regenerative medicine research[5]. However, the mechanisms of odontogenic proliferation and differentiation of DPSCs are not well understood. Long non-coding RNAs (lncRNAs) are a sort of RNAs that are longer than 200 nucleotides but do not have capacity of protein-coding, which could affect cancer occurrence and development through regulating gene expression at epigenetic, transcriptional, and post-transcriptional levels[6-8]. In addition, lncRNAs are also involved in the regulation of stem cell proliferation and differentiation and other biological processes[9,10]. However, the biological functions and mechanisms of lncRNAs in DPSCs are largely unknown. Colon cancer-associated transcript 1 (CCAT1) is located at chromosome 8q24.21, which had been found to be involved in the processes of metastasis, migration, progression, and proliferation in some cancers, such as ovarian cancer, prostate cancer, squamous cancer, thyroid carcinoma, gastric cancer, etc.[11-16]. Surprisingly, our previous study showed that CCAT1 was upregulated in DPSCs; however, the roles and functions of CCAT1 in DPSCs are currently poorly understood so far. In this study, we aimed to explore the functional roles of CCAT1 in DPSCs, as well as to disclose the molecular mechanisms. Firstly, we measured the CCAT1 levels in periodontal ligament cells (PDLCs), DPSCs, differentiated main population (MP) cells and stem-cell-enriched side population (SP) cells in DPSCs. Secondly, the proliferation

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ability and differentiation functions were measured after transfection with lentiviral CCAT1. At last, we assessed the regulatory relationship between CCAT1 and miR-218 and the miR-218-mediated roles of CCAT1. Our study, therefore, uncovered a critical function of lncRNA CCAT1 in cell proliferation and differentiation in DPSCs.

Patients and Methods

Cell Isolation and Cell Culture

PDLCs and DPSCs were isolated from the premolars and third molars extracted from 6 adults. All subjects received written informed consent according to guidelines recommended by our hospital Ethics Committee. DPSCs were divided into differentiated main population (MP) cells and stem-cell-enriched side population (SP) cells. The isolation and culture methods of DPSCs and PDLCs were used according to the method reported by Gronthos et al[17]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 15% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C and 5% CO_2 culture incubator.

Construction of Lentivirus and Cell Transfection

The full-length of human CCAT1 cDNA was synthesized and subcloned into a lentivirus (Shanghai GenePharma Co., Ltd., Shanghai, China), resulting in CCAT1 overexpression. Lentiviral lnc-CCAT1 and lnc-NC were added into DPSCs for 24 h. Next, the transfection efficiency was observed under an inverted fluorescent microscope. A stable cell line of DPSCs with CCAT1 overexpression was constructed after 1 to 2 weeks. Cells were seeded in 6-well plates (2×10^6/well) until reached 70-80%. Before transfection, the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), serum-free DMEM and miR-218 NC or miR-218 mimic were mixed and incubated for 30 min. They were then added into DPSCs with complete medium containing 15% fetal bovine serum (FBS). At indicated time point after transfection, cells were harvested for further study.

RNA Extraction and Quantitative Real-Time PCR

Total RNA of PDLCs and DPSCs was extracted by using RNAiso Plus (TaKaRa, Otsu, Shiga, Japan) according to its protocol. Reverse transcription was performed by using PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the protocol. PCR primers were synthesized by GenePharma (ShangHai Gene Pharma, ShangHai, China) and sequences were listed in Table I. mRNA expressions were detected by SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan). The mRNA expression of CCAT1 was normalized to GAPDH and miR-218 was normalized to U6 and $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expressions.

MTT Assay

MP and SP cells were respectively transfected with lentiviral NC, lentiviral CCAT1, or co-transfected with lentiviral CCAT1 and miR-218 for 48 h. Next, cells were seeded on 96-well plates (5×10^3/well) and cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12) medium at 37°C and 5% CO_2 for 3 d, 7 d, and 14 d. Afterward, 10 µl 5 g/l of MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 4 h, and proliferation of MP and SP cells were measured by MTT assay. The absorbance (OD) value of each well was measured at 490 nm with a microplate reader (Thermo Fisher, Waltham, MA, USA). Cell viability (P%) =OD (experimental group)/OD (control group)×100%. Every experiment was repeated for three times.

