LincRNA-Cox2 targeting miR-150 regulating the proliferation and apoptosis of chondrocytes in osteoarthritis

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Abstract
Background: Osteoarthritis (OA) is a joint disease characterized by progressive cartilage degradation and inflammation, but the detailed pathogenesis of OA is still unclear. Here, we aimed to investigate the role of LincRNA-Cox2 in OA progression and the potential mechanism.

Methods: OA mouse model and IL-1β-induced injury of mouse chondrocytes were conducted. Si-Cox2 was transfected into chondrocytes for elucidating the effect of LincRNA-Cox2 on OA. qR-TPCR was used to detect the expression of LincRNA-Cox2 and miR-150. Cell proliferation and apoptosis were analyzed by MTT assay and Annexin V/PI stain respectively. Western blot was used to evaluate the protein levels in chondrocytes.

Results: High levels of LincRNA-Cox2 were observed in both cartilage tissues of OA and IL-1β-treated chondrocytes. Knockdown of LincRNA-Cox2 promoted the proliferation and inhibited apoptosis of chondrocytes. Mechanically, LincRNA-Cox2 directly target to miR-150, acting as a ceRNA, and the effect of si-Cox2 on proliferation and apoptosis in chondrocytes was reversed by miR-150 inhibitor. Moreover, LincRNA-Cox2 had ability to activate wnt/β-catenin pathway to regulate chondrocytes proliferation and apoptosis.

Conclusion: Silencing LincRNA-Cox2 plays a protective role in OA by enhancing the proliferation and suppressing apoptosis of chondrocytes, which was related with increase of miR-150 and activation of Wnt/β-catenin pathway.

Background
Osteoarthritis (OA) is the most frequent degenerative joint disease with leading cause of pain and disability in middle-aged and older people, and this has turned into a worldwide health concern [3]. OA is mainly characterized by degeneration of articular cartilage and inflammatory response [7]. The degeneration of articular cartilage is influenced by multiple factors, such as aging, obesity, strain, trauma, and inflammatory disease. As this disease is being more prevalent and still hard to cure, more clinical and experimental research are urgently needed for exploring the molecular mechanisms of OA.

During the past decades, deregulation and dysfunction of mRNAs, lncRNAs and miRNAs [11, 28] is
evident in OA. It is reported that IncRNAs, transcripts in length longer than 200 nucleotides, are involved in OA progression by regulating cartilage degradation [18]. Also, studies have demonstrated the therapeutic potential of noncoding RNAs including long noncoding RNAs (IncRNAs) in the treatment of OA [26]. Long intergenic noncoding RNAs (lincRNAs), a subclass of IncRNAs, are emerged as a type of key regulators of mammalian gene expression. Several thousand lincRNAs have been identified in the mouse genome [4, 15]. Moreover, lincRNAs are reported to be associated with human inflammatory diseases and tumorigenesis [22, 5]. LincRNA-Cox2 is one of the better characterized lincRNAs and were reported to regulate transcription of distinct classes of immune genes in the inflammatory response, thus are regarded as an immune-inducible lincRNA [2]. In the previous study, Elling R et al found that LincRNA-Cox2 could regulate critical innate immune genes, dependently or independently of Ptgs2 [6]. Moreover, Tong et al demonstrate a novel mechanism of epigenetic modulation by LincRNA-Cox2 on Il12b transcription, suggesting an important role for lincRNAs in regulation of intestinal epithelial inflammatory responses [23]. However, the role of LincRNA-Cox2 in cartilage degradation and the development of OA remains to be unclear.

Chondrocytes are the important type of cells in cartilage and their growth, differentiation, and apoptosis are regulated to maintain a dynamic equilibrium. Therefore, their dysfunction is responsible for OA development [17]. The apoptosis and growth of chondrocytes were also found to be modulated by IncRNA, such as IncRNA XIST, PVT1 and PART-1 [19, 14, 13]. In this study, we investigated the effect of LincRNA-Cox2 on the proliferation and apoptosis of chondrocytes supporting an important role for LincRNA-Cox2 in the development of OA.

Methods

**Isolation and culture of primary mouse chondrocytes**

Primary murine chondrocytes were isolated from newborn mice as described previously [8]. Briefly, 5-day-old C57BL/6 mice were sacrificed by intraperitoneal injection of pentobarbital and then isolated articular cartilage. The isolated articular cartilage was digested with 3 mg/mL collagenase D for 90 min at 37 °C under 5% CO2 and 0.5 mg/mL collagenase D overnight at 37 °C. After centrifugation at 400 g for 10, the supernatant was discarded and cells precipitation was resuspended in DMEM
supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. Then seed chondrocytes on a culture dish at density of 8×10^3 cells per cm. Change the culture medium after 2 d of culture, and the isolated chondrocytes reach confluence by 6-7 d. Only passage 1 to 3 were used for further experiments.

