MEG3 inhibits proliferation and promote apoptosis in osteoarthritis chondrocytes by miR-361-5p/wnt/β-catenin axis

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Abstract

Background: This study aimed to investigate the role of long noncoding RNA (lncRNA) maternally expressed 3 (MEG3) and related molecular mechanisms in osteoarthritis (OA).

Methods: Patients with OA and patients undergoing thigh amputation were involved in OA group and control group, respectively. Cartilage tissues of all patients were isolated and cultured. Based on different transfection, MEG3 cells were grouped into Blank, pcDNA3.1-NC, pcDNA3.1-MEG3, si-NC, si-MEG3, pcDNA3.1-NC + mimics NC, pcDNA3.1-MEG3 + mimics NC, pcDNA3.1-NC + miR-361-5p mimics and pcDNA3.1-MEG3 + miR-361-5p mimics group. The cells transfected with pcDNA3.1-NC and pcDNA3.1-MEG3, and then cultured with XAV939 was named as pcDNA3.1-NC +XAV939 group and pcDNA3.1-MEG3 + XAV939 group respectively. The RT-qPCR was used to detect the expression of MEG3 and miR-361-5p. Moreover, Western blot, luciferase reporter assay, RIP, CCK-8 and flow cytometry analysis were performed to reveal the morphology, proliferation and apoptosis in cartilage cells. Finally, the histological analysis and immunostaining were performed on OA rat model. Results: The expression of lncRNA MEG3 and miR-361-5p in OA was significantly decreased and increased respectively than that in normal. Meanwhile, MEG3 was competitive binding with miR-361-5p in OA chondrocytes. Moreover, the Western blot and CCK-8 assay showed that MEG3 might inhibit cell proliferation and promote cell apoptosis via Wnt/β-catenin pathway. Finally, rat model analysis showed that MEG3 contributed to the cartilage matrix degradation. Conclusion: MEG3 and miR-361-5p might down-regulated and up-regulated respectively in the chondrocytes of OA patients. Furthermore, MEG3 might inhibit cell proliferation and promote cell apoptosis via miR-361-5p/Wnt/β-catenin axis in OA chondrocytes.

Background
Osteoarthritis (OA) is the most common degenerative disease of human articular cartilage, which increases with age and even induces serious pain and disability\(^1\). It lead to various pathological changes including articular cartilage degradation, synovial inflammation and subchondral osteoblast activation\(^2\). Globally, approximately 250 million people (3.6% of the population) have OA of the knee\(^3\). Since various genetic, biological, and biomechanical components contribute to the development of OA\(^4\), and the molecular mechanisms underlying the progression of OA is not completely understood.

Long noncoding RNAs (IncRNAs) are important class of pervasive genes play important regulatory roles in oncogenic pathways\(^5\text{-}9\). The biological function of IncRNAs is proved to be associated with knee OA progression\(^10\). As a member of IncRNA, maternally expressed 3 (MEG3) acts as a growth suppressor in tumor cells\(^11\). Recently, MEG3 is found to be a potential target for OA, which was confirmed to participate in various cancer including lung cancer\(^12\), breast cancer\(^13\) and esophageal cancer\(^14\). Su et al. showed that MEG3 was down-regulated and inversely associated with vascular endothelial growth factor A levels in OA\(^15\). Actually, the biological function of MEG3 is realized by targeting certain pathways such as wnt/\(\beta\)-catenin signaling pathway\(^16\). During this process, the wnt/\(\beta\)-catenin signaling related factors such as Matrix metallopeptidase 1 (MMP-1) and MMP-13 are highly overexpressed in OA\(^17\). Cui et al. showed that MEG3-mediated wnt/\(\beta\)-catenin signaling pathway controls the inhibition of tunicamycin-mediated viability in glioblastoma\(^18\). Xia et al. indicated that the downregulation of MEG3 enhances cisplatin resistance of lung cancer cells through activation of the wnt/\(\beta\)-catenin signaling pathway\(^17\).

