European Review for Medical and Pharmacological Sciences 2019; 23: 9222-9229

SNHG16 promotes the progression of osteoarthritis through activating microRNA-93-5p/CCND1 axis

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Abstract. – OBJECTIVE: This study aims to investigate whether SNHG16 (small nucleolar RNA host gene 16) can promote the progression of osteoarthritis (OA) by regulating the microRNA-93-5p/Cyclin D1 (CCND1) axis, thereby finding new therapeutic targets for the treatment of OA.

PATIENTS AND METHODS: A total of 23 OA patients and 23 patients undergoing lower extremity amputation were enrolled in this study. We collected their cartilage tissues from knee joint for isolating chondrocytes. The relative levels of SNHG16, CCND1 and microRNA-93-5p in cartilage tissues of OA patients and controls were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The regulatory effect of SNHG16 on proliferative potential of chondrocytes was evaluated by Cell Counting Kit-8 (CCK-8) and colony formation assay, respectively. Cell cycle progression was examined using flow cytometry. Dual-Luciferase reporter gene assay was conducted to verify the binding between SNHG16 with microRNA-93-5p and microRNA-93-5p with CCND1. Rescue experiments were performed to elucidate whether SNHG16 regulated CCND1 expression by targeting microRNA-93-5p.

RESULTS: The expressions of SNHG16 and CCND1 upregulated, while microRNA-93-5p downregulated in cartilage tissues of OA patients relative to controls. Correlation regression analyses showed a negative expression correlation between SNHG16 and microRNA-93-5p, as well as CCND1 and microRNA-93-5p in OA patients. On the contrary, SNHG16 expression was positively correlated to CCND1 expression in OA. The knockdown of SNHG16 suppressed viability, cloning ability and cell cycle progression, but induced apoptosis in chondrocytes. Dual-Luciferase reporter gene assay showed that SNHG16 could bind to microRNA-93-5p. SNHG16 knockdown markedly upregulated the expression of microRNA-93-5p. Moreover, the knockdown of microRNA-93-5p reversed the inhibited viability due to SNHG16 knockdown. Transfection of microRNA-93-5p mimics markedly inhibited CCND1 expression. Importantly, CCND1 overexpression reversed the inhibitory effect of SNHG16 knockdown on chondrocyte viability.

CONCLUSIONS: SNHG16 promotes the development of OA by regulating microRNA-93-5p/CCND1 axis.

Key Words:
Osteoarthritis, MicroRNA-93-5p, CCND1, SNHG16.

Introduction

Osteoarthritis (OA) is characterized by joint degeneration and destruction, as well as bone hyperplasia at the edge of the subchondral bone plate, which frequently affects middle-aged and elderly people. The incidence of OA is higher in females than males, with the total prevalence of 4%. In people over 60 years, the incidence of gonarthritis is as high as 42.8%[1].

Long non-coding RNAs (lncRNAs) are RNAs with the length ranging from 200 nt to 100 000 nt. They could exert epigenetic, transcriptional, and post-transcriptional regulations on gene expressions[2]. LncRNA has been identified in multiple biological processes, including embryonic development, cellular behaviors, chromatin remodeling, immune response and heat shock response[3]. LncRNA-SNHG16 (small nucleolar RNA host gene 16) is a potential diagnostic biomarker for early-stage lung cancer, which promotes cell proliferation by regulating the cell cycle progression[4].

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MicroRNAs belong to the small ncRNAs with 1825 nucleotides in length and participate in various diseases[5]. They are involved in normal cartilage development, including chondrogenic differentiation and osteoclast activity. Under pathological circumstances, certain microRNAs have been identified to be specifically expressed in cartilage tissues, exerting a vital role in intra-articular cartilage balance, endochondral ossification and arthritis pathogenesis[6]. MicroRNA is considered to be a crucial link to the biological function of lncRNA[7].

Cyclin D1 (CCND1) is a positive regulator of the cell cycle progression, which is responsible for controlling cell cycle transition from the G phase to S phase. CCND1 upregulation greatly enhances the potentials of cell growth[8]. Hence, we speculated that CCND1 may be important in regulating the development of OA. In this work, we investigated the potential role of SNHG16 in promoting chondrocyte proliferation *via* microRNA-93-5p/CCND1 axis. We aim to provide a theoretical basis for improving clinical outcomes of OA.

