NEAT1 attenuates osteoarthritis development by sponging miR-424-5p and up-regulating SMAD7 expression

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Abstract

Background: Osteoarthritis is extensively accepted as a chronic joint disease primarily characterized by destruction of articular cartilage, yet with rare molecular mechanism underlying its development and limited therapeutic targets in clinic. We here to investigate the role of NEAT1 and its molecular basis in OA development.

Methods: Rat tissues and chondrocytes with osteoarthritis were used to detect the mRNA level of SMAD7 and miR-424-5p in which the correlation between them was also analyzed; Overexpression and knockdown of miRNA-424-5p were implemented to detect the regulation on SMAD7; The level of inflammatory factors and cell apoptosis of chondrocytes were detected with the opposite treatment on miR-424-5p; The potential interacting IncRNA was identified through online tool and RIP was employed to demonstrate the association between them; The biological coordination between NEAT1 and miR-425-5p in osteoarthritis was explored via the effects on inflammatory factors and cell apoptosis of chondrocytes.

Results: Significantly, our results showed that levels of miR-424-5p are negatively correlated to expression of SMAD7 in rat cartilage tissues and chondrocytes, and overexpression of miR-424-5p was demonstrated to down-regulate SMAD7 expression. Additionally, the results also showed that miR-424-5p regulated expression of pro-inflammatory cytokines and apoptosis in chondrocytes via a SMAD7-dependent manner. Moreover, the potential interacting IncRNA of miR-424-5p was figured out, and NEAT1 was proved to inversely mediate miR-424-5p expression leading to up-regulation of SMAD7. Further, NEAT1 was also estimated to work concert with miR-424-5p orchestrating expression of pro-inflammatory cytokines and chondrocytes apoptosis to ameliorate development of OA.

Conclusions: We here demonstrate that NEAT1 could attenuate OA development via
sponging miR-424-5p and thus up-regulating SMAD7 expression. Our data suggest that NEAT1-miR-424-5p-SMAD7 axis might be a potential therapeutic target for OA patients.

**Background**

OA as a human chronic joint disease is characterized by a systemic dysfunction of articular cartilage, which has developed to be a public concerned healthy trouble [1] [2]. At the molecular level, several pro-inflammation cytokines produced in chondrocytes have been documented to be involved in OA development via disrupting repair process of cartilage, including IL-6 [3, 4] and TNFα [5]. Significantly, it is also reported that SMAD7 which is tightly associated with inflammatory process elicits suppressor role in OA development through mediating expression of these pro-inflammatory cytokines [6, 7]. Nevertheless, the upstream regulators underlying OA development and mediating SMAD7 expression is poorly understood.

In recent years, massive studies have revealed that miRNAs described as an endogenous small molecule RNA with 20–25 nucleotides in length [8] exert crucial roles in human diseases, including cancers [9], immune-related [10] and cardiovascular diseases [11]. At the molecular level, it is estimated that miRNAs mediate genetic post-transcriptional expression via degrading mRNA or suppressing protein translation of target genes [8], which hints that miRNA could be a potential upstream signaling of OA. Intriguingly, it is found that miRNA could control OA development by targeting OA-related factors. For instance, it is reported that miR-21 controls progression of OA by virtue of inhibiting GDF-5 in chondrocytes [12]. However, the potential regulatory miRNA of OA is largely elusive. MiRNA-424-5p located in Xq26.3 has been verified to be a tumor promoter in several cancers, yet with poor research about its downstream regulatory signaling molecules. At the cellular level, it has been documented that miR-424-5p could repress tumor cell metastasis in esophageal squamous cell carcinoma [13], and it was also found that it
could promote invasion and metastasis of hepatocellular carcinoma cells. At the molecular level, association between miR-424-5p and SMAD7 was established in tumor cells, which suggests a putative link of miR-424-5p to SMAD7-related OA development [13]. Clearly, the mechanistic involvement of miR-424-5p in OA development is expected to explore.

