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Long non-coding RNA DANCR induces chondrogenesis by regulating the miR-1275/MMP-13 axis in synovial fluid-derived mesenchymal stem cells

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Abstract. – OBJECTIVE: The aim of this study was to examine the role of lncRNA-differentiation antagonizing non-protein coding RNA (DANCR) and its underlying mechanisms in chondrogenesis, more specifically in synovial fluid-derived mesenchymal stem cell (SFMSCs).

MATERIALS AND METHODS: The expression levels of DANCR in SFMSCs were measured by qRT-PCR. Luciferase reporter assay and RIP assay were used to investigate the direct target of DANCR and miR-1275 in SFMSCs. The expression of matrix metallopeptidase 13 (MMP13, also known as *chondrogenic marker*) protein was examined by Western blot. Cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8) assay, while chondrogenic differentiation was explored by sGAG assay.

RESULTS: Our data indicated that DANCR can promote SFMSCs proliferation and chondrogenesis. In addition, miR-1275 was indicated as a direct target of DANCR. MiR-1275 was negatively regulated by DANCR via competing endogenous RNA (ceRNA) mechanism. Moreover, our data revealed that miR-1275 could bind to MMP13 and regulate its expression.

CONCLUSIONS: Our findings suggested that DANCR was involved in SFMSCs proliferation and chondrogenesis. Mechanistically, DANCR functions as a sponge RNA for miR-1275 that regulates the expression of target gene MMP13. These data provide a therapeutic option for Osteoarthritis (OA).

Key Words:
DANCR, Synovial fluid-derived mesenchymal stem cell, Chondrogenesis, MiR-1275, MMP13.

Introduction

Osteoarthritis (OA) is a chronic, degenerative joint disease that commonly affects knees, temporomandibular joint (TMJ), and hip joint[1,2]. OA is usually characterized by progressive reductions in the extracellular matrix of joint cartilage and bone, which eventually leads to joint dysfunction[3]; cartilage tissues have a limited self-repair capacity and have a low abundance of regenerated chondrocytes in injured cartilage sites. Yet, existing therapies only focus on relieving symptoms instead of curing the disease[4,5]. Thus, there is an urgent need to explore and develop new therapies for articular cartilage repair.

Mesenchymal stem cells (MSCs) are types of cells that possess the capacity of self-renewal and multipotential differentiation in various adult tissues, including fat, bone, cartilage, and muscle[6]. Due to their chondrogenic differentiation capability, human MSCs are considered ideal candidates for cell-based strategies for articular cartilage repair[7]. Synovial fluid-derived MSCs (SFMSCs) are type of MSCs that are capable of differentiating into chondrocytes, osteoblasts, and adipocytes and could be easily obtained by routine arthroscopic examination or arthrocentesis[8,9]. Previous studies[10,11] have shown that SFMSCs could reduce the inflammatory intra-articular environment in rheumatoid arthritis joints. Long non-coding RNAs (lncRNAs) are type of RNA with length exceeding 200 nucleotides that cannot be translated into protein[12]. Authors[13,14] revealed that lncRNAs are essential for various functions including differentiation, cell proliferation, metabolize, and metastasis. For example, Gong et al[15] found that lncMyoD could regulate the differentiation by blocking IMP2-mediated mRNA translation in skeletal muscle. Furthermore, Li et al[16] showed that lncRNA UCA1 increases glutamine metabolism in bladder can-

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cer by regulating miR-16 expression. Ji et al[17] found that lncRNA MALAT1 promotes colorectal cancer growth and metastasis by regulating SFPQ and PTBP2 expression. Moreover, few authors[18-20] have evaluated the role of lncRNA in cartilage formation, such as ZBED3-AS1, lncRNA-HIT, and HOTAIR. In the present study, we examined the role of lncRNA-DANCR and its underlying mechanisms in chondrogenesis. MicroRNAs are a class of small non-coding RNAs with about 19-22 nucleotides in length, which are important components of epigenetic regulation system[21]. Recently, researches reported that miRNAs might have critical roles in chondrogenic differentiation. For example, Mao et al[22] showed that miR-92a-3p regulated ADAMTS-4 and ADAMTS-5 expression in chondrogenesis. Furthermore, Kureel et al[23] found that miR-376c/Wnt3 axis might have important roles in osteoblastogenesis. However, the role and underlying mechanisms of miR-1275 in chondrogenesis have not yet been reported. In the present study, we discovered that DANCR could serve as a molecular sponge for miR-1275 and regulate MMP-13 expression in chondrogenic differentiation of SFMSCs. These data suggested that DANCR might serve as an important therapeutic target for stem cell therapy in OA.

Materials and Methods

Cell Isolation and Culture

Synovial fluid samples were collected from ten osteoarthritis patients (6 females and 4 males) by joint puncture. SFMSCs were isolated from synovial fluid according to the previous study[24,25]. SFMSC experiments were carried out at passages 4 to 6. The present study was approved by the Ethics Committee of the Jinling Hospital of Nanjing University. The written informed consent was obtained from all the participants in this research.