Patients and Methods

Isolation and Chondrocytes from Cartilage Tissues and Cell Culture

Cartilage tissues were taken from 23 OA patients undergoing knee arthroplasty. Enrolled OA patients were diagnosed based on the KOA (knee osteoarthritis) diagnosis and treatment guidelines proposed by the Orthopedics, Chinese Medical Association in 2007[7]. Those who underwent knee arthroplasty for other diseases were excluded. Cartilage degeneration tissues at the edge of the knee cartilage leakage area were harvested. Normal cartilage tissues were taken from 23 patients who underwent lower extremity amputation in our hospital during the same period. All of them were non-OA patients, and their knee joint function was previously normal. This study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. Signed informed consents were obtained from all participants before the study.

Chondrocytes were isolated from cartilages tissues of OA patients as previously described[9]. Cartilages tissues were washed with ice-cold Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) containing penicillin-streptomycin 3 times and cut into about 1 mm × 1 mm × 1 mm using a sterile aseptic scissor. Tissues were digested with 0.25% trypsin for 40 min. After incubation with type II collagenase for 4-6 h, the mixture was centrifuged at 3 000 r/min for 10 min. The precipitate was cultured as chondrocytes.

Cell Culture and Transfection

Chondrocyte cell line C28/I2 was provided by the Cell Bank, Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and placed in a 5% CO_2 at 37°C. Cell passage was performed at the confluence of 80%.

C28/I2 cells in the exponential growth phase were digested, inoculated into a 6-well plate to 60% of confluence. Cells were transfected with si-SNHG16, si-NC, microRNA-93-5p mimics, microRNA-93-5p inhibitor or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The medium was replaced 6 h later.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). The purity of RNA sample was measured by an ultraviolet spectrophotometer and stored at -80°C until use. The complementary deoxyribose nucleic acid (cDNA) was reversely transcribed, and the SYBR Green method was used for Polymerase Chain Reaction (PCR) detection. The relative expressions of SNHG16, CCND1, microRNA-93-5p were examined. Primer sequences were as follows: SNHG16, F: 5'-CCCAAGCTTGCGTTCTTTTCGAGGTCGGC-3', R: 5'-CCGGAATTCTGACGGTAGTTTCCCAAGTT-3'; CCND1, F: 5'-TGCTGGTTTTCTACCCAACG-3, R: 5'-AGTGCTTGGAAATGGAATGG-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'; MicroRNA-93-5p, F: 5'-AGATGCTCTAGAGCGCATGCCCTGAGCCAGACCTCACTGCTGCACTTTC-3', R: 5'-AGCAGGAATTCCTTGGAGAGCAGCAATCTTAGCACCTTGGAAAGTG CAG-3'; U6, F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'.

Cell Proliferation Assay

Cells were cultured in a 96-well plate at a density of 5×10^4/mL, with 100 μL per well. After

cell culture for 24 h, 36 h, 48, and 72 h, 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, respectively. Absorbance at 450 nm wavelength was recorded after incubation for 1 h using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Colony Formation Assay

Cells were digested for preparation of single cell suspension, inoculated in 6-well plates with 2.5×10^3 cells per well and cultured for 7 days. They were fixed with 95% ethanol, dyed with 1% violet crystal and captured under a microscope. We selected 7 random fields in each sample for calculating colonies containing at least 10 cells.

Cell Apoptosis Assay

2.5×10^3 cells were washed with PBS twice, digested and fixed in pre-cold 70% ethanol at 4°C for 30 min. Subsequently, cells were induced with 5 mL of Annexin V-FITC (fluorescein isothiocyanate) and 1 mL of Propidium Iodide (PI; 50 mg/mL) for 5 min. Apoptosis was determined using flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

Dual-Luciferase Reporter Gene Assay

C28/I2 cells were inoculated in 24-well plates with 3×10^5 cells per well. Cells were co-transfected with wild-type or mutant-type plasmids with microRNA-93-5p mimics or miR-NC using Lipofectamine 2000. At 24 h later, Luciferase activity was determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Western Blot

Total protein was extracted for determining protein expression. The protein sample was quantified by bicinchoninic acid (BCA; Pierce, Waltham, MA, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by chemiluminescence (ECL; Millipore, Billerica, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS 16.0; SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. Normally distributed measurement data were represented as mean ± standard deviation ($\bar{x} \pm s$). Differences between the two groups were analyzed by the *t*-test. Correlation analysis was conducted by Spearman correlation test. $p<0.05$ was considered as statistically significant.