**Induction of OA mouse model**

10-week-old C57BL/6J male mice were purchased from Animal Center of the Chinese Academy of Sciences (Shanghai, China) and housed in plastic cages with free access to drinking water and a pellet based diet. Experimental OA model was induced by surgical destabilization of the medial meniscus (DMM) as described previously [12]. Briefly, under general anesthesia, the medial collateral ligament and the medial meniscus of the right knee were resected under a microscope. After surgery, the mice were randomly divided into the following groups: sham group, OA group. 8 weeks after surgery, mice were sacrificed for detection of LincRNA-Cox2 expression.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

qRT-PCR was performed to detect the expression of mouse LincRNA-Cox2 and miR-150 in chondrocytes after transfection and IL-1β treatment. Total RNA of chondrocytes was extracted using TRIzlo reagent (Thermo Fisher Scientific, MA, USA). After transcription to cDNA, qRT-PCR was performed by SYBR Green Master Mix (Applied Biosystems, CA, USA) and the setting parameters are as follows: firstly 95 °C for 10 min, then 95 °C for 15 s and 60 °C for 30 s lasting 40 cycles. The cycle threshold (Ct) values were obtained, normalized to the level of GAPDH and compared with the control. Data were quantified using the 2^{-\Delta\Delta CT} method. The primer sequences of mouse LincRNA-Cox2 were as follows: forward 5’-AAGGAAGCCTTGGCGTTGTGA-3’; reverse 5’-GAGAGGTGAGGAGTCTTATG-3’.

**Proliferation assay**

The proliferation capability of chondrocytes was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT assay). 5×10^3 cells were seeded into 96-well plates for 24 h, then cells were transfected with si-NC, si-Cox2, inhibitor NC and miR-150 inhibitor, respectively. 48 h after transfection, 20 uLMTT (5 mg/ml) (Sigma, CA, USA) was added, and the cells were incubated for 4 h at
37 °C under 5% CO₂. Subsequently, the supernatant was discarded and 200 ml DMSO was added. Finally, the OD490 nm value was measured to evaluate the proliferation capability of chondrocytes.

**Apoptosis assay**

Apoptosis of chondrocytes was determined using Annexin V FITC Apoptosis Detection Kit (BD Bioscience, NJ, USA). After IL-1β treatment and/or relevant transfection, cells were collected, washed with phosphate-buffered saline (PBS) and resuspended in 1 Binding Buffer at a concentration of 1×10⁶ cells/mL. Then 5 µl of annexin V FITC and PI were added. After incubation for 15 min at room temperature in the dark, quantification of apoptotic cells was analyzed by flow cytometry (BD Bioscience, NJ, USA). Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

**Western blotting**

After the indicated treatment, chondrocytes were collected and washed with PBS, then lysed on ice with RIPA lysis buffer supplemented with 10 mM of PMSF (Beyotime, Nanjing, China) for 15 min. Total protein was quantified using the BCA Protein Assay Kit (Solarbio, Beijing, China). Then, proteins in equal amounts were subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Bio‐Rad Laboratories, CA, USA). After blocking with 5% skimmed milk for 2 h at room temperature, membranes were incubated with specific primary antibodies against Ki67, PCNA, Bax, Capase-3, Capase-9, GSK-3β, p- GSK-3β (ser9), β-catenin, connlin D1, c-Myc, MMP-7 and GAPDH (Santa Cruz, CA, USA) overnight at 4 °C. Then membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature, and protein bands were visualized using electrochemiluminescence (ECL) plus (GE Healthcare; Buckinghamshire, England, UK) according to the manufacturer’s instructions. Densitometry analysis of bands was performed using Image J software (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as an endogenous protein for normalization.

**Cell transfection**

The miR-150 mimic, miR-150 inhibitor and their negative control (Scramble and anti-NC) were synthesized by GenePharma Co (Shanghai, China). The full-length wide-type LincRNA-Cox2 sequences
was constructed into the pEX-2 plasmid (GenePharma, Shanghai, China). An empty pEX-2 plasmid was transfected as a negative control. siRNA specific for LincRNA-Cox2 was constructed into U6/Neo plasmid (GenePharma, Shanghai, China). An empty U6/Neo plasmid with non-targeting sequences was transfected as a negative control (NC). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, CA, USA) depending on the manufacturer’s descriptions. After 48 h, the transfection efficiency was detected by qRT-PCR.