Meanwhile, pervious study shows that miR-361-5p inhibits the mobility of cancer cells through suppressing epithelial-mesenchymal transition via the wnt/\(\beta\)-catenin pathway\(^19\).
Interestingly, MEG3 is proved to be down-regulated and inversely associated with VEGF levels in OA. Jin et al. indicated that the down-regulation of MEG3 leaded to OA progression via certain miRNA-target gene axis. Although the effect of MEG3 in cancer progression via certain genes or pathways are mentioned in previous studies, the potential molecular mechanism of MEG3 in OA is still unclear.

In this study, the mechanism of lncRNA MEG3 and related pathways in OA was explored based on cartilage cells obtained from patients with OA (OA group) and artificial joint replacement (normal group). The real-time fluorescence quantitative polymerase chain reaction (RT-qPCR), Western blot, luciferase reporter assay, RNA immunoprecipitation (RIP), cholecystokinin (CCK-8) and flow cytometry analysis were performed to reveal the morphology, proliferation and apoptosis in cartilage cells. Finally, the histological analysis and immunostaining were performed on OA rat model. The study might provide a new target and theoretical basis for OA treatment.

Methods

Patients and grouping

The cartilage tissue of OA patients came from the knee joints of 30 patients who had undergone total knee arthroplasty. Meanwhile, the healthy cartilage tissue came from 20 patients who had not undergone OA or RA (rheumatoid arthritis). All patients voluntarily signed the informed notice. The current study obtained the approval of Ethics Committee of the hospital (ethic vote 198/203).

Cell culture

The femoral articular cartilage of the femur was obtained and cut. Then, it was digested by 0.2% type II collagenase, oscillation resolving for 40min at 37°C, washed by D-Hanks, and diluted in DMEM/F12 (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine
serum (FBS), 100 u/ml penicillin (Gibco, Rockville, MD, USA), 100 mg/ml streptomycin (Gibco, Rockville, MD, USA). Then cells were culture in a saturated humidity incubator (37°C, 5% CO₂). The solution was changed every two days until the chondrocytes grew into sheets and covered more than 85% of the wall of the bottle. The cells of 2 or 3 generations were enrolled for the further investigation.

Cell transfection
PcDNA3.1-MEG3 overexpression vector, si-MEG3 (Sangon Biotech, Shanghai, China), miR-361-5p mimics and miR-361-5p mimics NC (Guangzhou Reeber) were constructed, followed by cells transfection according to the instructions of the transfection reagent. Based on different transfection, osteoarthritic chondrocytes were divided into Blank group, pcDNA3.1-NC group, pcDNA3.1-MEG3 group, si-NC group, si-MEG3 group, pcDNA3.1-NC + mimics NC group, pcDNA3.1-MEG3 + mimics NC group, pcDNA3.1-NC + miR-361-5p mimics group and pcDNA3.1-MEG3 + miR-361-5p mimics group. All transfection was carried out by Lipfectamine 2000 transfection kit (Invivrogn™ USA) according to the instructions. Then, the cells transfected with pcDNA3.1-NC and pcDNA3.1-MEG3 were cultured for 48 h by adding 10 μmol/l of Wnt/β-catenin signaling pathway inhibitor XAV939 (Tocris Bioscience), which was named as pcDNA3.1-NC + XAV939 group and pcDNA3.1-MEG3 + XAV939 group respectively. After 48 hours of transfection, cells were stimulated with IL-1β (10 ng / ml) for 24 hours. Finally, cells of each group were collected for following experiments.

Real-time fluorescent quantitative PCR
Total RNA (500 ng) from sample of each group was extracted and quantified using TRIzol reagent, and cDNA template was synthesized by reagent kit (invitrogen, San Diego, USA) according to the manufacturer's instructions. GAPDH was used as reference. The primers were shown in Table 1. The reaction conditions were as follows: 95°C for 3 min, 39 cycles
at 95 °C for 10 s, 55 °C. Fluorescence signals were collected at the end points of each cycle extension, followed by the amplification curve investigation. Relative expression of candidate genes were calculated by $2^{-\Delta\Delta CT}$ method.$^{23}$