Cell Transfection

The sequence of DANCR was obtained from Sangon (Shanghai, China) and cloned into a pcDNA3.1 plasmid to construct DANCR overexpression vector (DANCR). siRNAs (si-DANCR, si-MMP-13, si-NC), miR-1275 mimics, anti-miR-1275, and anti-miR-NC were obtained from GenePharma (Shanghai, China). Transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

sGAG Assay

sGAG assay was performed by following manufacturer's protocols and previous studies[26]. The sGAG levels were quantified at the absorbance of 656 nm.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cells with TRIzol (Invitrogen) following the manufacturer's instructions. cDNA synthesis was performed by using the iScript™cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). RNA expression was measured by quantitative Real Time-PCR using the SYBR method (TaKaRa, Dalian, China). GAPDH and U6 nuclear RNA were used as internal controls. The relative expression was calculated by $2^{-\Delta\Delta Ct}$ method.

The primer sequences used in the experiment were as follows: DANCR: Forward Primer: 5'-GCCACTATGTAGCGGGTTTC-3', Reverse Primer: 5'-ACCTGCGCTAAGAACTGAGG-3'; Sox9: Forward Primer 5'-CTGGGAACAACCCGTCTA-3', Reverse Primer: 5'-GGGTAATGCGCTTGGATA-3'; Col II: Forward Primer 5'-GGCTCCCAACACCGCTAAC-3', Reverse Primer: 5'- GATGTTCTGGGAGCCCTCAGT-3'; MMP13: Forward Primer 5'-GCCAGATGGGTTTTGAGAC-3', Reverse Primer: 5'-GTGATGCCTGGGGACTGTT-3'; miR-1275: Forward Primer 5'-GTGCAGGGTCCGAGGT-3', Reverse Primer: 5'-GCCGCTAGCTTATCGACTAC-3'.

CCK8 Assay

Cell proliferation was determined by Cell Counting Kit-8 (CCK-8, Beyotime, China). Briefly, 2,000 SFMSCs cells with upregulation of downregulation of DANCR were plated in 96-well plates and incubated for 1-7 days. At each time point, the cells were incubated in 10 μl of sterile CCK-8 solution (X mg/mL) for 2 h at 37°C. The absorbance at 450 nm was determined using a microplate reader (SpectraMax Plus 384).

Luciferase Reporter Assay

SFMSCs (3 × 104/well) were seeded in 24-well plates. After 24h, SFMSCs were co-transfected with miR-1275 mimics and DANCR-WT or DANCR-MUT (MMP-13-WT or MMP-13-MUT) to confirm the interaction between miR-1275 and DANCR or MMP-13. After 48 h, Luciferase intensity was detected with Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Assay

RNA immunoprecipitation assay was performed by imprint RNA immunoprecipitation kit (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's protocols 19.

Western Blot

The total protein was extracted from cells using RIPA lysis buffer following manufacturer's protocol and previous studies[15]. Briefly, after being separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA), protein samples were transferred into polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were then blocked with 5% nonfat dry milk, and then cultured with primary antibodies against Sox9, col II, MMP-13 and GAPDH (1:1000, Abcam, Cambridge, UK) at 37°C overnight. Consequently, samples were incubated with the secondary antibody for 2h. The immunoreactive proteins were visualized by ECL (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Data were represented as mean ± standard deviation (SD) from three separate experiments. All statistical assays were determined by using SPSS 18.0 software. Student's *t*-test was used for differences between two groups, One-way ANOVA followed Tukey's post-hoc test for multiple comparisons. A $p < 0.05$ was considered statistically significant.

Results

Characterization and Differentiation of SFMSCs

After 72 h cultivation, individual spindle-type cells adhered to culture plates (Figure 1A). After osteogenic and adipogenic induction for 14 days, Alizarin red staining or Oil Red O staining were performed to examine the presence of calcium deposits or lipid drops in SFMSCs (Figure 1B and 1C). In addition, flow cytometry was used to analyze the surface markers of SFMSCs; CD90, CD44, CD105, CD73 were expressed in SFMSCs, while CD45, CD34, CD19, and HLA-DR were not detected (Figure 1D). Thus, these data suggested that SFMSCs had the characteristics of MSCs.

DANCR Promotes SFMSCs' Proliferation

To examine the effects of DANCR on SFMSCs chondrogenesis, two SFMSCs were constructed: one with up-regulated DANCR and the other one with down-regulated DANCR. The transfected efficiency was determined by qRT-PCR (Figure 2A and 2B). Furthermore, CCK-8 assay demonstrated that the overexpression of DANCR remarkably increased SFMSCs proliferation ability, while the DANCR inhibition had an opposite effect (Figure 2C and 2D).

DANCR Promotes SFMSCs' Chondrogenesis

Furthermore, to evaluate the effects of DANCR on chondrogenic differentiation, we determined the expression of chondrogenic-specific markers Sox9 and Col II. Briefly, the DANCR overexpression of DANCR increased the mRNA and protein levels Sox9 and Col II in SFMSCs, while DANCR knockdown had an opposite effect (Figure 3A-C). Moreover, we measured the accumulation of glycosaminoglycan (GAGs), which are a group of extracellular matrix polysaccharides with important roles in cell signaling, proliferation and cell differentiation and found that the GAG/DNA ratio was significantly increased in SFMSCs with DANCR overexpression, while it was decreased in SFMSCs with DANCR downregulation (Figure 3D). These data suggested that DANCR might promote SFMSCs' chondrogenesis.