LncRNAs recognized as a class of noncoding RNAs that are over 200 nucleotides in length [14]. It has been reported that IncRNAs work in concert with proteins and other effectors in complex regulatory network serving indispensable and complementary roles over the past decade, especially the miRNAs [15]. Importantly, dysregulation of IncRNAs contributes to pathological conditions, cancers [16] and immune-related diseases [17] included. Across these IncRNAs, NEAT1 that is found to be the architectural component of a paraspeckle structure [18] has been reported to be a crucial molecule in differential biological processes, including cellular differentiation [19] and stress response [20] via paraspeckles pathway. At the molecular level, NEAT1 functioned as a ceRNA interacting with has-miR-377-3p downregulating its target [21], and NEAT1 is also known to coordinate with SIN3A corepressor complex involved in epigenetic regulation in breast cancer [22]. Although the massive studies on NEAT1 function, its potential targets remain to be further investigated.

In this time research, we found an unexpected regulatory axis of NEAT1 in OA development. Importantly, we showed that NEAT1 acted as an upstream regulator of miR-424-5p and suppressed its negative regulation on SMAD7. Moreover, NEAT1 coordinated with miR-424-5p promoting expression of pro-inflammatory cytokines and apoptosis of chondrocytes, suggesting that both of them could be the potential therapeutic target of OA patients.

Methods
Antibodies and reagents

Antibodies used were αSMAD7, αGAPDH, and αTP53 from Proteintech; αTNF-α, αIL-6, αBax, and α-cleaved caspase-3 from Cell Signaling Technology; Control siRNA, siRNA for NEAT1 and SMAD7, control-miRNA, miR-424-5p mimics and anti-miR-424-5p were synthesized from Sangon Biotech (Shanghai, China); Fresh bovine serum was obtained from Biological Industries (Israel) and DMEM was obtained from Gibco (USA); Trizol and lipofectamine 3000 transfection were obtained from Invitrogen (USA).

Cell culture and transfection

Rat chondrocytes were isolated from rat cartilage and then seeded into culture plate; Human C28/I2 cell line was obtained from the American Type Culture Collection (ATCC). Cells were maintained in DMEM medium supplemented with 10% FBS in a humidified incubator equilibrated with 5% CO₂ at 37 °C. All transfections were carried out with Lipofectamine 3000 reagent based on the manufacturer’s instruction. Each experiment was done in triplicate and repeated at least 3 times. The sequences of mimic and inhibitor of miR-424-5p, siRNA of NEAT1 and SMAD7 were provided in table 1.

Plasmid construction

The sequences of NEAT1 and 3’-UTR of SMAD7 containing the putative binding sites of miR-424-5p were synthetized from Sangon Biotech, which were then cloned into the pmirGLO Dual-Luciferase vector; NEAT1 overexpression vector was constructed into pcDNA3.1 vector by Sangon Biotech.

Western blot analysis

Cell lysates were obtained using RIPA buffer and centrifuged at 10000 g for 10 min. These proteins were separated by 10% SDS-PAGE for 2 h and then transferred to NC membranes (Millipore) for 90 min. After that, membranes were blocked by 5% skim milk in TBST for
1 h at room temperature. Then membranes with differential proteins were cut into single bands for differential antibodies at 4 °C overnight. The next day, these membranes were washed with TBST with three times for 15 min, and then they were incubated with HRP-conjugated secondary antibodies (Proteintech) for 1 h at room temperature. Finally, these membranes were exposed using luminol buffer (Santa).

RNA extraction and RT-qPCR

Tissues or cells were treated with TRIzol and chloroform and then centrifuged with 10000 g for 10 min at 4°C. The supernatant was collected and isopropanol with same volume was added and mixed, which was then centrifuged with 10000 g for 10 min at 4°C. After that, precipitates were collected and washed with 75% ethanol for two times. RNA was dissolved with nuclease-free water in 70°C for 10 min; 1000 ng RNA in total was used for reverse transcription with RT-PCR kit (Transgene) and then the cDNA was detected for the expression of SMAD7 and miR-424-5p with SYBR Green (Roche) on 7500 real-time PCR system (Applied Biosystems). GAPDH or U6 was used as internal reference and the results were calculated using $2^{-\Delta\Delta^{ct}}$ method. The primers used were presented in table 2.