Results

Relative Expressions of SNHG16, CCND1 and MicroRNA-93-5p in OA

We found that expression levels of SNHG16 and CCND1 significantly increased in cartilage tissues of OA patients relative to controls (Figure 1A, 1B). However, microRNA-93-5p expression was markedly reduced in cartilage tissues of OA patients (Figure 1C). Correlation regression analyses showed a negative expression correlation between SNHG16 and microRNA-93-5p, as well as CCND1 and microRNA-93-5p in OA patients (Figure 1D, 1F). On the contrary, SNHG16 expression was positively correlated to CCND1 expression in OA (Figure 1E).

SNHG16 Accelerated Proliferative Rate but Inhibited Apoptosis of C28/I2 Cells

To observe the regulatory effect of SHHG16 on chondrocytes, C28/I2 cells were first transfected with si-SNHG16 to downregulate SNHG16 expression (Figure 2A). After knockdown of SNHG16 in C28/I2 cells, CCK-8 assay showed a decrease in cell viability (Figure 2B). Colony formation assay confirmed that the cell cloning ability was remarkably reduced after SNHG16 knockdown (Figure 2C). Transfection of si-SNHG16 markedly arrested chondrocytes in G1/G0 phase, suggesting an inhibited cell cycle progression (Figure 2D). Moreover, C28/I2 cells with SNHG16 knockdown presented an accelerated apoptotic rate (Figure 2E).

SNHG16 Regulated Chondrocyte Viability by Targeting MicroRNA-93-5p

A potential binding site was found between SNHG16 and microRNA-93-5p (Figure 3B). To elucidate the potential role of microRNA-93-5p in the development of OA, transfection efficacy of microRNA-93-5p mimics and inhibitor was verified by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) (Figure 3A). Subsequently, Dual-Luciferase reporter gene assay showed that the Luciferase activity decreased in chondrocytes co-transfected with SNHG16