DANCR Directly Interacts with MiR-1275

To explore whether miRNAs were involved in DANCR-facilitated SFMSCs proliferation and chondrogenesis, miRcode was used to identify miRNAs that interacted with DANCR. Figure 4A showed that miR-1275 harbored DANCR with the complementary binding at 3'-UTR. Furthermore, QRT-PCR showed that miR-1275 expression was increased in SFMSCs transfected with sh-DANCR, while it was decreased in SFMSCs transfected with DANCR plasmids (Figure 4B). RNA immunoprecipitation (RIP) assay showed that DANCR and miR-1275 were largely captured by anti-Ago2 compared with the control group (Figure 4C). Moreover, dual-luciferase reporter assay showed that miR-1275 mimics significantly reduced the luciferase activity of DANCR-WT group (Figure 4D).

MiR-1275 Reversed the Effects of DANCR on SFMSCs Proliferation and Chondrogenesis

Next, we explored the effects of DANCR/miR-1275 axis on proliferation and chondrogenesis in SFMSCs. Our data showed that DANCR overexpression increased the SFMSC proliferation

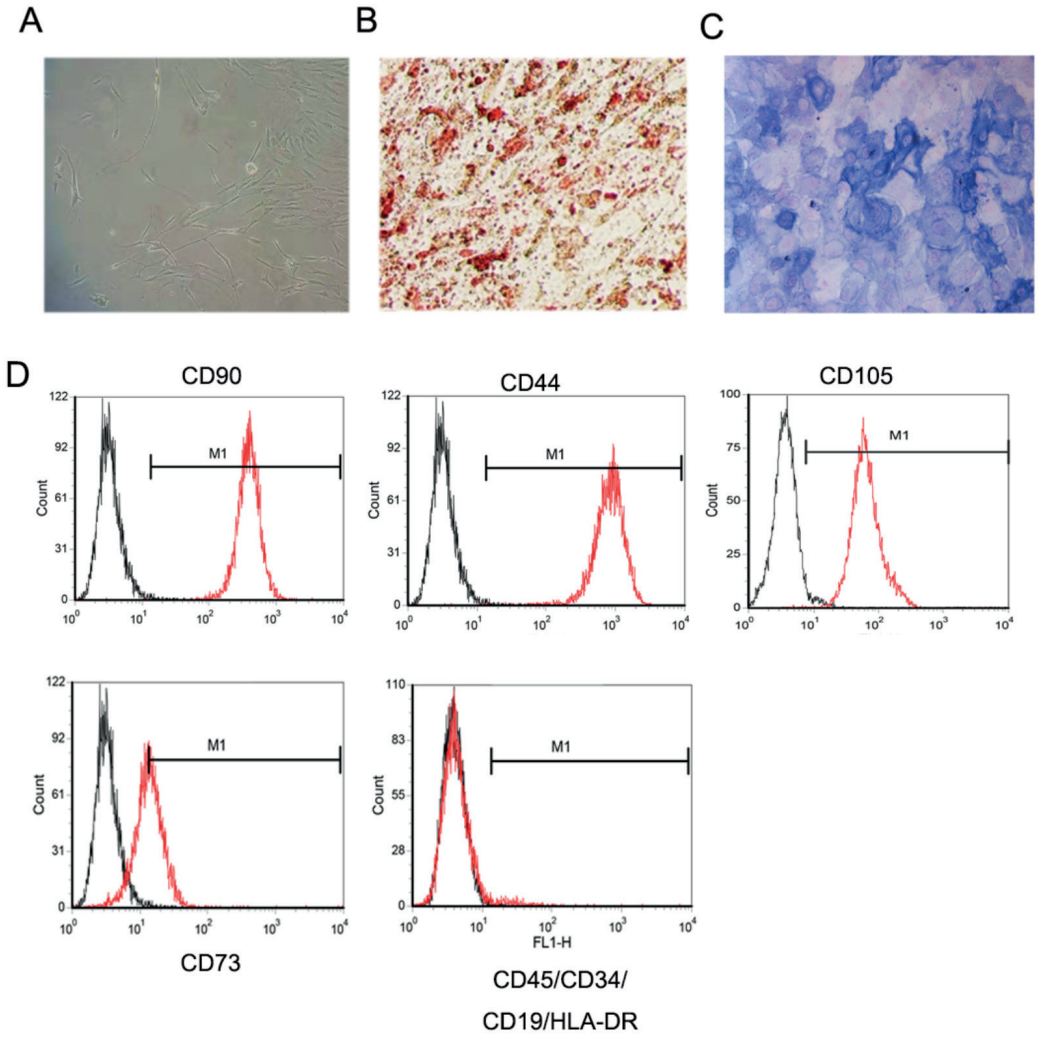

Figure 1. Characterization and differentiation of synovial fluid-derived mesenchymal stem cells. (SFMSCs). **A,** Morphology of SFMSCs in monolayer culture. **B,** Alizarin Red S staining. **C,** Red Oil staining. **D,** Surface marker expression of SFMSCs. magnification, ×200.

ability and up-regulated Sox9 and Col II, while miR-1275 mimics rescued the effects (Figure 5A, 5C). Moreover, down-regulation of miR-1275 enhanced the effects of DANCR overexpression on SFMSCs proliferation and chondrogenesis (Figure 5B, 5D).