Luciferase reporter assay

The plasmids with NEAT1-wild type or NEAT1-mutant were transfected into C28/I2 cell cells with miR-NC, miR-424-5p mimics, anti-miR-NC or anti-miR-424-5p. Similarly, the SMAD7-wild type or SMAD7-mutant were transfected into C28/I2 cell cells with miR-NC and pcDNA3.1 or miR-16 mimics or/and NEAT1. The relative luciferase activity was analyzed by the Dual-Glo Luciferase Assay System according to manufacturer’s instruction (Promega).

Enzyme-Linked Immunosorbent Assay (ELISA)

Secreted interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) were examined by ELISA. Supernatant of cells with differential treatment was collected after transfection for 48 h.
IL-6 and TNFα protein levels were evaluated with an ELISA kit using anti-human IL-6 and TNFα antibodies (BD) according to the manufacturer’s instructions. All measurements were performed in duplicate using a FLUO star OPTIMA plate reader (BMG Labtech).

**Flow cytometry**

The Annexin V-FITC Apoptosis Detection Kit (Abcam) was applied to detect apoptosis of rat chondrocytes and C28/I2 cells via a flow cytometer (FACSCalibur, Becton–Dickinson). After that, these data were analyzed by Cell-Quest software for the apoptotic rates.

**Statistical analysis**

All data in this time research were analyzed by SPSS 19.0. Data were shown as the mean ± standard deviation (mean ± SD) and analyzed using t-test. The Pearson’s correlation coefficient analysis was used to analyze the correlations between SMAD7 and miR-424-5p. A p-value less than 0.05 was considered to be statistically significant.

**Results**

**MiR-424-5p was upregulated in OA tissues and negatively correlated to SMAD7 expression in OA tissues and chondrocytes**

Previous research has shown that SMAD7 is demonstrated to be a downstream target of miR-424-5p in cancer cells [13]. Considering the critical role of SMAD7 in OA development, we then addressed the potential role of miR-424-5p in OA tissues. Initially, rat cartilage tissues in sham and OA groups were stained with HE to verify the occurrence of OA and the morphology of tissues were estimated to be altered in OA (Fig. 1A). Then mRNA and protein levels of SMAD7 were further detected to assess the OA development. The results showed that mRNA level of SMAD7 decreased in rat cartilage tissues with OA compared to sham (Fig. 1B). In agreement with expression variety in tissues, mRNA and protein levels of SMAD7 were reduced in OA in contrast to sham of rat cartilage tissues (Fig. 1C). and
chondrocytes (Fig. 1D). To further corroborate association between miR-424-5p and SMAD7 in chondrocytes, levels of miR-424-5p were checked and the correlation between them was analyzed statistically. Notably, levels of miR-424-5p were negatively correlated to levels of SMAD7 \((r=-0.8421, \ p < 0.001, \text{Fig. 1E})\), hinting the putative regulation of miR-424-5p in OA. Then the expression of miR-424-5p in sham and OA of rat cartilage tissues and chondrocytes was interrogated. Oppositely, the expression of miR-424-5p in OA was up-regulated compared to sham of rat cartilage tissues (Fig. 1F) and chondrocytes (Fig. 1G). Taken together, our results suggest a plausible role of miR-424-5p in the development of OA.