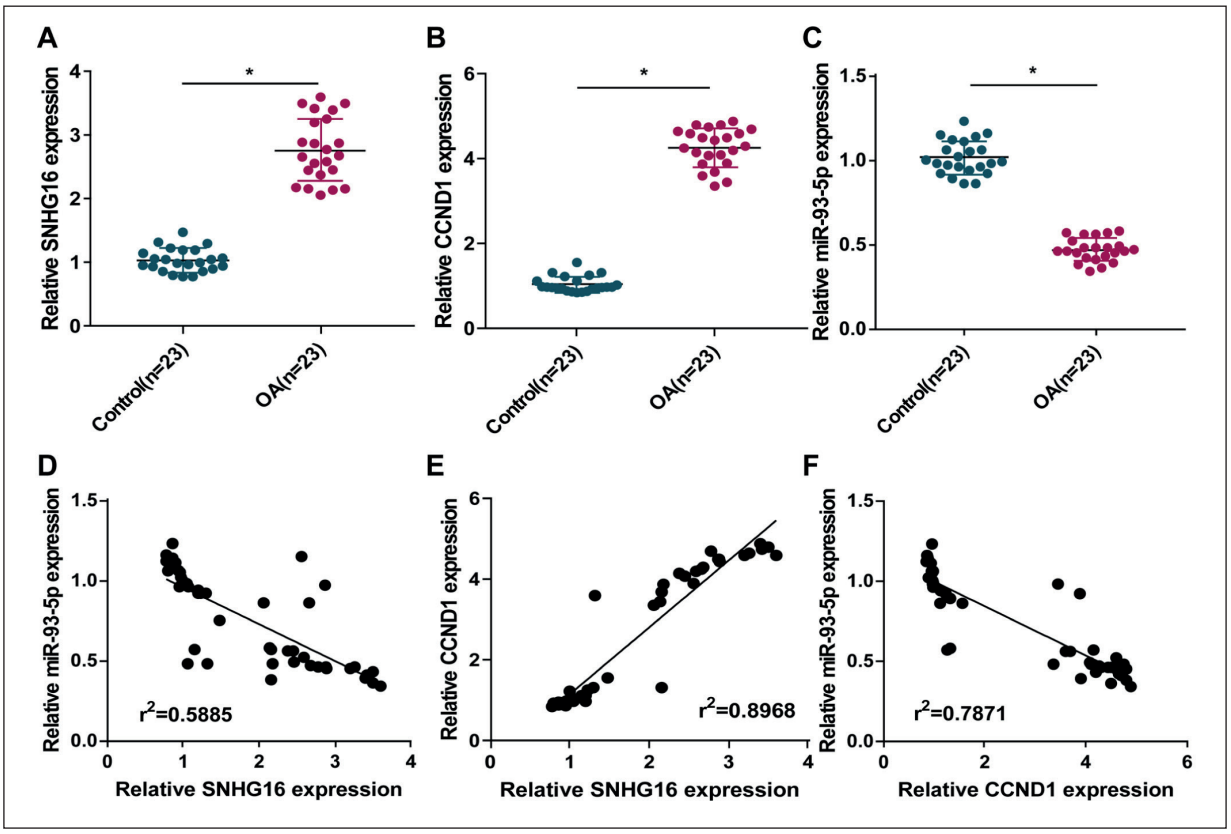

Figure 1. Relative expressions of SNHG16, CCND1 and miR-93-5p in OA. **A,** Relative expression of SNHG16 was higher in OA tissues than controls. **B,** Relative expression of CCND1 was higher in OA tissues than controls. **C,** Relative expression of miR-93-5p was lower in OA tissues than controls. **D,** Correlation regression between miR-93-5p and SNHG16 in OA. **E,** Correlation regression between CCND1 and SNHG16 in OA. **F,** Correlation regression between miR-93-5p and CCND1 in OA. OA, osteoarthritis.

WT and microRNA-93-5p mimics, confirming the binding of microRNA-93-5p to SNHG16 (Figure 3C). Transfection of si-SNHG16 markedly upregulated microRNA-93-5p expression in C28/I2 cells (Figure 3D). Moreover, the decreased cell viability by SNHG16 knockdown was partially reversed after co-transfection of si-SNHG16 and microRNA-93-5p inhibitor (Figure 3E). We may conclude that SNHG16 regulated chondrocyte viability by targeting microRNA-93-5p.

MicroRNA-93-5p Participated in the Development of OA Through Binding to CCND1

Subsequently, we predicted the binding site of microRNA-93-5p to CCND1 and further explored the interaction between microRNA-93-5p with CCND1 in OA (Figure 4A). After constructing the Luciferase reporter plasmids CCND1 MT and CCND1 WT, Dual-Luciferase reporter gene assay showed decreased Luciferase activity in C28/I2 cells co-transfected with microRNA-93-5p mimics and CCND1 MT (Figure 4B). The overexpression of microRNA-93-5p in chondrocytes markedly downregulated the mRNA and protein expression of CCND1. On the contrary, the knockdown of microRNA-93-5p upregulated CCND1 expression at both mRNA and protein level (Figure 4C, 4D). The downregulated CCND1 expression in C28/I2 cells with microRNA-93-5p knockdown was partially reversed by SNHG16 knockdown (Figure 4E, 4F). The CCK-8 assay indicated that co-transfection of si-SNHG16 and microRNA-93-5p reversed the inhibited viability of C28/I2 cells transfected with si-SNHG16 (Figure 4G). The above results indicated that SNHG16 promoted the progression of OA by targeting microRNA-93-5p to regulate CCND1 expression.