Table I. Sequences of primers for RT-PCR.

Genes	Primer sequences
CCAT1	Forward: 5'- TTTATGCTTGAGCCTTGA -3' Reverse: 5'- CTTGCCTGAAATACTTGC -3'
miR-218	Forward: 5'-ACACTCCAGCTGGGTTGT-3' Reverse: 5'-TGGTGTCGTGGAGTCG-3'
GAPDH	Forward: 5'-GGAGTCCACTGGTGTCTTCA-3' Forward: 5'-GGGAACTGAGCAATTGGTGG-3'
U6	Forward: 5'-CGCTTCGGCAGCACATATACT -3' Forward: 5'-CGCTTCACGAATTTGCGTGTC-3'

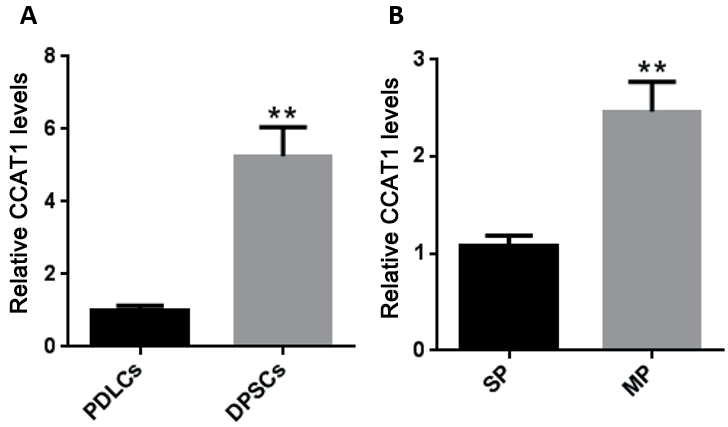

Figure 1. CCAT1 was upregulated in DPSCs. ***A,*** The mRNA levels of CCAT1 in PDLCs and DPSCs were detected by RT-PCR. ***B,*** The mRNA levels of CCAT1 in MP cells and SP cells from DPSCs were detected by RT-PCR. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$.

Cell Differentiation Assay

MP and SP cells were seeded on 96-well plates (5×10^3/well), and were respectively transfected with lentiviral NC, lentiviral CCAT1, or co-transfected with lentiviral CCAT1 and miR-218 for 48 h. After culturing for 0 d, 3 d, 7 d, and 14 d, the relative mRNA expressions of collagen I, Osteopontin (OPN) and Osteocalcin (OCN) were detected by using ELISA kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Luciferase Assay

The potential binding site of CCAT1-wt and mutant sequence CCAT1-mut was synthesized into pmiR-GLO (Promega, Madison, WI, USA). MP and SP cells were cultured overnight after being seeded into a 48-well plate, co-transfected with the CCAT1-wt or CCAT1-mut reporter gene plasmid and miR-218 mimic for 24 h. Plasmids with 200 ng were mixed with Lipofectamine 2000 and DMEM for 30 min, then the mixtures were added into MP and SP cells for 24 h. The cells were lysed and the activities of Firefly luciferase and Renilla luciferase were measured by using a Promega luciferase assay (Promega, Madison, WI, USA). Data were normalized against the activity of the Renilla luciferase gene and the ratio of these two revealed the relative activity of luciferase.

Statistical Analysis

All data were analyzed by SPSS 21.0 (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Data were expressed as means±SD. The methods of one-way ANOVA and Student's *t*-test were used to analyze these data, and multiple comparisons between the groups were performed by SNK method. *p*-value <0.05 was considered statistically significant.

Results

CCAT1 Was Upregulated in DPSCs

For the first time, we detected the expressions of CCAT1 in PDLCs, DPSCs, MP cells, and SP cells in DPSCs by RT-PCR. Results showed that the CCAT1 level in DPSCs was significantly increased compared to that in PDLCs ($p<0.05$) (Figure 1A). Furthermore, the expression of CCAT1 in the MP cells was significantly higher than that in SP cells ($p<0.05$) (Figure 1B). These results indicated that the expression of CCAT1 was significantly up-regulated in DPSCs, especially in the differentiated MP cells.