**MiR-424-5p negatively regulated expression of SMAD7**

As stated above, negative correlation of miR-424-5p with SMAD7 was established in OA tissues and chondrocytes. We then asked that whether miR-424-5p could target SMAD7 for repression in chondrocytes. We first examined the expression levels of SMAD7 with the transfection of miR-424-5p mimic and anti-miR-424-5p in rat chondrocytes and human C28/I2 cells. After transfection for 48 h, elevated expression of miR-424-5p (Fig. 2A) and reduced expression of SMAD7 (Fig. 2B) in miR-424-5p mimic group were verified by qPCR in rat and human chondrocytes, respectively. Conversely, indicated overtly decreased expression of miR-424-5p (Fig. 2A) and increased expression of SMAD7 (Fig. 2B) in anti-miR-424-5p group was observed. After that, luciferase reporter vectors containing the WT or MUT 3′UTR of SMAD7 (Fig. 2C) were transfected together with miR-424 mimic or anti-miR-424-5p in rat chondrocytes and human C28/I2 cells. Importantly, the luciferase activity of WT-SMAD7 decreased evidently in miR-424-5p mimic group, while elevated luciferase activity was found in anti-miR-424-5p group of rat chondrocytes (Fig. 2D) and human C28/I2 cells (Fig. 2E) in contrast to control group. Nevertheless, there was no difference of the luciferase activity between miR-424-5p mimic and anti-miR-424-5p group
with the Mut-SMAD7 vector in rat chondrocytes (Fig. 2D) and human C28/I2 cells (Fig. 2E).

To gain detailed orchestration of miR-424-5p on SMAD7, protein levels of SMAD7 was examined with the transfection of miR-424-5p mimic and anti-miR-424-5p. The results showed that SMAD7 protein levels elevated in anti-miR-424-5p group, while it decreased in group of miR-424-5p mimic, all compared to control group in rat chondrocytes (Fig. 2F) and human C28/I2 cells (Fig. 2G). Together, our results support that SMAD7 is a target of miR-424-5p in chondrocytes, further sustaining the putative function of miR-424-5p in OA development.

MiR-424-5p mediated expression of pro-inflammatory cytokines and apoptosis of chondrocytes via a SMAD7-dependent manner

To further clarify the effects of miR-424-5p on SMAD7 expression in chondrocytes cells, we then checked alteration of SMAD7-related inflammatory reaction and cell apoptosis. First, protein levels of secreted IL-6 and TNFα cytokines were detected via ELISA with the transfection of siNC or/and mock, anti-miR-424-5p or/and si-SMAD7 in rat chondrocytes and human C28/I2 cells. The results showed that IL-6 and TNFα levels decreased after the transfection of anti-miR-424-5p, which could be partially rescued by co-knockdown of SMAD7 (Fig. 3A). Consistently, mRNA levels of IL-6 and TNFα reduced with the inhibition of miR-424-5p, which was attenuated by simultaneous depletion of SMAD7 in rat chondrocytes and human C28/I2 cells (Fig. 3B). Moreover, western blot results also demonstrated that IL-6 and TNFα levels decreased by virtue of anti-miR-424-5p, which could be offset by further knockdown of SMAD7 in rat chondrocytes (Fig. 3C) and human C28/I2 cells (Fig. 3D). These results suggest that miR-424-5p could regulate inflammatory reaction via a SMAD7-dependent manner.

Given that apoptosis of chondrocytes has been reported to be associated with SMAD7 and
OA development, apoptosis of rat chondrocytes and human C28/I2 cells was investigated. These cells were transfected with siNC or and mock, anti-miR-424-5p or and si-SMAD7 and the apoptosis was examined by flow cytometry. Intriguingly, the data showed that cell apoptosis rates notably decreased with the treatment of anti-miR-424-5p, while this effect was markedly ameliorated by SMAD7 knockdown (Fig. 3E). Additionally, apoptosis markers were also detected by western blot with antibodies against Bax, Cleaved caspase-3 and TP53. Consistently, protein levels decreased after transfection of anti-miR-424-5p, which could be counteracted by co-knockdown of SMAD7 in rat chondrocytes (Fig. 3F) and human C28/I2 cells (Fig. 3G). Taken together, these results suggest that miR-424-5p might exert function of pro-apoptosis through mediating SMAD7 in chondrocytes.