**Luciferase reporter assay**

The regulatory relation between MEG3 and miR-361-5p was predicted based on StarBase (http://starbase.sysu.edu.cn/). A wild type (MEG3-WT) or mutant (MEG3-MUT) fragment of the MEG3 3'UTR containing miR-361-5p was synthesized. Then, the wild type or mutant sequences were cloned into the pmirGLO reporter vector (Promega, Madison, WI, USA). After inoculation of human chondrocytes in 24-well plates (5 × 105/well) for 24 hours, miR-361-5p mimic or miR-361-5p NC, MEG3-WT or MEG3-MUT were co-transfected into human chondrocytes by Lipofectamine 3000 (Thermo Fisher Scientific). Finally, the luciferase intensity was measured by Dual Luciferase Reporter Assay Kit (Promega, E1910, WI, USA) 48 hours after transfection.

**RIP assay**

RIP was determined by using Magna RIP™ RNA binding protein immunoprecipitation kit (Millipore, Bedford, MA, USA). Briefly, the cultured chondrocytes were collected and suspended in RIP lysis buffer (Solarbio). Then, cell extract was incubated overnight with RIP buffer containing human anti-Ago2 antibody beads (Millipore) (Input and normal IgG served as controls). The next day, the magnetic beads were incubated with proteinase K. Total RNA was subsequently isolated from the extract using TRIzol reagent. Finally, relative enrichment of MEG3 and miR-361-5p was determined by RT-qPCR analysis.

**Western blot**

Cultured cells were lysed with 100 μl/50mL protein lysate RIPA. Briefly, the extracted protein was quantified by bicinchoninic acid (BCA) method. A total of 50 g proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10%
polyacrylamide gels, and transferred to polyvinylidenefluoride membranes. After blocked with 5% Skim milk/BSA, the membrane was incubated with primary antibodies including β-catenin (#8480), Non-phospho (Active) β-Catenin (#19807), MMP-1 (#54376), MMP-13 (#94808), cyclinD1(#2922), c-Myc (#9402), PCNA (#13110), Bax (#5023) and Bcl-2 (#4223) antibody (1:1000, Cell Signaling, Boston, USA), as well as Ki67 (ab92742), ADAMTS-5 (ab41037) and Aggrecan (ab36861) (Abcam, Cambridge, MA, USA) at 4°C overnight. The β-actin was used as internal control. After washed by TBST for three times, the membranes were incubated with horseradish peroxidase-labeled sheep anti-rabbit secondary antibody (1:5000, Biyuntian biotechnology) at room temperature for 1 h. Finally, the protein was stained with diaminobenzidine (DAB) kit. All experiments were repeated for 3 times.

**CCK-8 assay**

All cells were seeded in 24-well plates (2 × 10³ cells/well) and incubated at 37 °C with 5% CO₂. After being incubated for 0, 24, 48, 72 h, a total of 10 μL CCK-8 (Sigma-Aldrich) were added to each well and then incubated for 4 h. OD₄₉₀ value was measured by enzyme-linked immunosorbent assay.

**Flow cytometry assay**

Flow cytometry was performed to detect the apoptosis of transfected cells. Simply, the cells digested by trypsin was mixed with 200 μL Annexin V-FITC, incubated for 10 min in dark, and then washed with 200 μL PBS and 10 μL PI. Cell cycle progression was subsequently monitored based on flow cytometry (Cell Lab Quanta, Beckman Coulter) and the data were analyzed using Multi-Cycle AV software (Phoenix Flow Systems, SanDiego, CA).