**Dual luciferase activity assay**

The 3’UTR target site was generated by PCR and the luciferase reporter constructs with the LincRNA-Cox2 sequences carrying a putative miR-150-binding site into pMiR-report vector were amplified by PCR. Cells were co-transfected with the reporter construct, control vector and miR-150 or scramble using Lipofectamine 3000 (Life Technologies, USA). Reporter assays were done using the dual-luciferase assay system (Promega) following to the manufacturer’s information.

**Statistical analyses**

All results were observed from at least three independent experiments. Statistical analysis was carried out using SPSS 19.0 and data were presented as the mean ± SD as indicated. Statistical differences between two groups were determined by two-tailed Student’s t-test. Differences among more than two groups in the above assays were estimated by one-way ANOVA. The linear relationship among levels of LincRNA-Cox2 and miR-150 in OA mice was analyzed by Spearman’s correlation coefficient. \( P< 0.05 \) was considered statistically significant.

**Results**

**LincRNA-Cox2 expression is up-regulated in cartilage tissues of OA and IL-1β-induced chondrocytes**

To detect the expression of LincRNA-Cox2 in cartilage tissues of OA, qRT-PCR was performed in cartilage specimens from 10 OA mice and 10 normal mice. The results demonstrated that the expression of LincRNA-Cox2 was markedly higher in OA cartilage tissues than that in normal tissues \( (P< 0.05, \text{Fig. 1A}) \). Moreover, it was also observed that the expression of LincRNA-Cox2 was significantly up-regulated in chondrocytes stimulated by IL-1β at 10 and 20 ng/mL \( (P< 0.05, \text{Fig. 1B}) \).
Knockdown of LincRNA-Cox2 promotes proliferation and inhibits apoptosis in IL-1β-induced chondrocytes

To explore the effect of LincRNA-Cox2 on the proliferation and apoptosis in IL-1β-induced chondrocytes, its abundance was knocked down using siRNA (si-Cox2) in IL-1β-treated (10 ng/mL) chondrocytes ($P<0.05$, Fig. 2A). Moreover, the data of MTT assay showed that knockdown of LincRNA-Cox2 markedly restored the cell viability decreased by treatment of IL-1β in chondrocytes ($P<0.05$, Fig. 2B). The protein levels of major proliferation-related genes including Ki67 and PCNA were significantly increased after silencing LincRNA-Cox2 in IL-1β-induced chondrocytes ($P<0.05$, Fig. 2C). In addition, after transfection with si-Cox2, a lower proportion of apoptotic cells was shown in chondrocytes with treatment of IL-1β ($P<0.05$, Fig. 2D), and the protein levels of Bax, cleaved Caspase-3 (c-Caspase-3) and cleaved Caspase-9 (c-Caspase-9) were also down-regulated ($P<0.05$, Fig. 2E).

LincRNA-Cox2 directly targeted miR-150

We predicted that there were putative complementary sequences of LincRNA-Cox2 and miR-150 using starBase 2.0 ($P<0.05$, Fig. 3A). To confirm the potential relationship between LincRNA-Cox2 and miR-150, we constructed the luciferase reporter vectors wt-Cox2 and mut-Cox2 and transfected them into chondrocytes. The results demonstrated that overexpression of miR-150 induced a notably reduction of luciferase activity and knockdown of miR-150 led to an increase of luciferase activity in wt-Cox2 group, while little effect was observed on the activity in mut-Cox2 group ($P<0.05$, Fig. 3B). Additionally, the effect of LincRNA-Cox2 on miR-150 in chondrocytes were evaluated and results showed that the expression of miR-150 was significantly increased by interference of LincRNA-Cox2 ($P<0.05$, Fig. 3C). Also, the expression of LincRNA-Cox2 in chondrocytes was reduced by overexpression of miR-150, while increased by knockdown of miR-150 ($P<0.05$, Fig. 3D). In cartilage tissues of OA mouse model, miR-150 expression was significantly decreased ($P<0.05$, Fig. 3E) and negatively correlated with LincRNA-Cox2 expression ($P<0.05$, Fig. 3F).