CCAT1 Promoted Cell Proliferation and Differentiation of DPSCs

To explore the functions of CCAT1 in DPSCs, lentiviral CCAT1 was constructed, which was respectively transfected into MP cells and SP cells of DPSCs; CCAT1 expression was detected by RT-PCR. Results showed that the CCAT1 expressions were significantly increased after lentiviral CCAT1 transfection ($p<0.05$) (Figure 2A). These cells were collected at 0 d, 3 d, 7 d, and 14 d to determine the OD570 value. The MTT assay showed that CCAT1 overexpression significant-

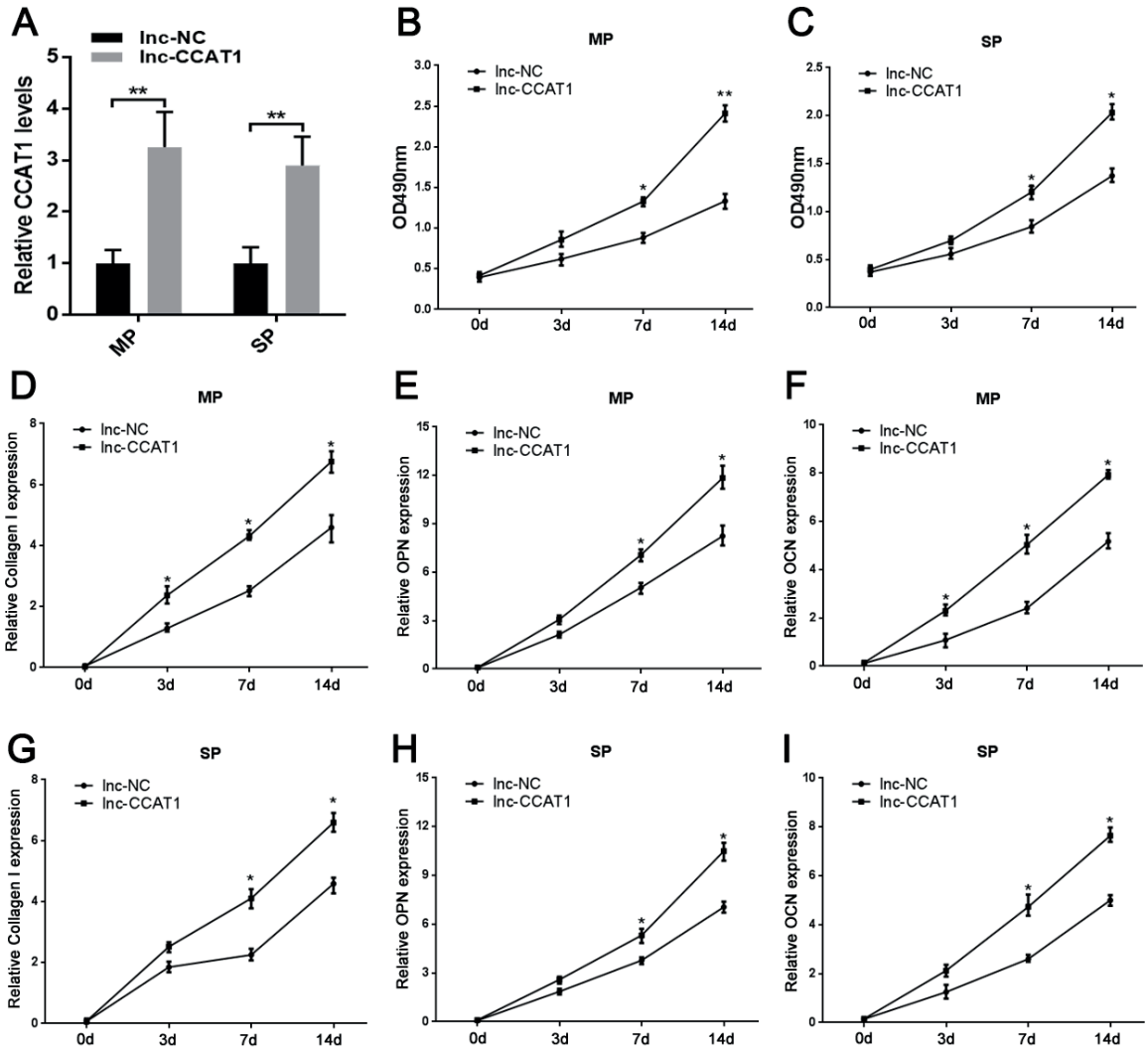

Figure 2. CCAT1 promoted cell proliferation and differentiation of DPSCs**. A,** Lentiviral CCAT1 was respectively transfected into MP cells and SP cells, CCAT1 expression was detected by RT-PCR. **B, C,** MTT assay was performed to evaluate cell proliferation activity after lentiviral CCAT1 transfection. **D-I,** Expressions of collagen I, OPN and OCN were detected by ELISA to evaluate cell differentiation after lentiviral CCAT1 transfection. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01.

ly promoted the cell proliferation activity of MP cells and SP cells after 7 d and 14 d, compared to the control (p<0.05) (Figure 2B, C). To further validate the effects of CCAT1 on the differentiation process of DPSCs, cell differentiation assay was performed and the expressions of collagen I, OPN and OCN, which were the important genes for cell differentiation of DPSCs, were detected by ELISA. Results showed that the expressions of collagen I, OPN and OCN were significantly increased after transfections in both MP cells and SP cells (p<0.05) (Figure 2D-I). These results indicated that CCAT1 promoted cell proliferation and differentiation. However, the molecular mechanism remained unknown.

miR-218 was Downregulated in DPSCs, which was Negatively Correlated with CCAT1

In order to explore whether CCAT1 was correlated miRNA, StarBase v2.0 database was used and miR-218 was one of the target genes of CCAT1, which was an important regulator among cell proliferation. Therefore, we also detected the miR-218 expressions of PDLCs and DPSCs by RT-PCR. Results showed that miR-218 was

downregulated in DPSCs, compared with PDLCs (p<0.05) (Figure 3A). To further explore the relationship between CCAT1 and miR-218, correlation analysis was performed. Results showed that miR-218 was negatively correlated with CCAT1 (p<0.05) (Figure 3B), indicating that miR-218 might be regulated by CCAT1.