**OA rat model construction**
Animal experiments were carried out according to the guidelines for animal experiments in our hospital. Male Sprague-Dawley (SD) rats (200-250 g) were purchased from the Experimental Animal Center of Shandong University. All rats were anesthetized with intramuscular injection of sodium pentobarbital (0.05 mg/g, Chuangdong Co., Chongqing, China). The experimental OA of 10-week-old SD rats was traversed by the medial collateral ligament and destabilized by the medial meniscus (DMM). One week after the operation, si-NC, si-MEG3 (1×10^9 PFU, 20 μl) was injected into the knee joint of the recipient rat (n = 6 for each group, 20 μL per joint) twice a week for 4 weeks. Eight weeks after the operation, the rats were sacrificed with cervical dislocation method (external force dislocated the cervical vertebra of rat and disconnects the spinal cord from the cerebrospinal cord), and then the knee joints were harvested. All experiment was performed in Experimental Center of Taishan Medical College. This study was approved by the Laboratory Animal Ethics Committee of Taishan Medical College (No. 2019146), and all experiments were in accordance with the guide for the care and use of laboratory animals established by United States National Institutes of Health (Bethesda, MD, USA).

*Histological analysis and immunostaining*

Rat cartilage was fixed in 4% paraformaldehyde, embedded in paraffin and cut into sample slice (5μm/slice). In order to assess the extent of cartilage destruction, Safranin ‘O’ staining was performed. Histological scores were performed according to the International Osteoarthritis Research Association (OARSI) grading system, which ranging from 0 (normal) to 6 (>80% represented the cartilage loss). Scores were determined from multiple serial sections of the knee joint of each mouse.

*Statistical Analysis*

Data were analyzed using version 18.0 (SPSS, Chicago, IL, USA) and represented as the
mean ± standard deviation (SD). Significant differences between two groups were assessed using Student’s t-test, while least significant difference between means (LSD)-t multiple comparison test was processed for more than two groups. P < 0.05 was considered as statistically significant.

All rats were anesthetized with intramuscular injection of sodium pentobarbital (0.05 mg/g, Chuangdong Co., Chongqing, China).

All rats were sacrificed with cervical dislocation method (external force dislocated the cervical vertebra of rat and disconnects the spinal cord from the cerebrospinal cord).

Results

MEG3 regulated OA cell proliferation, apoptosis, and cartilage matrix degradation

RT-q PCR was used to detect the expression of MEG3 in OA group and normal group. The results showed that compared with Normal group, the expression of MEG3 in chondrocytes of OA group was significantly decreased (P < 0.001) (Figure 1A). After transfection with pcDNA3.1-MEG3 or MEG3 siRNA into OA chondrocytes, the expression of MEG3 in pcDNA3.1-MEG3 group was significantly increased compared with that in pcDNA3.1-NC group (P < 0.05). Moreover, compared with the si-NC group, the expression of MEG3 in the si-MEG3 group was significantly decreased (Figure 1B) (P < 0.05). CCK-8 method was used to detect cell proliferation at different time points. The results showed that the cell proliferation ability of the pcDNA3.1-MEG3 group was significantly decreased than that of the pcDNA3.1-NC group (P < 0.05) (Figure 1C), while the cell proliferation ability of the si-MEG3 group was significantly increased compared with the si-NC group (Figure 1D).

Furthermore, Western blot was used to detect the expression of cell proliferation protein PCNA and marker Ki67 in each group. Compared with the pcDNA3.1-NC group, the expression of PCNA and the marker ki67 in the pcDNA3.1-MEG3 group was significantly
decreased, and the expression of the PCNA and the marker ki67 in the pcDNA3.1-MEG3 group was significantly increased (Figure 1E). In addition, apoptosis of cells was quantified by Annexin V/PI double staining. The results showed that the apoptosis rate of pcDNA3.1-MEG3 group was significantly up-regulated compared with pcDNA3.1-NC group, and the apoptosis rate of si-MEG3 group was significantly lower than that of si-NC group (Figure 1F). We further passed Western. The expressions of apoptosis-related proteins Bcl-2 and Bax were detected by blot. The results showed that compared with pcDNA3.1-NC group, Bcl-2 was up-regulated and Bax was down-regulated in pcDNA3.1-MEG3 group. Compared with si-NC group, Bcl-2 was down-regulated and Bax was up-regulated si-MEG3 group (Figure 1G).