MMP-13 is a Target Gene of MiR-1275

TargetScan was used to search the downstream targets of miR-1275. MMP-13 was identified as a potential target of miR-1275 (Figure 6A). Dual-Luciferase reporter assay showed that miR-1275 mimics reduced the luciferase activity of MMP13-WT group (Figure 6B). Moreover, QRT-PCR showed that MMP-13 mRNA expression was significantly reduced in SFMSCs transfected with miR-1275 mimics (Figure 6C). In addition, Western blot results were consistent with the QRT-PCR data (Figure 6D). These data suggested that MMP-13 could act as a target of miR-1275 in SFMSCs.

DANCR-Mediated SFMSCs Proliferation and Chondrogenesis by Regulating MMP-13

Finally, we explored the effects of DANCR on MMP-13 expression. Results showed that DANCR inhibition reduced the MMP-13 protein expression, while DANCR overexpression had an opposite effect (Figure 7A, 7B). Next, we examined the effects of MMP-13 on SFMSCs proliferation and chondrogenesis (Figure

7C). CCK-8 assay showed that DANCR overexpression enhanced SFMSCs proliferation, while MMP-13 inhibition abolished the effects (Figure 7F). Additionally, Western blot indicated that DANCR overexpression enhanced Sox9 and Col II expression; while MMP-13 suppression reversed the effects (Figure 7D, 7E). These data detected that DANCR-mediated SFMSCs proliferation and chondrogenesis by regulating the miR-1275/MMP-13 axis.

Discussion

Differentiation antagonizing non-protein coding RNA is located on human chromosome 4 close to annotated genes USP46 and ERVMER34-1[27]. Recently, a number of studies have reported that DANCR has a critical role in tumor progression. Liu et al[28] disclosed that DANCR upregulation in colorectal cancer was associated with advanced

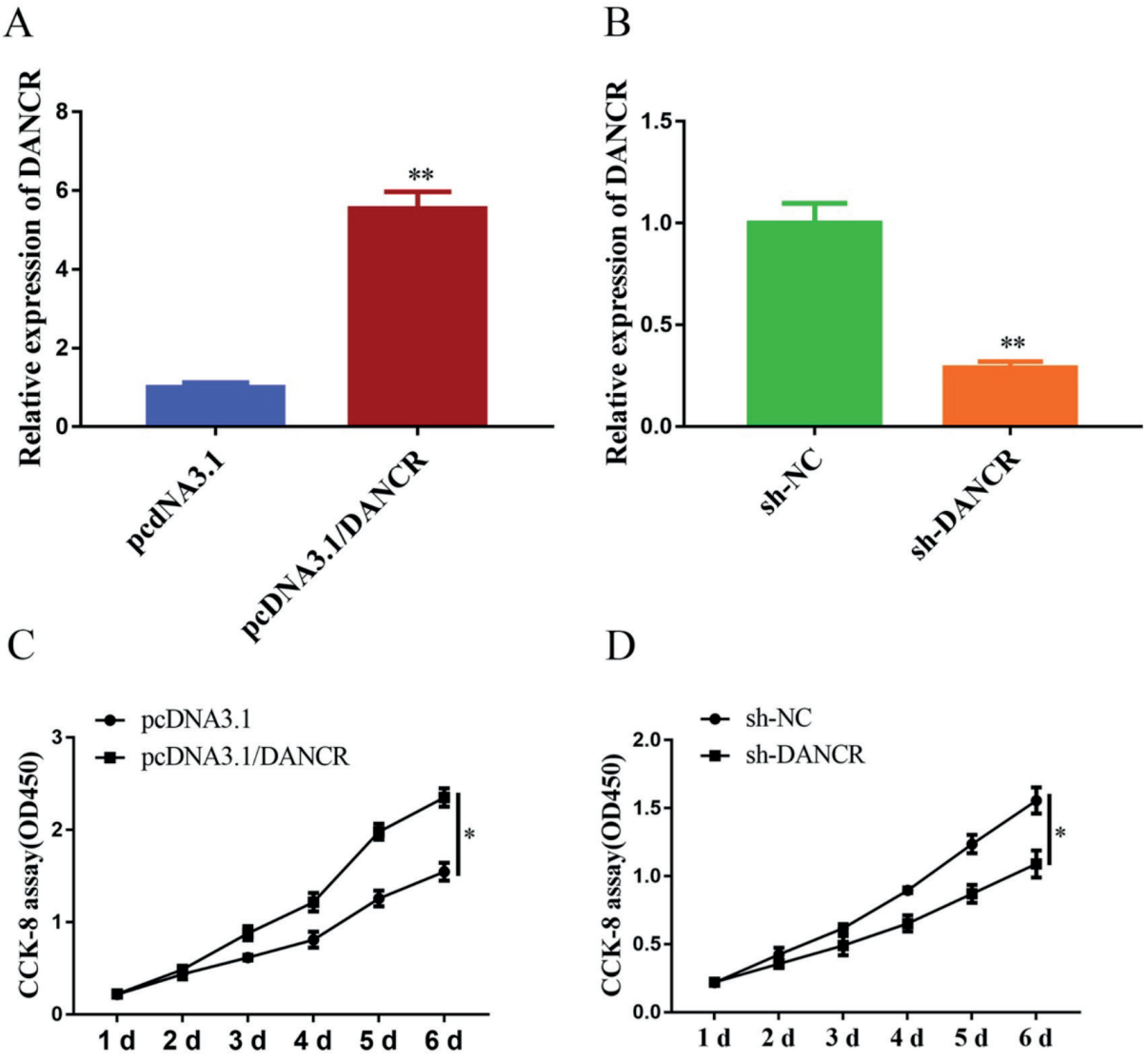

Figure 2. Regulatory effects of DANCR on SFMSCs proliferation. **A-B,** DANCR expression in SFMSCs transfected with pcDNA3.1/DANCR or sh-DANCR. **C,** DANCR overexpression increased SFMSCs cells proliferation. **D,** DANCR inhibition reduced SFMSCs cells proliferation. *$p< 0.05$