CCAT1 Could Directly Bind with miRNA-218 in DPSCs

Researches demonstrated that lncRNAs might act as a competing sponge in regulating the biological functions of miRNAs. Our previous results showed that miR-218 was negatively correlated with CCAT1, so we suspected that CCAT1 regulated cell proliferation and differentiation through interaction with miR-218. To further investigate the association of miR-218 and CCAT1, the potential binding site was predicted. Next, CCAT1-wt luciferase reporter vector and CCAT1-mut 3'UTR luciferase reporter vector were synthesized (Figure 4A) and luciferase reporter assay was performed. Compared with the control, the luciferase activity of DPSCs that co-transfected with miR-218 mimic and CCAT1-wt was significantly decreased (p<0.05), and it was reversely increased in CCAT1-mut, compared with CCAT1-wt (p<0.01) (Figure 4B, C). These results revealed that miR-218 could directly bind to CCAT1. Furthermore, CCAT1 overexpression suppressed miR-218 expression and CCAT1 inhibition reversely facilitated miR-218 expression in both MP cells and SP cells (Figure 4D, E). Additionally, we also transfected miR-218 mimic and miR-218 inhibitor into MP cells and SP cells. The results showed that miR-218 mimic inhibited CCAT1 expression and miR-218 inhibitor increased CCAT1 expression (Figure 4F, G). All above, these results suggested that miR-218 directly bound to CCAT1 at the recognition sites.

CCAT1 Promoted Cell Proliferation and Differentiation via miR-218 in DPSCs

To further verify that CCAT1 regulated cell proliferation and differentiation by targeting miR-218, lnc-CCAT1 and miR-218 mimic were transfected into MP cells and SP cells. The OD570 value was determined after 0 d, 3 d, 7 d, and 14 d. Results showed that CCAT1 significantly promoted the proliferation after 7 d and 14 d compared with the control group, while the overexpression of miR-218 significantly inhibited cell proliferation (p<0.05) (Figure 5A, B). Cell differentiation assay was performed and the expressions of collagen I, OPN and OCN were detected by ELISA. Results showed that the expressions of collagen I, OPN and OCN were significantly increased after CCAT1 transfections, while they were repressed after miR-218 mimic transfection (p<0.05) (Figure 5C-H). Above all, these results indicated that CCAT1 promoted cell proliferation and differentiation via targeting miR-218 in DPSCs.