Based on the rat OA model, Western blot was used to detect the expression of cartilage matrix proteins MMP1, MMP13, ADAMTS-5, Collagen II and Aggrecan in chondrocytes (Figure 1H). Compared with pcDNA3.1-NC group, MMP1, MMP13, ADAMTS-5 was up-regulated, while Collagen II and Aggrecan expression were down-regulated in cDNA3.1-MEG3 group. Compared with si-NC group, MMP1, MMP13, ADAMTS-5 was down-regulated, while Collagen II and Aggrecan was up-regulated in si-MEG3 group.

**MEG3 competitive binding miR-361-5p in OA chondrocytes**

The binding sites of MEG3 and miR-361-5p were predicted using StarBase (Figure 2A). The luciferase reporter assay demonstrated that MEG3 regulated miR-361-5p, MEG3-Wt and miR-361-5p mimics co-transfected cells with significantly reduced luciferase activity (Figure 2B). Meanwhile, the RIP assay showed that compared with IgG control group, MEG3 and miR-361-5p were significantly overexpressed in the Ago2 pellet (Figure 2C). After transfection of pcDNA3.1-MEG3 or MEG3 siRNA into OA chondrocytes, RT-qPCR results showed that miR-361-5p expression was down-regulated when compared with pcDNA3.1-NC group, while miR-361-5p was significantly up-regulated when compared with si-NC
Moreover, the expression of MEG3 in OA group and normal group were detected by RT-qPCR. The results showed that compared with normal group, the expression of miR-361-5p was significantly (P < 0.001) up-regulated in chondrocytes of OA group (Figure 2E). Furthermore, the Spearman correlation analysis showed a negative correlation between expression of MEG3 and miR-361-5p (r = -0.529, p = 0.0026) (Figure 2F).

The effect of MEG3 on proliferation, apoptosis and cartilage matrix degradation of OA chondrocytes was inhibited by miR-361-5p

The result of RT-qPCR showed that compared with pcDNA3.1-NC + mimics NC group, the expression of miR-361-5p was decreased in pcDNA3.1-MEG3 + mimics NC group, while the expression of miR-361-5p was increased in pcDNA3.1-NC + miR-361-5p mimics group (Figure 3A). Moreover, the cell proliferation ability of pcDNA3.1-NC + miR-361-5p mimics group was stronger than that of pcDNA3.1-NC + mimics NC group, while the proliferation ability of pcDNA3.1-MEG3 + miR-361-5p mimics group was higher than that of pcDNA3.1-NC + mimics NC group (Figure 3B). The expression of PCNA and ki67 in NA3.1-NC + miR-361-5p mimics group was decreased than that in pcDNA3.1-NC + mimics NC group (Figure 3C). Furthermore, the apoptosis rate of pcDNA3.1-NC + miR-361-5p mimics group was down-regulated compared with pcDNA3.1-NC + mimics NC group (P<0.05) (Figure 3D). Compared with pcDNA3.1-NC + mimics NC group, Bcl-2 and Bax was significant down-regulated and up-regulated respectively in pcDNA3.1-NC+miR-361-5p mimics group (P<0.05) (Figure 3E). In addition, compared with pcDNA3.1-NC + mimics NC group, MMP1, MMP13, ADAMTS-5 and Collagen II/Aggrecan were significant down-regulated and up-regulated respectively in pcDNA3.1-NC+miR-361-5p mimics group (all P<0.05) (Figure 3F).

MEG3 regulated the expression of Wnt/β-catenin signaling pathway

Western blot was used to detect the expression of Wnt/β-catenin pathway protein in
chondrocytes of each group. The results showed that compared with the si-NC group, the expression levels of Wnt/β-catenin pathway proteins (Active-β-catenin, β-catenin, Wnt3a, Wnt5a, cyclin D1, c-Myc) were significantly up-regulated in pcDNA3.1-NC and pcDNA3.1-MEG3 groups (all P < 0.05). Compared with the pcDNA3.1-MEG3 group, the expression level of Wnt/β-catenin pathway protein was significantly down-regulated in the si-MEG3 group (Figure 4A). Moreover, the transfection of miR-361-5p mimics reduced the expression of Wnt/β-catenin pathway related protein, and co-transfection of pcDNA3.1-MEG3 with miR-361-5p mimics counteracted the effect of MEG3 overexpression (Figure 4B). Furthermore, the expression level of Wnt/β-catenin pathway protein was significantly down-regulated in pcDNA3.1-MEG3 + XAV939 group than other groups (all P < 0.05) (Figure 4C).