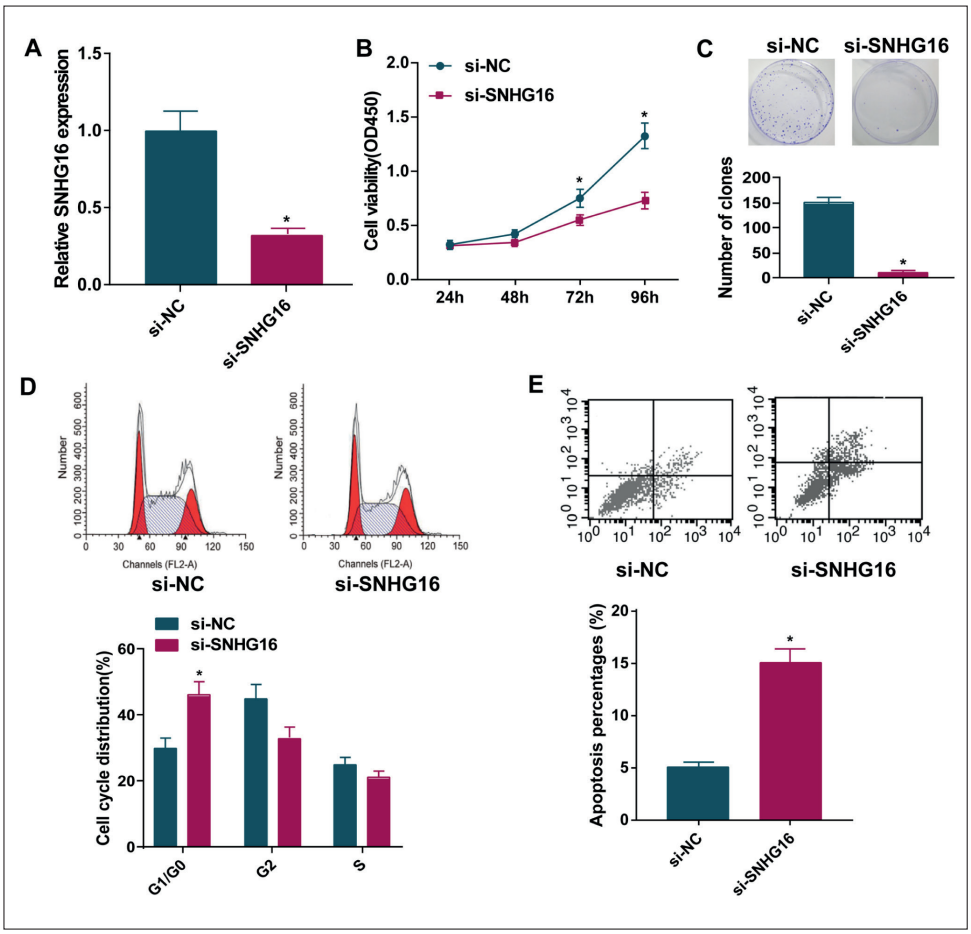

Figure 2. SNHG16 accelerated proliferative rate but inhibited apoptosis of C28/I2 cells. **A,** Transfection of si-SNHG16 downregulated SNHG16 expression in C28/I2 cells. **B,** CCK-8 assay showed that cell viability in C28/I2 cells transfected with si-SNHG16 at 24, 48, 72 and 96 h was inhibited relative to those transfected with si-NC. **C,** Colony formation assay showed that cell cloning ability was significantly reduced in C28/I2 cells transfected with si-SNHG16 relative to those transfected with si-NC. **D,** Cell cycle progression of C28/I2 cells transfected with si-SNHG16 or si-NC. **E,** Apoptosis of C28/I2 cells transfected with si-SNHG16 or si-NC.