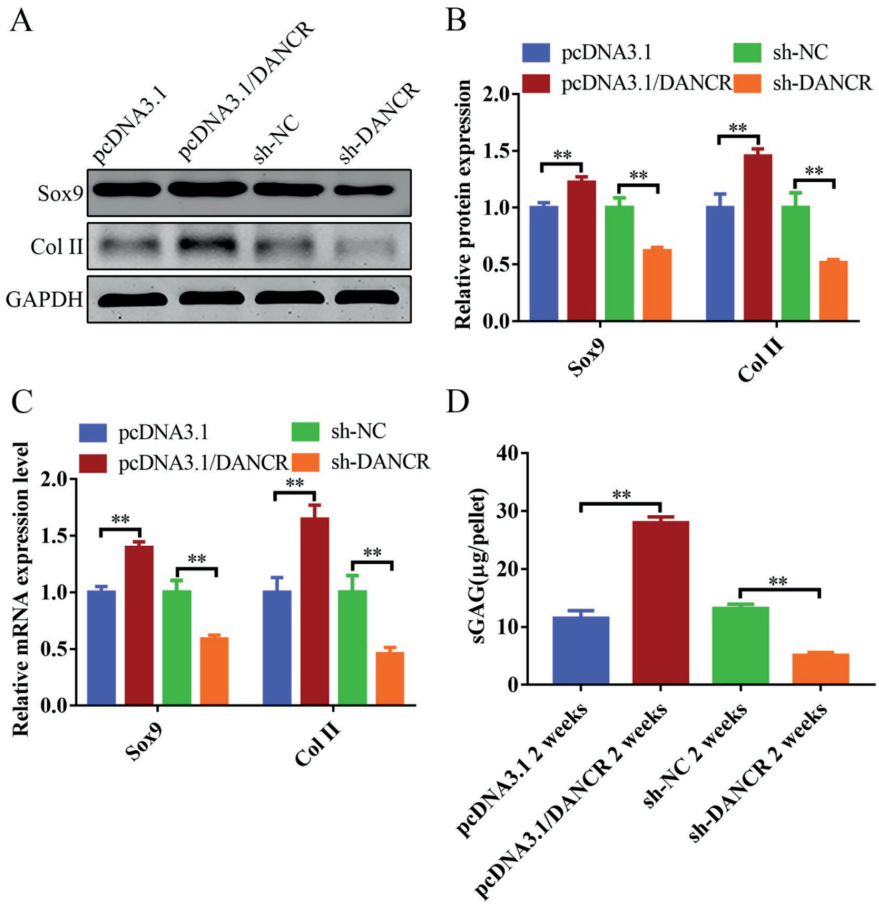

Figure 3. DANCR promoted SFMSCs' chondrogenesis. **A-B,** The effects of DANCR on Sox9 and Col II protein expression analyzed by Western blot. **C,** The effects of DANCR on Sox9 and Col II mRNA expression analyzed by qRT-PCR. **D,** sGAG contents in pellets of control vector, decreased expression group and overexpression group. * $p< 0.05$