Wnt pathway inhibitor reversed the role of MEG3 in OA chondrocytes

After treatment with XAV939, the proliferation (Figure 5A) and apoptosis (Figure 5B) in pcDNA3.1-MEG3 + XAV939 group was significant increased and decreased respectively (all P < 0.05) than those in pcDNA3.1-MEG3 group. Meanwhile, compared with pcDNA3.1-MEG3 group, the expression of Bcl-2 and Bax was up-regulated and down-regulated respectively in pcDNA3.1-MEG3 + XAV939 group (Figure 5C). Moreover, compared with pcDNA3.1-MEG3 group, the expression of MMP1, MMP13 and ADAMTS-5 were down-regulated, while Collagen II and Aggrecan were up-regulated in pcDNA3.1-MEG3 + XAV939 group (Figure 5D).

MEG3 contributed to the cartilage matrix degradation in rats

Compared with the control group, the expression of MEG3 OA group was decreased. Meanwhile, compared with the OA group, the expression of MEG3 in OA + si-MEG3 group was decreased (Figure 6A). Injection of si-MEG3 effectively reduced the cartilage damage of the operation, protected the cartilage from degradation, and reduced the loss of
proteoglycan and joint soft cell (Figure 6B). Moreover, the interference with MEG3 inhibited cartilage bone marrow matrix degradation in the rat OA model (Figure 6C).

Discussion

Although OA is the most common form of arthritis affecting about 237 million people worldwide, the detail molecule mechanism of OA is still not fully investigated until now. In the current study, the RT-qPCR analysis showed that the expression of lncRNA MEG3 and miR-361-5p in OA was significantly decreased and increased respectively than that in normal. Meanwhile, StarBase prediction showed that the MEG3 was competitive binding miR-361-5p in OA chondrocytes. Moreover, the Western blot and CCK-8 assay showed that MEG3 might inhibit cell proliferation and promote cell apoptosis via Wnt/β-catenin pathway.

As a maternally expressed imprinted gene, MEG3 is expressed in many normal tissues but lost in multiple cancer cell lines. A previous study shows that the downregulation of MEG3 contributes to nickel malignant transformation of human bronchial epithelial cells. Ying et al. showed that the downregulated MEG3 activates autophagy and increases cell proliferation in bladder cancer. Downregulated of MEG3 is associated with poor prognosis and promotes cell proliferation in gastric cancer. A previous study shows that the low expression induced by gene knockdown can inhibit apoptosis of chondrocyte in OA rat model. Actually, the low expression of MEG3 has also been revealed in OA. MEG3 has been proved to participate in the development of OA via miR-16/AMAD7 interaction. A previous study indicates that MEG3 is down-regulated and inversely associated with vascular endothelial growth factor A levels in OA. In the current study, the RT-qPCR analysis showed that the expression of MEG3 in OA group was significantly decreased than
that in normal group. Thus, we speculated that MEG3 might down-regulated in the chondrocytes of patients with OA. MEG3 has been proved to participate in the process of cell proliferation and apoptosis in previous studies. Xu et al. revealed that knockdown of MEG3 induced proliferation of rat chondrocytes and inhibited apoptosis. Sun et al. revealed that down-regulated MEG3 promoted cell proliferation in gastric cancer. A previous study shows that MEG3 can inhibit the proliferation of non-small cell lung cancer cells and induce apoptosis. Actually, the biological function of MEG3 is realized by involving certain signaling pathway. The wnt/β-catenin signaling plays key roles in several developmental and pathological processes. The previous mechanics, inflammatory mediators and aging collide studies in developmental, genetic and joint disease models indicate that wnt/β-catenin signaling is critically involved in these processes. In fact, the down-regulated MEG3 can activate in wnt/β-catenin signaling pathway, and then enhance cisplatin resistance in lung and glioma cancer. Liu et al. indicated that MEG3 inhibited the proliferation and metastasis of oral squamous cell carcinoma by regulating the wnt/β-catenin signaling pathway. Gao et al. showed that the decreased expression of MEG3 contributed to retinoblastoma progression and affected retinoblastoma cell growth by regulating the activity of wnt/β-catenin pathway. In adult β-catenin conditional activation rat, OA-like phenotype were shown in articular chondrocytes. In this study, the Western blot, CCK-8 and flow cytometry assay showed that MEG3 not only promoted the proliferation, but also inhibited apoptosis in OA chondrocytes cells. Meanwhile, StarBase prediction showed that MEG3 competitive binding miR-361-5p in OA chondrocytes. Moreover, the RT-qPCR analysis for expression of PCNA and ki67 showed
that the effect of MEG3 on proliferation, apoptosis and cartilage matrix degradation of OA chondrocytes was inhibited by miR-361-5p. Importantly, to reveal whether a target gene participates in certain pathway, the detection of core protein (β-catenin) and related factors (e.g. MMP-1, MMP-13) in wnt/β-catenin signaling pathway is needed. A previous study indicates that the low expression of MMP13 is an effective method for decrease articular cartilage loss in OA models. In the current study, the expression of MMP-1, MMP-13 and β-catenin detected by Western blot showed that EMG3 involved in the regulation of Wnt/β-catenin during OA progression. Thus, based on these results in current study, we speculated that MEG3 might inhibit cell proliferation and promote cell apoptosis via miR-361-5p/Wnt/β-catenin axis in OA chondrocytes.