Discussion

DPSCs are a kind of adult stem cells, which have the potential to differentiate into other different types of cells and to renew themselves[18]. Therefore, they are of vital importance in tissue engineering and regenerative medicine research[5]. However, the mechanisms of cell proliferation and differentiation of DPSCs are not fully understood. Recent studies[19,20] found that lncRNAs

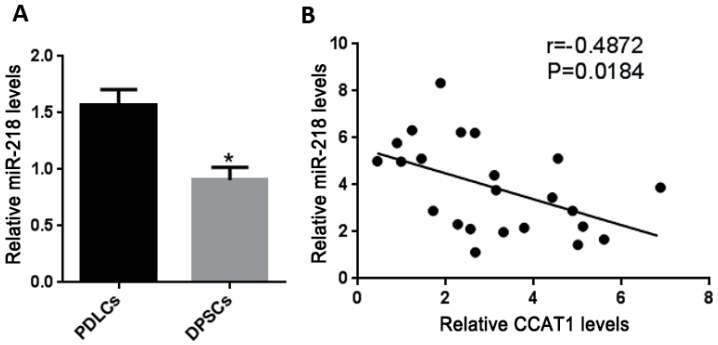

Figure 3. miR-218 was downregulated in DPSCs, which was negatively correlated with CCAT1. **A,** The mRNA levels of miR-218 in PDLCs and DPSCs were detected by RT-PCR. **B,** Correlation analysis was performed to evaluate the relationship between miR-218 and CCAT1. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05.