**NEAT1 was verified as a candidate interacting lncRNA of miR-424-5p and regulated SMAD7 expression**

Previous researches have shown that NEAT1 is in implicated in occurrence of inflammation, so we reasoned that NEAT1 might be involved in regulation of OA development. Expression levels of NEAT1 between OA and adjacent tissues were tested by qPCR and the data showed that NEAT1 had a higher expression in adjacent compared to OA tissues (Fig. 4A). Given that lncRNA is reported to interact with miRNA to function at the molecular level, we then investigated the association between miR-424-5p and NEAT1. Initially, miR-424-5p and SMAD7 expression levels were examined by qPCR after transfection of NEAT1 or siNEAT1. The results verified that expression levels of miR-425-5p decreased and elevated with the overexpression and knockdown of NEAT1, respectively, in which SMAD7 had an opposite expression profile (Fig. 4B). In addition, it is intriguingly found that NEAT1 had putative binding sites of miR-424-5p by virtue of starBase database (Fig. 4C). To demonstrate the interaction between NEAT1 and miR-424-
5p, luciferase reporter assay of WT and MUT-NEAT1 vector was performed in which the luciferase activity was tested with the transfection of anti-miR-424-5p or miR-424-5p into human C28/I2 cells. The results showed that the luciferase activity of WT-NEAT1 vector overtly decreased and elevated with the transfection of miR-424-5p mimics and anti-miR-424-5p, respectively, while MUT-NEAT1 vector had rare effects in luciferase activity assay following the similar treatment (Fig. 4D). To further validate the association of NEAT1 and miR-424-5p, RNA immunoprecipitation (RIP) assay with the antibodies against IgG and Argonaute2 protein that is documented to be a crucial part of the RNA induced silencing complex was employed [16]. Significantly, our data found that NEAT1 and miR-424-5p were largely immuoprecipitated with anti-Ago2 compared to IgG control group in human C28/I2 cells (Fig. 4E). Together, our results indicate NEAT1 could interact with miR-424-5p and regulate its expression.

To further interrogate the interaction of between NEAT1 and miR-424-5p, luciferase reporter assay of WT and MUT- SMAD7 and its expression with the transfection of pcDNA3.1 and mock, miR-424-5p mimics and pcDNA3.1, miR-424-5p mimics and NEAT1 and mock and NEAT1. Importantly, our data displayed that the luciferase activity of WT-SMAD7 was obviously enhanced by transfection of NEAT1. And the luciferase activity was reduced by overexpression of miR-424-5p reversely, which was then rescued by co-transfection of NEAT1 in human C28/I2 cells (Fig. 4F). However, there was little alteration in MUT-SMAD7 groups. Moreover, mRNA and protein levels of SMAD7 were also examined in human C28/I2 cells. The results showed that mRNA and protein levels of SMAD7 decreased after transfection of miR-424-5p mimics, which were counteracted by further overexpression of NEAT1 (Fig. 4G). Taken together, these results propose that NEAT1 is implicated in miR-424-5-mediated SMAD7 regulatory axis and positively regulate SMAD7 expression.
NEAT1 coordinated with miR-424-5p mediating expression of pro-inflammatory cytokines and apoptosis of chondrocytes