Conclusions

In conclusion, MEG3 and miR-361-5p might down-regulated and up-regulated respectively in the chondrocytes of OA patients. Furthermore, MEG3 might inhibit cell proliferation and promote cell apoptosis via miR-361-5p/Wnt/β-catenin axis in OA chondrocytes.

Abbreviations

| Term                          | Abbreviation |
|-------------------------------|--------------|
| osteoarthritis                | OA           |
| long noncoding RNA            | lncRNA       |
| Long noncoding RNAs           | IncRNAs      |
| maternally expressed 3        | MEG3         |

Declarations

Ethics approval and consent to participate: The ethics committee of The Third Hospital of Hebei Medical University approved the study. This study was written informed consent from the patients.

Consent for publication: Not applicable.
Availability of data and material: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: AYW, NXH and YFZ designed and analyzed the experiment, and was a major contributor in writing the manuscript. YZC, CHS, YL and YS performed the experiment. All authors read and approved the final manuscript.

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Table 1 The amplification primer used for current RT-qPCR analysis
| Name of primer | Sequences |
|----------------|-----------|
| LncRNA MEG3    | 5′-CTGCCCATCTACACCTCACG-3′<br>5′-CTCTCCGCCTCTTGCTAGGGGCT-3′ |
| GADPH          | 5′-TGCACCACAACTGCTTAGC-3′<br>5′-GGCATGCACTGTGGTCATGAG-3′ |
| miR-361-5p     | 5′-ATAAAGTGTAGCAGATGAG-3′<br>5′-TCAAGTACCCACAGTGCGGT-3′ |
| U6             | 5′-CTCGCTTCGGCAGCACA-3′<br>5′-AACGCTTCAGAATTTCGCT-3′ |

Notes: MEG3, maternally expressed 3; GADPH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, real-time fluorescence quantitative polymerase chain reaction.