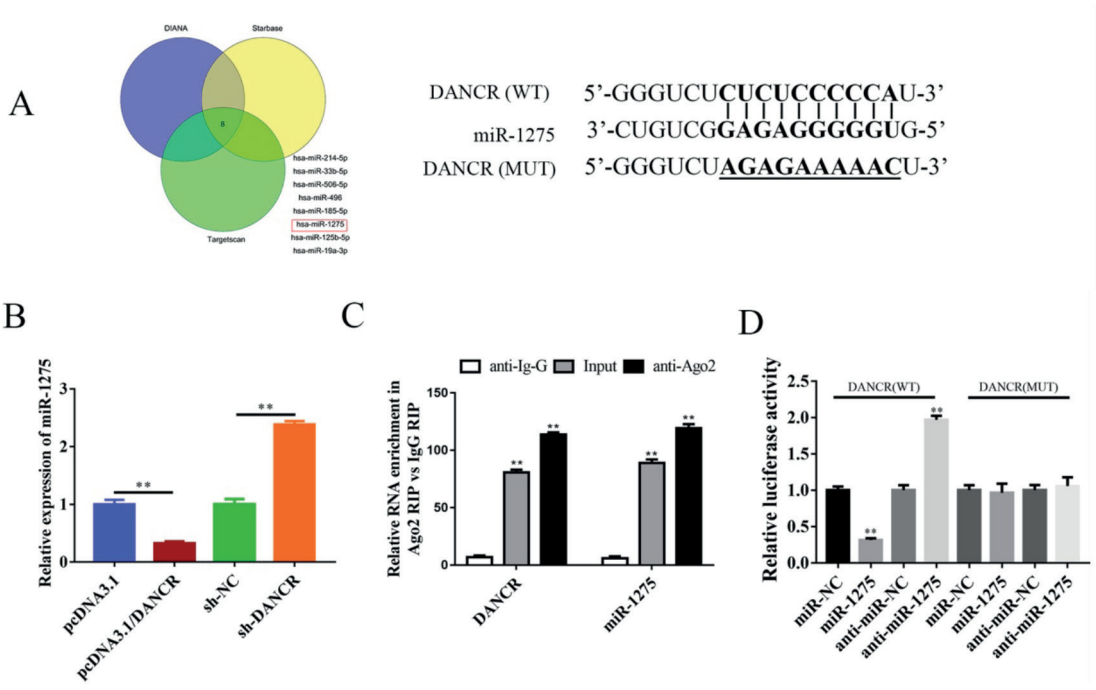

Figure 4. Direct interaction between DANCR and miR-1275. **A,** Bioinformatics evidence of miR-1275 binding onto 3'-UTR of DANCR. **B,** The effects of DNACR on miR-1275 expression in SFMSCs. **C,** Amount of DANCR and miR-1275 in SFMSCs were detected by RIP assay. **D,** Dual-luciferase reporter assay in SFMSCs cells transfected with miR-1275 mimics or miR-1275 inhibitors. *$p<0.05$

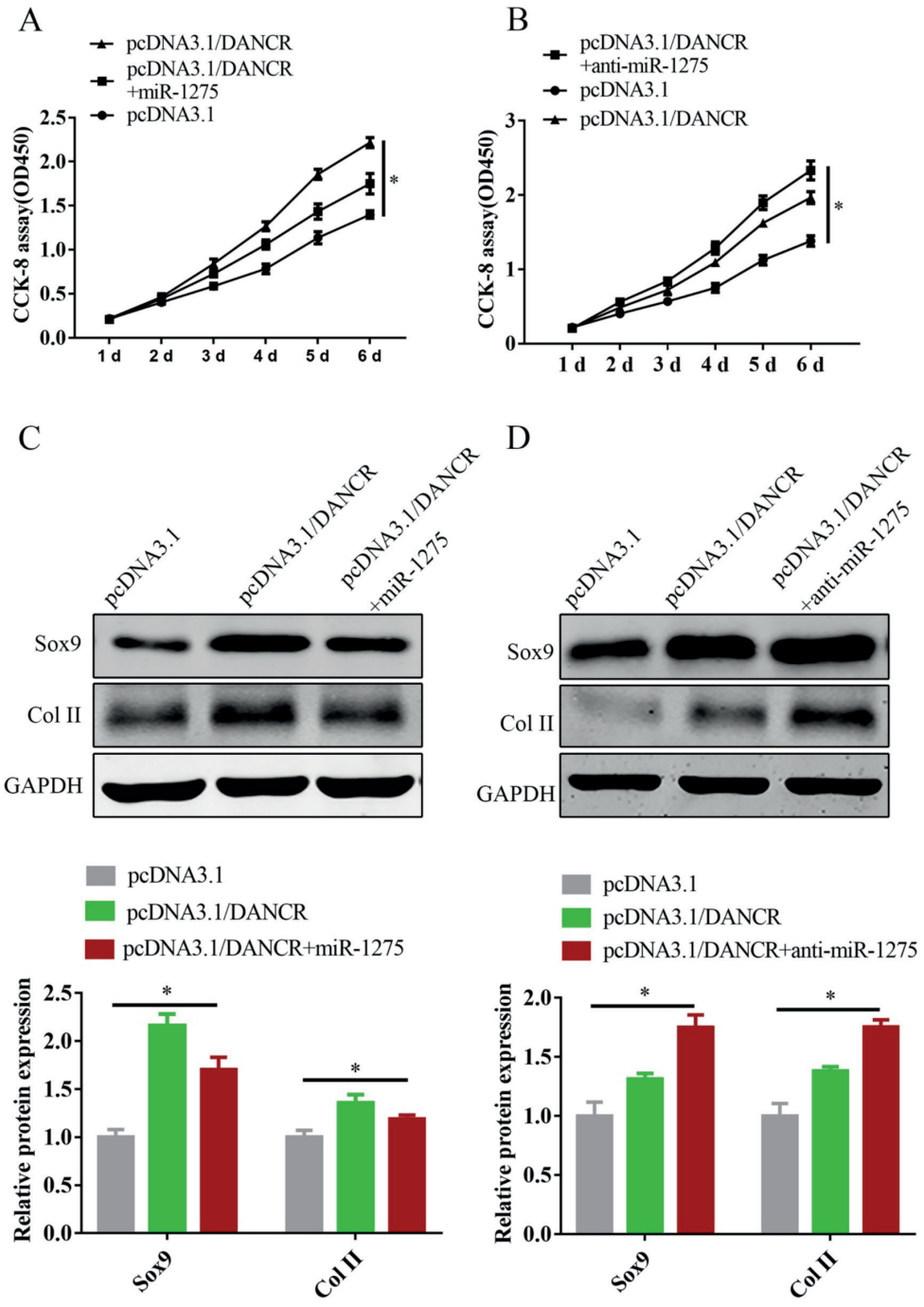

Figure 5. MiR-1275 reversed the effects of DANCR on SFMSCs proliferation and chondrogenesis. **A,** MiR-1275 mimics abolished the effects of DANCR overexpression on SFMSCs proliferation. **B,** MiR-1275 inhibitors enhanced the effects of DANCR overexpression on SFMSCs proliferation. **C,** MiR-1275 mimics abolished the effects of DANCR overexpression on Sox9 and Col II protein levels. **D,** MiR-1275 inhibitors enhanced the effects of DANCR overexpression on Sox9 and Col II protein levels. *$p<0.05$