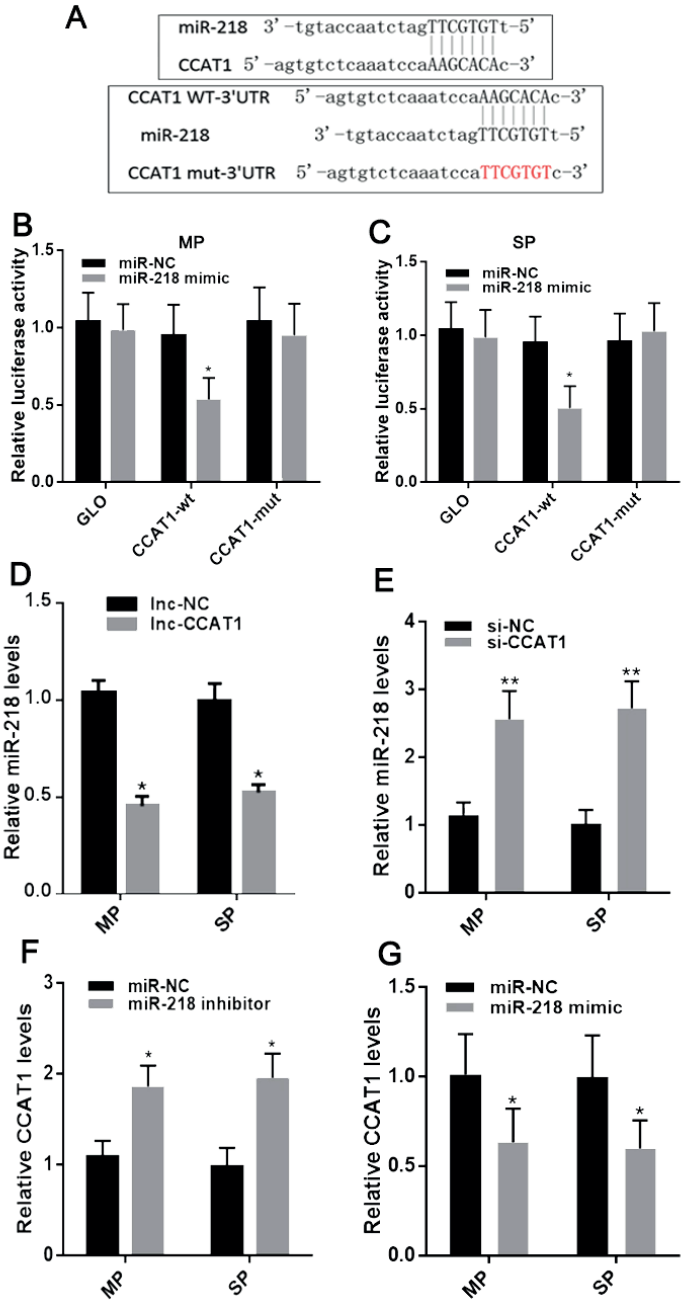

Figure 4. CCAT1 could directly bind with miRNA-218 in DPSCs. ***A,*** The potential binding site was predicted, then CCAT1-wt and CCAT1-mut 3'UTR luciferase reporter vector were synthesized. ***B-C,*** Relative luciferase activity was detected by luciferase reporter assay. ***D-E,*** MiR-218 expression was detected after lentiviral CCAT1 and si- CCAT1 transfection by RT-PCR. ***F-G,*** CCAT1 expression was detected after miR-218 mimic and miR-218 inhibitor by RT-PCR. Data are shown as mean ± SD based on at least three independent experiments, *$p<0.05$, **$p<0.01$.

are closely related to biological processes, such as cell proliferation and migration of many cancer cells or stem cells. However, whether lncRNAs played some roles in cell proliferation and differentiation of DPSCs remained unknown. CCAT1 had been found to be involved in the processes of metastasis, migration, progression, and proliferation in some cancers. Yang et al[21] reported that lnc-CCAT1 promoted cell proliferation, migration and invasion of thyroid cancer cells by down-regulating the expression of miR-143. Yu et al[22] reported that CCAT1 could be activated by

c-Myc to promote cell proliferation and migration of pancreatic cancer cells. Li et al[23] showed that CCAT1 knockdown led to inhibition of cell growth, invasion and peritoneal metastasis in gastric cancer. However, the role of CCAT1 in DPSCs remained unknown. In this study, we found CCAT1 in DPSCs was significantly higher than that in PDLCs, and MP was higher than that of SP cells. However, the functions of CCAT1 in DPSCs were unknown. To explore the functions of CCAT1 in DPSCs, lentiviral CCAT1 was constructed and transfected into DPSCs. The MTT assay and cell differentiation assay showed that CCAT1 overexpression significantly promoted the cell proliferation and expressions of collagen I, OPN and OCN, which indicated that CCAT1 promoted cell proliferation and differentiation in both MP and SP cells of DPSCs. Xu et al[24] reported that CCAT1 promoted the symmetric division of non-small cell lung cancer stem cells through Wnt signaling pathway. Our results were somewhat consistent with it. However, the molecular mechanisms remained unknown. MiRNAs are kinds of ncRNAs, approximately 20 nucleotides in length, which regulate gene expression by inhibiting translation of target genes in 3'UTR. It