To further elucidate the putative effects of NEAT1 on OA development, pro-inflammatory cytokines expression and chondrocytes apoptosis were checked via gain-function of NEAT1 and miR-424-5p. Human C28/I2 cells were transfected with vector of pcDNA3.1 and mock, miR-424-5p mimics and pcDNA3.1, miR-424-5p mimics and NEAT1 and mock and NEAT1, and the secreted IL-6, TNFα cytokines and mRNA of them were detected through ELISA and qPCR. The results showed that IL-6 and TNFα levels increased after the transfection of miR-424-5p mimics, which could be offset by overexpression of NEAT1 together (Fig. 5A). Similarly, mRNA levels of IL-6 and TNFα elevated with the overexpression of miR-424-5p, which was attenuated by simultaneous transfection of NEAT1 (Fig. 5B). Moreover, western blot results also proved that IL-6 and TNFα levels increased by virtue of overexpression of miR-424-5p mimics, which could be rescued by further transfection of NEAT1 (Fig. 5C). Importantly, overexpression of NEAT1 induced the decreased expression of IL-6 and TNFα across these experiments (Fig. 5A, 5B, 5C). Additionally, apoptosis rates of these cells were examined and the data showed that cell apoptosis rates apparently elevated treatment of miR-424-5p mimics, of which this effect was markedly rescued by overexpression of NEAT1 (Fig. 5D). In addition, apoptosis markers were also checked by western blot with antibodies against Bax, Cleaved caspase-3 and TP53. Correspondingly, protein levels decreased after transfection of miR-424-5p mimics, which could be offset by further transfection of NEAT1 (Fig. 5E). Significantly, overexpression of NEAT1 induced the elevated apoptosis rates and proteins markers significantly in human C28/I2 cells (Fig. 5D, 5E). Taken together, these results suggest that NEAT1 coordinate with miR-424-5p mediating expression of inflammatory cytokines and cell apoptosis in chondrocytes.
Discussion

OA has developed to be a public concerned problem [2], yet with rare molecular mechanism and limited therapeutic targets. In recent years, it has been documented that miR-425-5p could inhibit esophageal squamous cell carcinoma metastasis by targeting SMAD7 [13]. Importantly, SMAD7 that is proved to be an intracellular antagonist of TGF-β signaling pathway exerts crucial role in in several inflammatory diseases [23]. Previous studies have also revealed that SMAD7 negatively mediated expression of pro-inflammatory cytokines. Of note, OA development is accompanied by up-regulation of pro-inflammatory cytokines, which suggests the putative negative role of SMAD7 in OA. And it was further corroborated by several reports [7, 24, 25]. Thus, the underlying mechanisms of miR-424-5p in OA development is expected to illustrated. In this time research, we revealed how miR-424-5p was involved in regulation of OA development and its mediation on proinflammatory cytokines and cell apoptosis. Additionally, the putative interacting lncRNA of miR-424-5p in OA development was also identified.

A series of studies have uncovered that miR-424-5p plays important role in cancer cell proliferation and invasion. For instance, miR-424-5p inhibits breast cancer proliferation and invasion via targeting DCLK1 [26]. Although massive researches on exploration of miR-424-5p in tumor progression, the other pathological function of it is largely elusive. Our results showed that expression of miR-424-5p was up-regulated in OA tissues compared to normal and negatively correlated to expression of SMAD7, which hinted a negative regulation of miR-424-5p in OA development. Additionally, our data found that miR-424-5p targeted SMAD7 for inhibition in chondrocytes which is consistent to previous study. And we also verified that miR-424-5p positively regulated expression of proinflammatory cytokines and chondrocytes apoptosis. Intriguingly, as we further introduced si-SMAD7 after transfection of anti-miR-424-5p, phenotypes induced by
knockdown of miR-424-5p were counteracted, suggesting its molecular orchestration on OA development could be achieved by SMAD7. These data suggested that miR-424-5p could be a putative therapeutic target for OA patients in clinic.

In recent years, IncRNA has been reported to exert significant role in human pathological progress, including OA. For instance, GAS5 has been documented to contribute to OA development [27], and it is also shown that down-regulation of MEG3 promotes development of OA [28]. Momentously, function of IncRNA was achieved partly through miRNA and it was reported that IncRNA could repress miRNAs by interacting with them. Across these IncRNAs, NEAT1 was screened to be an interactor of miR-424-5p via online tool and further experiments showed that NEAT1 acted to negatively regulate miR-424-5p. And it was manifested that NEAT1 also mediated expression of SMAD7 through miR-424-5p. At the molecular level, NEAT1 interacted with miR-424-5p repressing its downstream signaling molecule, which was first discovered ever. In addition, our results that expression of NEAT1 was higher in OA tissues compared to normal and NEAT1 coordinated with miR-424-5p inhibiting expression of pro-inflammatory cytokines and apoptosis of chondrocytes also suggested a potential role of NEAT1 in OA development. Similarly, previous study has shown that NEAT1 could inhibit osteopontin-mediated synoviocyte proliferation repressing OA development [29], which further sustained our results.