Figures
Figure 1

The effect of MEG3 in OA chondrocyte proliferation, apoptosis and cartilage matrix degradation. A, the expression of MEG3 in normal group and OA group detected by RT-qPCR; data expressed as mean ± standard deviation (SD). B, expression of MEG3 by RT-qPCR after transfection of OA chondrocytes with pcDNA3.1-MEG3 or si-MEG3. C, CCK-8 showed the proliferation of pcDNA3.1-MEG3 transfected cells; lncRNA MEG3 overexpression inhibited the proliferation of OA chondrocytes. D, the proliferation of si-MEG3 cells detected by CCK-8 assay. E, the expression of PCNA and marker ki67 was detected by Western Blot after transfection of OA chondrocytes with pcDNA3.1-MEG3 or si-MEG3. F, the apoptosis
detected by flow cytometry of OA chondrocytes. G, the expression of apoptosis protein Bcl-2 and Bax in OA chondrocytes detected by Western Blot; H, Western blot detect the expression of cartilage matrix proteins (MMP1, MMP13, ADAMTS-5, Collagen II, Aggrecan) in chondrocytes of each group. *, P<0.05 compared with Blank or pcDNA3.1-NC group. & P<0.05, compared with the Blank or si-NC group.
MEG3 competitive binding miR-361-5p in OA chondrocytes. A, the binding sites of MEG3 and miR-361-5p predicted by bioinformatics analysis. B, luciferase reporter gene assay showed that the mimic of microRNA-361-5p reduced the luciferase activity of MEG3-Wt in chondrocytes. C, the concentration of MEG3 and miR-361-5p in IgG or Ago2 immunoprecipitates detected by RIP and RT-qPCR. D, the expression of miR-361-5p detected by RT-qPCR after transfection of OA chondrocytes with pcDNA3.1-MEG3 or si-MEG3; *, P<0.05 compared with Blank group or pcDNA3.1-NC group. &, P<0.05 compared with si-NC group. E, RT-qPCR detection of miR-361-5p expression in normal and OA groups; normal group vs. OA group, p < 0.001. F, correlation analysis of MEG3 and miR-361-5p expression in OA chondrocytes. Data expressed as mean ± standard deviation (SD).
Figure 3

The effect of miR-361-5p on MEG3 in OA chondrocytes. A, RT-qPCR detection of miR-361-5p expression. B, cell proliferation detected by CCK-8 assay. C, detection of PCNA, Ki67 expression by Western Blot. D, flow cytometry was used to detect the apoptosis of OA chondrocytes in each group. E, the expression of apoptotic proteins Bcl-2 and Bax detected by Western Blot. F, Western blot analysis of cartilage matrix protein expression in chondrocytes of each group. #, P<0.05 when compared with Blank group or pcDNA3.1-NC+mimics NC group.
Figure 4

The expression of Wnt/β-catenin signal pathway regulated by MEG3. A, he expression of MMP-1 and MMP-13 in chondrocytes of each group detected by
Western blot; *P<0.05 when compared with Blank group or pcDNA3.1-NC group; & P < 0.05 when compared with the si-NC group. B, Western blot analysis of pcDNA3.1-MEG3, miR-361-5p mimics transfection of wnt/β-catenin protein expression in chondrocytes; #, P<0.05 when compared with Blank group or pcDNA3.1-NC + mimics NC group. C, Western blot was used to detect the expression of wnt/β-catenin protein in chondrocytes of all groups with or without wnt inhibitor. *, p<0.05 when compared with the pcDNA-MEG3 NC group; #, p<0.05 when compared to pcDNA-MEG3.
Wnt pathway inhibitor reversing MEG3 regulation of proliferation and apoptosis of OA chondrocytes. A, CCK-8 detected the proliferation of chondrocytes; B, cell apoptosis in each group. C, Flow cytometry detected the apoptosis of chondrocytes and the statistical analysis of the apoptotic rat. D, Western Blot was used to detect the expression of apoptosis protein Bcl-2 and Bax in chondrocytes.

E, the expression of MMP1, MMP13, ADAMTS-5, Collagen II and Aggrecan in chondrocytes detected by Western Blot. *, P < 0.05 when compared with the pcDNA-MEG3 NC group; #, P < 0.05 when compared with pcDNA-MEG3.
MEG3 aggravated cartilage matrix degradation in rats. A, MEG3 expression in cartilage tissue samples of each group. B, Safranin O staining and OARSI grade (Bar = 100 μm) of cartilage tissue samples from each group. C, the expression of MMP1, MMP13, ADAMTS-5, Collagen II and Aggrecan proteins in rat cartilage tissue samples. *, P < 0.05 when compared with the control group; #, P <0.05 when compared with the OA group.

Supplementary Files

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