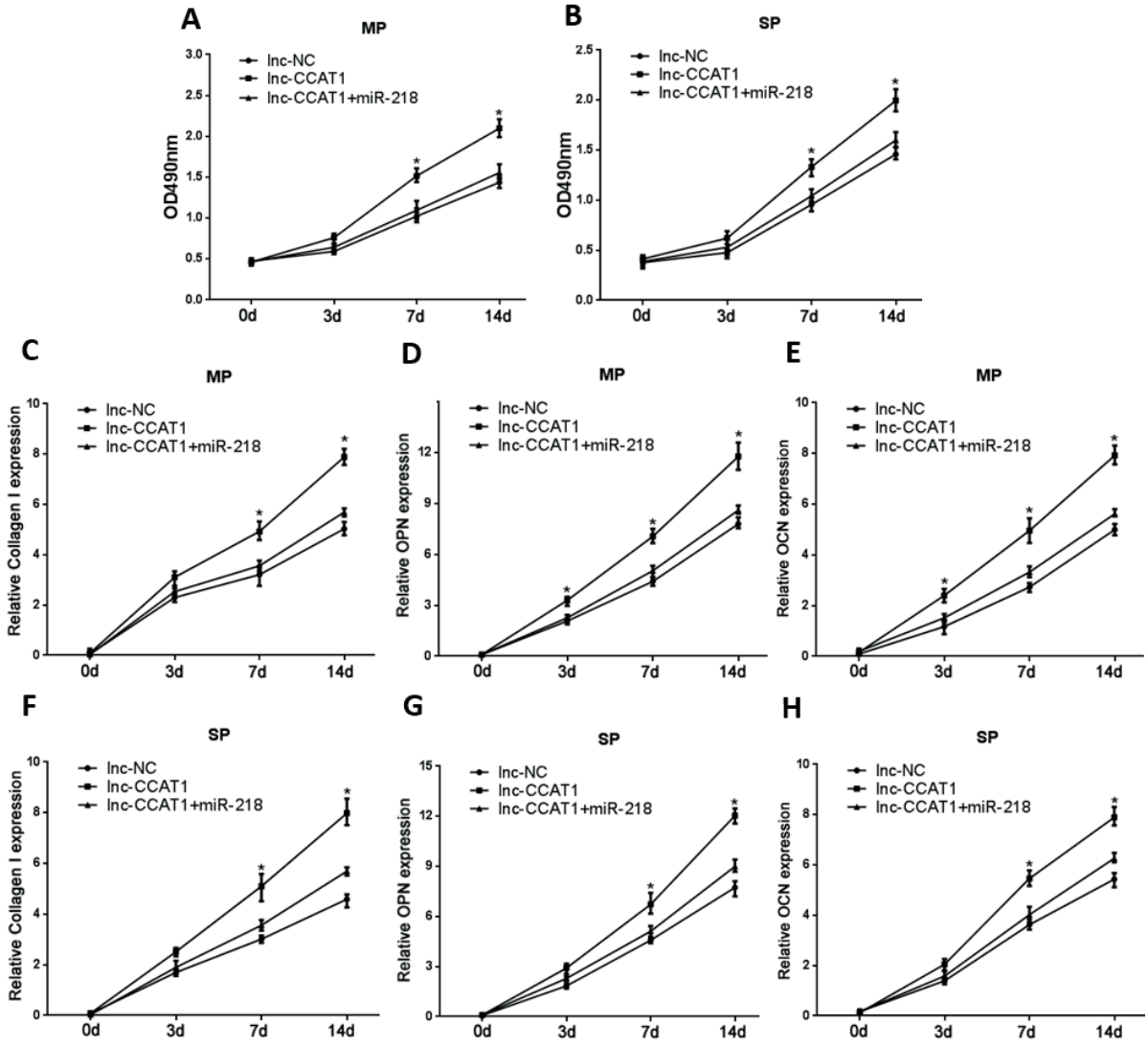

Figure 5. CCAT1 promoted cell proliferation and differentiation via miR-218 in DPSCs. A, B, MTT assay was performed to evaluate cell proliferation activity after lentiviral CCAT1 and miR-218 mimic transfection. **C-H,** Expressions of collagen I, OPN and OCN were detected by ELISA to evaluate cell differentiation. Data are shown as mean ± SD based on at least three independent experiments, *$p<0.05$.

has been reported that some miRNAs play a critical role in stem cells, such as cell proliferation, cell growth, differentiation, etc.[25]. miR-218 was reported to be involved in some cancers, and it was found to have tumor-suppressing qualities in bladder cancer cells[26]. Furthermore, miR-218 was associated with overall survival in breast cancer[27]. We used starBase v2.0 database to predict the target genes of CCAT1 and we found that miR-218 was one of them. After that, we detected the expressions of miR-218 in DPSCs. Results showed that miR-218 was downregulated in DPSCs, which was negatively correlated with CCAT1. To further confirm the relationship between CCAT1 and miR-218, luciferase reporter assay was performed. Results suggested that miR-218 could directly bind to CCAT1 at the recognition sites. To further verify that CCAT1 regulated cell proliferation and differentiation by targeting miR-218, lnc-CCAT1 and miR-218 mimic were transfected. MTT assay and cell differentiation assay showed that CCAT1 significantly promoted cell proliferation and differentiation, while they were significantly repressed after miR-218 mimic transfection. Above all, these results indicated that CCAT1 promoted cell proliferation and differentiation via targeting miR-218 in DPSCs.

Conclusions

For the first time, we found that lncRNA-CCAT1 was upregulated in DPSCs. Furthermore, CCAT1 promoted cell proliferation and differentiation by repressing the expression of miR-218, which might be a novel marker of DPSCs and might be a potential target for clinical application.

Conflict of interest

The authors declare no conflicts of interest.

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