Nevertheless, our data revealed a novel pathway of NEAT1-mediated OA development, which expands the regulatory net of NEAT1 in OA. More significantly, given that miR-424-5p was proved to be a bona fide interactor of NEAT1, function of these two molecules is expected to further discuss in future studies.

Of note, given the multitude of the cellular function of NEAT1 and miR-424-5p, their function in OA is possibly beyond what we explored in the current study. Additionally, the contribution of the other putative targets to NEAT1 or miR-424-5p-related OA development
remains to be identified in the future studies.

In summary, we report in the current study that NEAT1 attenuates osteoarthritis development by sponging miR-424-5p and up-regulating SMAD7, indicating that miR-424-5p and NEAT1 could be a novel therapeutic target of OA patients.

List Of Abbreviations
American type culture collection, ATCC; Competing endogenous RNA, CeRNA; Doublecortin like kinase 1, DLK1; Growth arrest-specific transcript 5, GAS5; Growth differentiation factor 5, GDF 5; Interleukin, IL; Long noncoding RNA, LncRNA; MEG3, Maternally Expressed Gene 3; Nuclear paraspeckle assembly transcript 1, NEAT1; Osteoarthritis, OA; RNA immunoprecipitation, RIP; Tumor necrosis factor α, TNFα.

Declarations

Ethics approval and consent to participate
This study did not include any personal or clinical participants.

Consent for publication
Not applicable.

Availability of data and material
Not applicable.

Competing interests
No potential conflict of interest was reported by the authors.

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Author contributions
DZ, LS and XW conceived and designed the experiments. DZ and LS analyzed the data and performed the experiments. DZ, LS and XW wrote the paper.
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Tables

Due to technical limitations, Tables 1-2 are provided in the Supplementary Files section.

Figures
MiR-424-5p was up-regulated in OA tissues and negatively correlated to SMAD7 expression in OA tissues and chondrocytes. (A) HE staining showed the morphological changes of OA model tissues. (B) Expression of SMAD7 in rat cartilage tissues were detected by qPCR, #p<0.01 represents the comparison to Sham group. (C) Expression of SMAD7 in rat chondrocytes were detected by qPCR, #p<0.01 represents the comparison to Sham group. (D) Western blot detected the expression of SMAD7 in OA cartilage cells, #p<0.01represents the comparison to Sham group. (E) MiR-424-5p was negatively correlated with SMAD7 compared to Sham group and the correlation coefficient was calculated. (F) QPCR were used to detect the higher expression of miR-424-5p in cartilage tissue and chondrocytes of OA, #p<0.01represents the comparison to Sham group.
MiR-424-5p negatively regulated expression of SMAD7. (A) qPCR was used to detect the expression of miR-424-5p by overexpression or knockdown of miR-424-5p (rat and human), #p<0.01 represents the comparison to Mock group. (B) qPCR was used to detect the expression of SMAD7 by overexpression or knockdown of miR-424-5p (rat and human), #p<0.01 represents the comparison to Mock group. (C) The conserved binding site of miR-424-5p to SMAD7 was showed. (D) Luciferase assay was used to detected 3'UTR luciferase activity of SMAD7 regulated by miR-424-5p in rat chondrocytes, #p<0.01 represents the comparison to Mock group. (E) Luciferase assay was used to detected 3'UTR luciferase activity of SMAD7 regulated by miR-424-5p in human C28/I2 cells, #p<0.01 represents the comparison to Mock group. (F) Western blot was used to examine the expression of SMAD7 in rat chondrocytes, #p<0.01 represents the comparison to Mock group. (G) Western blot was used to examine the expression of SMAD7 in human C28/I2 cells.
human C28/I2 cells, #p<0.01 represents the comparison to Mock group.