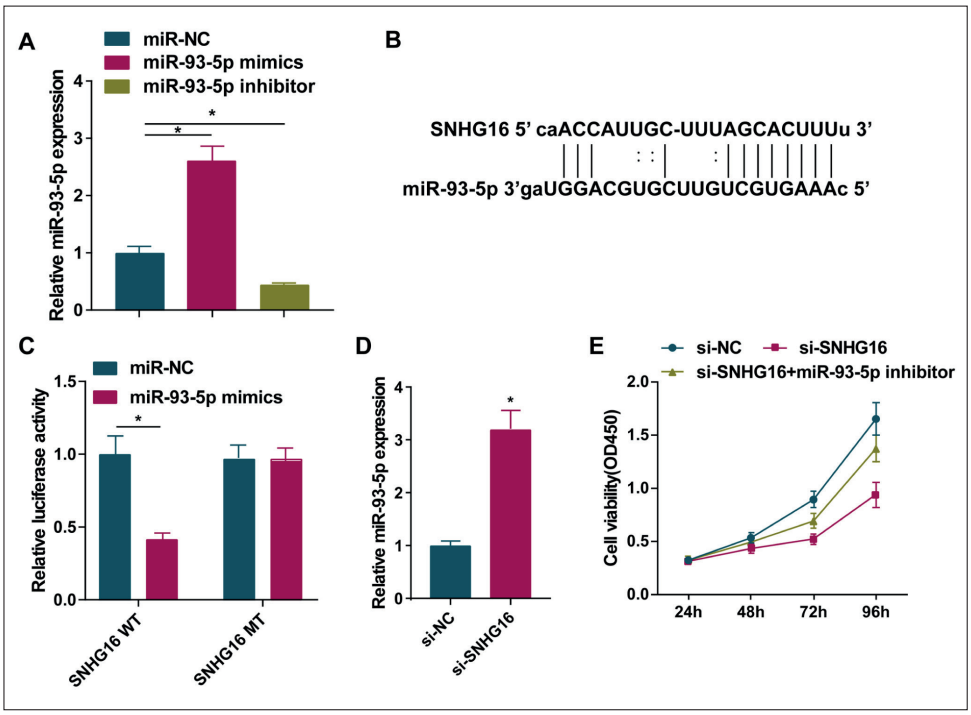

Figure 3. SNHG16 regulated chondrocyte viability by targeting miR-93-5p. **A,** Relative expression of miR-93-5p in C28/I2 cells transfected with miR-NC, miR-93-5p mimics or miR-93-5p inhibitor. **B,** Potential binding sites between SNHG16 and miR-93-5p. **C,** Dual-Luciferase reporter gene assay determined in C28/I2 cells co-transfected with miR-NC or miR-93-5p mimics and SNHG16 WT or SNHG16 MT. **D,** Relative expression of miR-93-5p in C28/I2 cells transfected with si-NC or si-SNHG16. **E,** CCK-8 assay showed cell viability in C28/I2 cells transfected with si-SNHG16, or si-SNHG16 or co-transfected with si-SNHG16 and miR-93-5p inhibitor at 24, 48, 72 and 96 h.