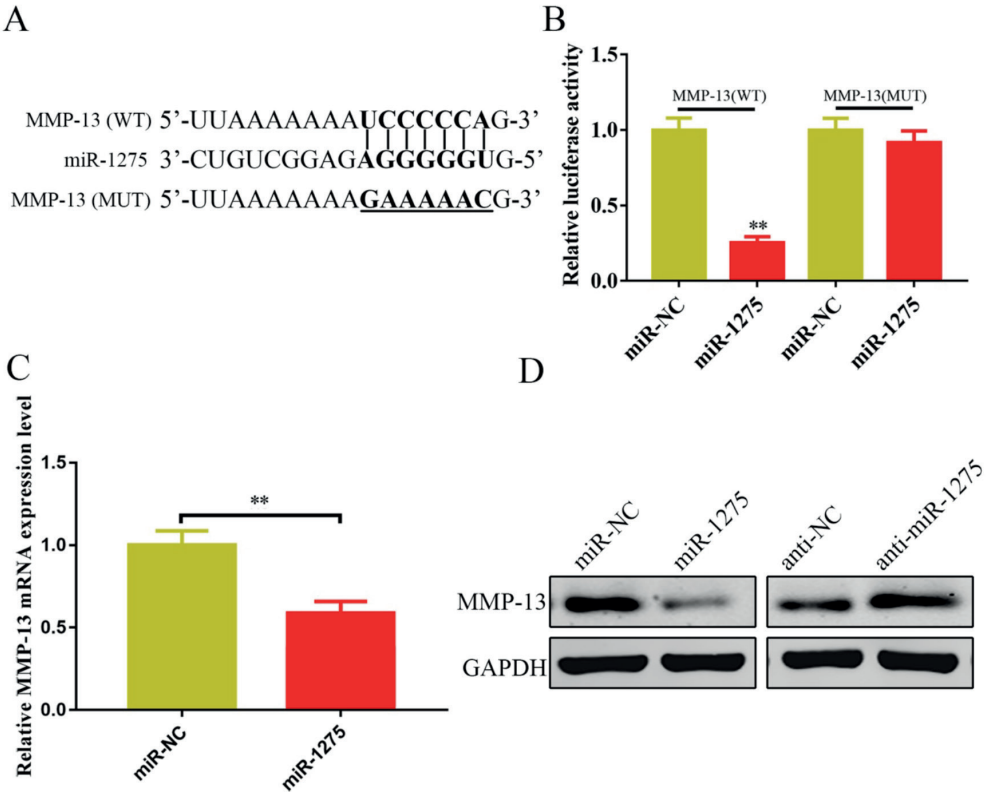

Figure 6. MMP13 is a direct target of miR-1275. **A,** Supposed miR-1275 binding sites in MMP13 sequences. **B,** Direct target sites as verified by dual-luciferase reporter assay. **C,** MiR-1275 mimics reduced MMP13 mRNA expression in SFMSCs. **D,** MMP13 protein level in SFMSCs transfected with miR-1275 mimics or miR-1275 inhibitors. *p<0.05

tumor progression and poor prognosis. Moreover, Jia et al[29] reported that DANCR promotes prostate cancer cell invasion by silencing TIMP2/3 expression. Nevertheless, so far, only few studies have investigated the role of DANCR in chondrogenesis. Our previous study showed that DANCR regulates the miR-1305/Smad4 axis in chondrogenic differentiation[30]. Hence, we speculated that DANCR might regulate chrondrogenic differentiation through other molecular mechanisms.

Recently, many authors have suggested that lncRNAs could serve as a ceRNA of miRNAs in a variety of diseases[31], including OA. For example, Xu et al[32] found that the MEG3/miR-16/SMAD7 axis might have a critical role in osteoarthritis progression. Furthermore, Li et al[33] found that lncRNA PVT1 serve as a miR-488-3p sponge in osteoarthritis to regulate chondrocyte apoptosis. However, the role and underlying mechanisms of DANCR in OA still remain to be determined. In the current study, we found that DANCR significantly promotes SFMSCs proliferation and chondrogenesis by directly interacting with miR-1275 in SFMSCs. Ectopic DANCR expression suppressed miR-1275 expression in SFMSCs.

MiR-1275 has been reported to be aberrantly expressed in various human cancers, such as nasopharyngeal carcinoma, lung cancer, and colorectal cancer[34-36]. Yet, the molecular mechanisms of miR-1275 on SFMSCs chondrogenesis remain unclear. In our study, we found that miR-1275 was negatively regulated by DANCR and miR-1275 mimics reversed the effects of DANCR overexpression on the SFMSCSs proliferation and chondrogenesis. These data suggested that miR-1275 has a critical role in SFMSCs progression.