Figure 3

MiR-424-5p mediated expression of pro-inflammatory cytokines and apoptosis of chondrocytes via a SMDA7-dependent manner. (A) ELISA assay was used to detect the expression of pro-inflammatory cytokines; #p<0.01 represents the comparison to Mock/si-NC group and Φp<0.01 represents the comparison to anti-miR-424-5p/si-NC group. (B) mRNA levels of pro-inflammatory cytokines was
detected by qPCR; #p<0.01 represents the comparison to Mock/si-NC group and Φp<0.01 represents the comparison to anti-miR-424-5p/si-NC group. (C) Western blot was used to detect the expression of pro-inflammatory cytokines in each group of rat chondrocytes. (D) Western blot was used to detect the expression of pro-inflammatory cytokines in each group of human C28/I2 cells. (E) Annexin V-FITC assay was used to detect the apoptosis of chondrocytes; #p<0.01 represents the comparison to Mock/si-NC group and Φp<0.01 represents the comparison to anti-miR-424-5p/si-NC group. (F) Western blot was used to detect the expression of apoptotic marker protein in each group of rat chondrocytes. (G) Western blot was used to detect the expression of apoptotic marker protein in each group of human C28/I2 cells.

Figure 4

NEAT1 was verified as a candidate interacting lncRNA of miR-424-5p and
regulated SMAD7 expression. (A) The expression of NEAT1 in OA was detected by qPCR, \#p<0.01 represents the comparison to Sham group. (B) The expression of miR-424-5p and SMAD7 was detected by qPCR after overexpression and knockdown of NEAT1, \#p<0.01 represents the comparison to pcDNA3.1 group. (C) Binding site of miR-424-5p on NEAT1 was shown. (D) Luciferase reporter assay was used to detect luciferase activity of NEAT1 with overexpression and knockdown of miR-424-5p, \#p<0.01 represents the comparison to Mock group. (E) RIP assay was examined by qPCR, \#p<0.01 represents the comparison to IgG group. (F) Luciferase reporter assay was used to detected the luciferase activity of SMAD7 3'UTR with the synergistic regulation by NEAT1 and miR-424-5p, \#p<0.01 represents the comparison to Mock+pcDNA3.1 group. (G) Expression of SMAD7 was examined by qPCR via synergistic regulation by NEAT1 and miR-424-5p, \#p<0.01 represents the comparison to Mock+pcDNA3.1 group. (H) Expression of SMAD7 was examined by western blot via synergistic regulation by NEAT1 and miR-424-5p.
NEAT1 coordinated with miR-424-5p mediating expression of proinflammatory cytokines and apoptosis of chondrocytes. (A) ELISA was used to detect the expression of pro-inflammatory cytokines via synergistic regulation by NEAT1 and miR-424-5p; #p<0.01 represents the comparison to Mock+pcDNA3.1 group and Фp<0.01 represents the comparison to miR-424-5p+NEAT1 group. (B) QPCR was used to detect the mRNA levels of pro-inflammatory cytokines via synergistic regulation by NEAT1 and miR-424-5p; #p<0.01 represents the comparison to Mock+pcDNA3.1 group and Фp<0.01 represents the comparison to miR-424-5p+NEAT1 group. (C) Western blot was used to detect the expression of pro-inflammatory cytokines via synergistic regulation by NEAT1 and miR-424-5p. (D) Annexin V-FITC/PI was used to examine apoptosis of chondrocytes with
synergistic regulation by NEAT1 and miR-424-5p; #p<0.01represents the comparison to Mock+pcDNA3.1 group and Фp<0.01 represents the comparison to miR-424-5p+NEAT1 group. (E) The expression of apoptotic marker protein in each group was detected by western blot with synergistic regulation of NEAT1 and miR-424-5p.

**Supplementary Files**

This is a list of supplementary files associated with the primary manuscript. Click to download.

* table 1.pdf
* table 2.pdf