Deficiency of miR-150 reverses the effect of LincRNA-Cox2 knockdown on IL-1β-induced injury in chondrocytes
To explore whether LincRNA-Cox2 exerted its function by miR-150 in chondrocytes, the rescue experiments were performed. As shown in Fig. 4A and B, knockdown of LincRNA-Cox2 enhanced the proliferation of IL-1β-treated chondrocytes and the expression of Ki67 and PCNA, while suppression of miR-150 blocked the effect effectively (*P* < 0.05). Moreover, the anti-apoptosis effect of LincRNA-Cox2 knockdown was also evidently reversed by miR-150 inhibitor (*P* < 0.05, Fig. 4C and D).

**LincRNA-Cox2 aggravates OA progression through wnt/β-catenin pathway**

In order to figure out the function of molecular mechanisms induced by LincRNA-Cox2/miR-150 axis, the wnt/β-catenin pathway related proteins including GSK-3β, pGSK-3β, β-catenin, cyclin D1, c-myc and MMP-7 were detected by western blot. The results showed that knockdown of LincRNA-Cox2 notably inhibited the expression of pGSK-3β, β-catenin, cyclin D1, c-myc and MMP-7, while the suppressing effect was reversed by miR-150 inhibitor (*P* < 0.05, Fig. 5).

**Discussion**

OA is a chronic, progressive, and degenerative disease, affecting multiple joint tissues, and results in great suffering including pain, stiffness, movement difficulty, and even progressive disabilities. The main characteristics of OA are degeneration of articular cartilage and chronic inflammatory response. However, despite the diverse etiology and pathogenesis of OA, the detailed pathogenesis has not yet been elucidated. Recent focus of the epigenetic regulating mechanisms of OA have revealed that numerous lncRNAs were served important functions in the development of inflammation related diseases including OA, such as lncRNA XIST and PVT1 [19, 14]. LincRNA-Cox2, a class of lncRNA localized to both the cytosolic and nuclear compartments, has ability to affect the expression of hundreds of inflammatory genes and regulate inflammatory response [2]. Besides, LincRNA-Cox2 has capacity to mediate neuroinflammation by regulating NLRP3 inflammasome and autophagy [24]. However, whether LincRNA-Cox2 involved in the pathogenesis of OA remains unclear. In this study, we found that the expression of LincRNA-Cox2 was markedly up-regulated both in vivo and in vitro OA model, indicating that LincRNA-Cox2 may play a role in OA development.

Chondrocytes are the only cells found in the cartilage and their dynamic equilibrium between growth, differentiation, and apoptosis was crucial to maintain the appropriate cycles of the biosynthesis and
degradation of the cartilaginous matrix [17]. Here, we investigated the role of LincRNA-Cox2 in viability of chondrocytes using IL-1β-induced chondrocytes, and the results demonstrated that LincRNA-Cox2 inhibited the viability of chondrocytes. In addition, the protein levels of Ki67 and PCNA, two main proliferation related proteins, were also suppressed by LincRNA-Cox2. Apoptosis is an important processes associated cell viability, and activation of Bax, c-Caspase 3 and c-Caspase 9 are responsible for monitor of cell apoptosis [25, 1]. In this study, we found that both the apoptosis cells and the expression of Bax, c-Caspase 3 and c-Caspase 9 are reduced after knockdown of LincRNA-Cox2, suggesting an important pro-apoptotic role of LincRNA-Cox2 in OA chondrocytes.

Recently, competing endogenous RNA (ceRNA) hypothesis attains more and more attentions as an alternative function for lncRNAs [21]. As an novel regulatory mechanism, the crosstalk between LncRNAs and miRNAs has been identified in various diseases including OA [10]. Zhang et al found that LncRNA MALAT1 promotes osteoarthritis by competing with miR-150-5p [27]. We hypothesized that LincRNA-Cox2 may act as an ceRNA to sponge miRNAs through which promoted the OA development. Our current study predicted a direct binding site between LincRNA-Cox2 and miR-150, and confirmed this prediction by luciferase activity assay. Furthermore, we found that the expression of miR-150 was decreased after silence of LincRNA-Cox2, while LincRNA-Cox2 can also negatively regulate miR-150 expression. Also, an inverse correlation between LincRNA-Cox2 and miR-150 was observed in OA cartilage tissues. These findings illustrated that LincRNA-Cox2 exerted its functions on the proliferation and apoptosis of chondrocyte by sponging miR-150. However, such effect of LincRNA-Cox2 was reversed by miR-150 inhibitor. To the best of our knowledge, we firstly revealed LincRNA-Cox2/miR-150 axis mediated OA progression.