Metalloproteinases are key regulators of OA and collagen degradation, and have been shown to participate in endochondral ossification[37,38]. Previous authors[39] reported that MMP-13 activity is required for hypertrophic chondrocyte differentiation, which is a central part of growth plate de-

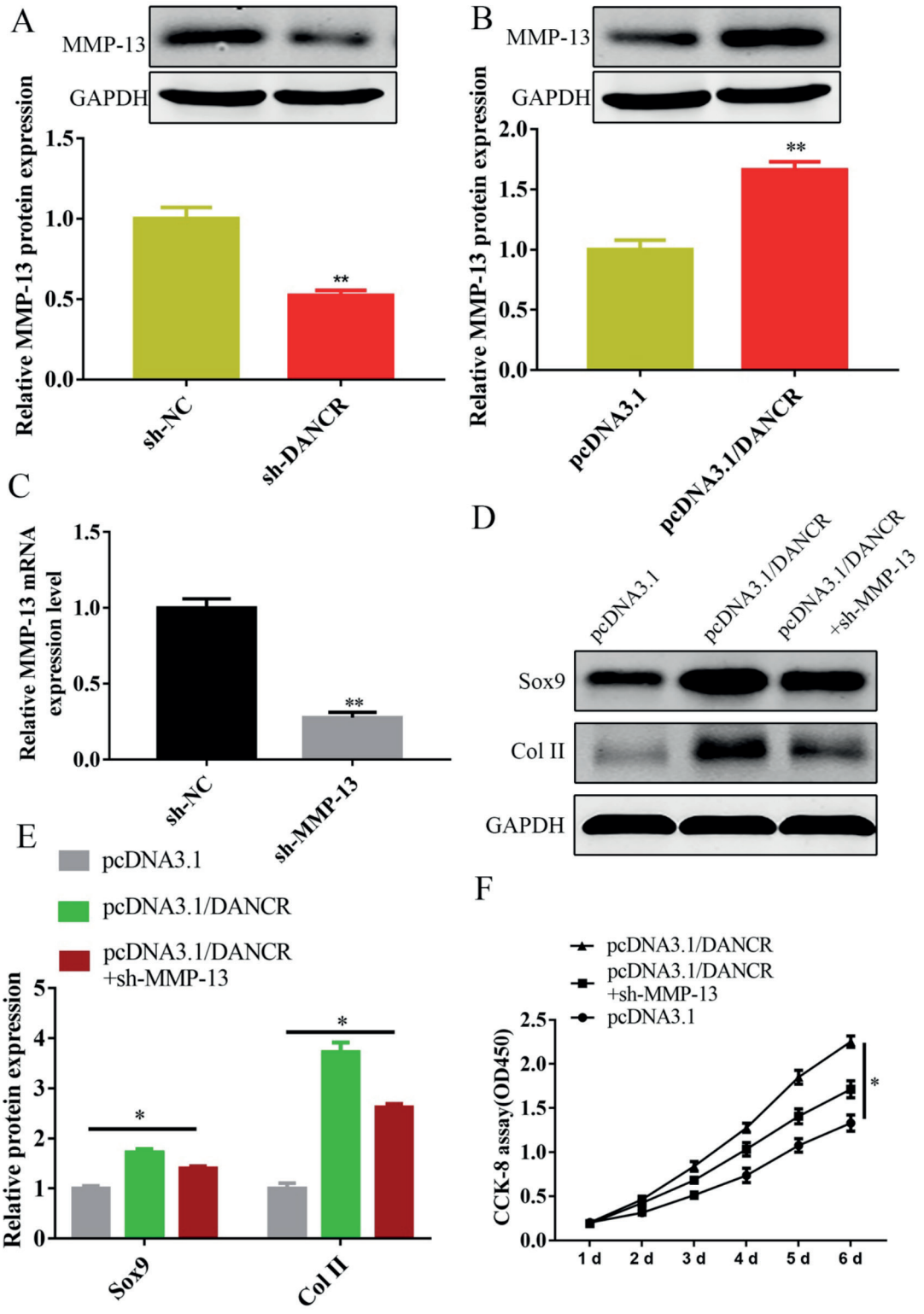

Figure 7. DANCR-mediated SFMSCs proliferation and chondrogenesis by regulating MMP-13 expression. **A,** DANCR inhibition reduced MMP13 protein levels in SFMSCs. **B,** DANCR upregulation increased MMP13 protein expression in SFMSCs. **C,** MMP13 was decreased in SFMSCs transfected with sh-MMP13. **D, E,** sh-MMP13 abolished the effects of DANCR overexpression on Sox9 and Col II protein expression in SFMSCs. **F,** sh-MMP13 abolished the effects of DANCR overexpression on SFMSCs proliferation. *$p<0.05$

velopment. Recently, several studies revealed that MMP-13 has an important role in OA cartilage. For example, Meng et al[40] found that miR-320 directly targets MMP-13 and in turn regulates chondrogenesis in mouse chondrocytes. Akhtar et al[41] noted that miR-27b might regulate the MMP13 expression in osteoarthritis chondrocytes. In the present study, we verified that MMP-13 is a direct target of miR-1275 and DANCR-mediated SFMSCs proliferation and chondrogenesis by regulating MMP-13 expression. All these data suggested that DANCR facilitated chondrogenic differentiation by regulating the miR-1275/MMP-13 axis.

Conclusions

In the present study, we demonstrated that DANCR was involved in the miR-1275/MMP-13 axis in SFMSCs proliferation and chondrogenesis. These findings might provide a potential therapeutic target for OA treatment.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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