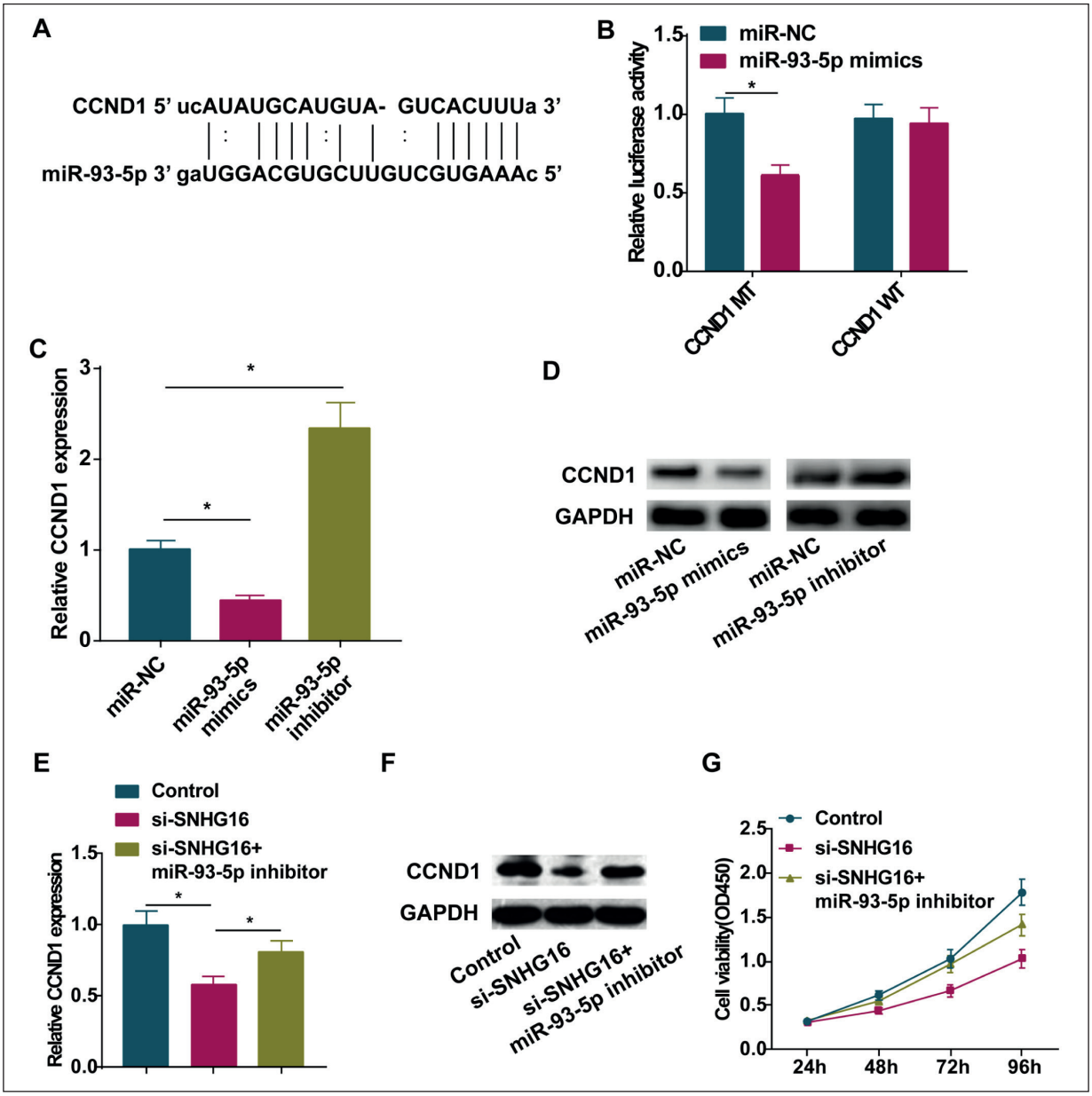

Figure 4. MiR-93-5p participated in the development of OA through binding to CCND1. **A,** Potential binding sites between CCND1 and miR-93-5p. **B,** Dual-Luciferase reporter gene assay determined in C28/I2 cells co-transfected with miR-NC or miR-93-5p mimics and CCND1 WT or CCND1 MT. **C,** Relative expression of CCND1 in C28/I2 cells transfected with miR-93-5p mimics or miR-93-5p inhibitor. **D,** Protein expression of CCND1 in C28/I2 cells transfected with miR-93-5p mimics or miR-93-5p inhibitor. **E,** Relative expression of CCND1 in C28/I2 cells, and those transfected with si-SNHG16 or si-SNHG16+miR-93-5p inhibitor. **F,** Protein expression of CCND1 in C28/I2 cells, and those transfected with si-SNHG16 or si-SNHG16+miR-93-5p inhibitor. **G,** Cell viability in C28/I2 cells, and those transfected with si-SNHG16 or si-SNHG16+miR-93-5p inhibitor.

Discussion

OA is the most common type of chronic progressive arthropathy and gradually increases with aging. More than 50% middle-aged and elderly people over 65 years suffer from OA. Generally, OA is characterized by degenerative changes in the internal compartment, manifesting as pain after medial knee exercise or even rest pain accompanied by hyperplasia around the joint and narrowed joint space. Anti-inflammatory, analgesic, physical therapy and joint replacement are

currently the common therapies for OA[10,11]. It is of great significance to elucidate the pathogenesis of OA and to develop novel therapeutic targets.

Long non-coding RNA SNHG16, also known as non-coding RNA expressed in aggressive neuroblastoma, was first discovered in neuroblastoma. SNHG16 has also been identified to promote the development of colorectal cancer and bladder cancer. SNHG16 is highly expressed in gastric cancer cells, and knockdown of SNHG16 significantly changes the cell cycle progression and cyclin expression in human gastric cancer cells. Uncontrolled cell cycle leads to cell proliferation, abnormal differentiation and disrupts cell proliferation and apoptosis. Therefore, we believed that SNHG16 exerts an important role in regulating the cell cycle progression of chondrocytes[12,13].

MicroRNA-93-5p is a kind of endogenous, non-coding RNA, which is widely distributed in cells. There are studies indicating that microRNA-93-5p is involved in cell cycle and apoptosis, and it also participates in tumorigenesis and tumor progression. For example, microRNA-93-5p is involved in the aging, osteoblast calcification and hyperglycemia. Meanwhile, it exerted a certain biological function in the occurrence and progression of various tumors[14,15].

CCND1 locates at 11q13, which contains 13388 bp and could encode 295 amino acids. Its main function is to regulate the cell cycle progression from the pre-synthesis phase of DNA to synthesis phase. Abnormal expression of CCND1 greatly affects the cell cycle progression[16].

In the present work, SNHG16 promoted chondrocyte proliferation in cartilage tissues of OA patients through microRNA-93-5p/CCND1 axis. To demonstrate whether SNHG16 exerted its regulatory role in OA by targeting microRNA-93-5p/CCND1 axis, Dual-Luciferase reporter gene assay and functional experiments were conducted. Our results discovered that SNHG16 promoted chondrocyte proliferation by absorbing microRNA-93-5p to regulate CCND1 expression.

Conclusions

We showed that SNHG16 promotes the development of OA by regulating microRNA-93-5p/CCND1 axis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgments

This work was supported by the General Scientific Research Project of Education Department of Liaoning Province (L2015326); Natural Science Fund Project of Science and Technology Department of Liaoning Province (20170540364).

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