Previous study indicated that Wnt/β-catenin pathway play a pivotal role in regulation of inflammation processes in various mammalian non-neuronal cells [20], and was also involved in OA development [9]. Moreover, silencing IncRNA HOTAIR regulated synoviocyte proliferation and apoptosis in osteoarthritis through inhibiting Wnt/β-catenin signaling pathway [16]. In this study, we found that knockdown of LincRNA-Cox2 inhibited β-catenin nuclear accumulation as well as other related proteins expression, while these proteins levels were return to increase by miR-150 inhibitor. Our data
demonstrated LincRNA-Cox2 triggered the activity of wnt/β-catenin pathway by sponging miR-150 in chondrocytes, suggesting a new sight in OA progression.

Conclusion
In conclusion, LincRNA-Cox2 was up-regulated in OA cartilage tissues and IL-1β-induced chondrocytes. The overexpressed LincRNA-Cox2 reduced the viability, enhanced apoptosis and aggravated chondrocytes injury, suggesting it could act as a useful marker and potential therapeutic target in OA. We further identified LincRNA-Cox2 exerted its effects partially through the LincRNA-Cox2/miR-150/wnt/β-catenin axis. This finding improved understanding of mechanism involved in OA progression and provided novel targets for the molecular treatment for OA.

Declarations

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Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no conflicts of interest.

Ethics approval and consent to participate
The protocol of this research has been approved by the Ethics Committee of our hospital. All patients have signed written informed consent.

Availability of data and material
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions
Jianping Cao designed the study; Meng Jiang, Kai Xu, Huafeng Ren, Mingmin Wang, and Ximin Hou performed the research, analyzed data, and wrote the paper.

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High expression of LincRNA-Cox2 in cartilage tissues of OA and IL-1β-induced chondrocytes.

(A) The expression of LincRNA-Cox2 in cartilage tissues of OA (n=10 per group). (B) The expression of LincRNA-Cox2 in the chondrocytes with treatment of IL-1β at different concentration (0, 1, 5, 10 and 20 ng/mL). *P< 0.05.
Figure 2

Silencing LincRNA-Cox2 promotes proliferation and inhibits apoptosis in IL-1β-treated chondrocytes. (A) The expression of LincRNA-Cox2 was detected in chondrocytes transfected with si-Cox2 or si-NC after treatment of IL-1β. (B) Chondrocytes proliferation detected by MTT assay. & Representative of comparison with Con, \( P < 0.05 \). # Representative of comparison with IL-1β+si-NC, \( P < 0.05 \). (C) The protein levels of Ki67 and PCNA in chondrocytes. (D) The apoptotic rate of chondrocytes with si-Cox2 or si-NC after treatment of IL-1β. (E) The protein levels of Bax, c-Caspase-3 and c-Caspase-9 in chondrocytes. *\( P < 0.05 \).
LincRNA-Cox2 directly targeted miR-150. (A) The predicted binding sites of LincRNA-Cox2 and miR-150. (B) Luciferase reporter assay of chondrocytes co-transfected with mimics NC, mimics miR-150, inhibitor NC or inhibitor miR-150 and luciferase reporters containing wt-LincRNA-Cox2 or mut-LincRNA-Cox2 transcript. (C) The expression of miR-150 in chondrocytes after silencing LincRNA-Cox2. (D) The expression of LincRNA-Cox2 in chondrocytes with mimics miR-150 or inhibitor miR-150. (E) The expression of miR-150 in cartilage tissues of OA (n=10 per group). (F) A negative correlation between LincRNA-Cox2 and miR-150 in cartilage tissues of OA (n=10 per group). *P< 0.05.
miR-150 inhibitor reverses the effect of LincRNA-Cox2 knockdown on IL-1β-induced injury in chondrocytes. (A) Chondrocytes proliferation with si-Cox2 or miR150 inhibitor after treatment of IL-1β. & Representative of comparison with IL-1β+si-NC+inhibitor NC, P< 0.05. # Representative of comparison with IL-1β+si-Cox2+inhibitor NC, P< 0.05. (B) Ki67 and PCNA levels in chondrocytes. (C) The apoptotic rate of chondrocytes after transfection and IL-1β stimulation. (D) The protein levels of Bax, c-Caspase-3 and c-Caspase-9 in chondrocytes. *P< 0.05.
Figure 5

The effect of LincRNA-Cox2/miR-150 axis on wnt/β-catenin pathway. The proteins expression of p-GSK-3β, GSK-3β, p-β-catenin, β-catenin, c-myc and cyclin D1 in chondrocytes after transfected with si-Cox2 or miR150 inhibitor. *P< 0.05.