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The role of lncRNA XIST/miR-211 axis in modulating the proliferation and apoptosis of osteoarthritis chondrocytes through CXCR4 and MAPK signaling

Lei Li, Guohua Lv, Bing Wang, Lei Kuang*

Department of Spine Surgery, The Second Xiangya Hospital of Central South University, Changsha, 410011, China

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ABSTRACT

Long noncoding RNAs (lncRNAs) participate in multiple diverse diseases, including osteoarthritis (OA). Here, we explored the role of lncRNA XIST in OA and identified the potential molecular mechanisms. The expression of XIST in cartilage samples in patients with OA was significantly upregulated. XIST knockdown remarkably suppressed IL-1β-suppressed OA chondrocyte proliferation while promoted IL-1β-induced cell apoptosis. By employing online tools, miRNAs related to CXCR4, a major contributor to chondrocyte apoptosis, and XIST were selected. miR-211 expression could be significantly inhibited by IL-1β stimulation, and miR-211 negatively regulated XIST expression and CXCR4 protein levels. Through direct binding, XIST served as a ceRNA for miR-211 to counteract miR-211-mediated CXCR4 repression, thereby modulating chondrocyte proliferation and apoptosis through downstream MAPK signaling. In OA tissues, miR-211 expression was significantly downregulated while CXCR4 mRNA expression was upregulated. miR-211 was negatively correlated with XIST and CXCR4, respectively, while XIST and CXCR4 was positively correlated in tissue samples. In conclusion, the study revealed that lncRNA XIST can promote the proliferation of OA chondrocytes and promote apoptosis through the miR-211/CXCR4 axis. Thus, lncRNA XIST might be considered as a potential therapeutic target for OA treatment.

1. Introduction

Osteoarthritis (OA), a degenerative joint disease which is characterized by progressive degenerative alterations in the articular cartilage and other joint tissues [1,2], chondrocyte hypertrophy and apoptosis [3,4] is reported to be the fourth leading cause of disability [5,6]. The pathology of OA can be controlled through the processing of both genetic and environmental information [2].

Chondrocyte apoptosis has been detected in OA cartilage participating in the initiation and development of OA [7,8]. Enhanced chondrocyte apoptosis has been reported to be significantly correlated with the severity of OA both *in vitro* and *in vivo* in studies of animals [9] and human [10,11]. Chondrocyte apoptosis can be modulated by several factors, including C-X-C chemokine receptor-4 (CXCR4), a specific receptor of chemokine stromal cell-derived factor-1 (SDF-1, also known as C-X-C motif chemokine ligand 12, CXCL12). The CXCL12/CXCR4 axis plays a pivotal role in the injury and repair of cartilage by acting as a chemoattractant of cells involved in inflammation and stem cell migration [12–15]. CXCL12/CXCR4-mediated aggrecanase upregulation occurs due to activation of the MAPK, NF-κB, and Wnt/β-catenin pathways during post-traumatic osteoarthritis [16]. Understanding the mechanism by which CXCR4 modulating OA chondrocyte apoptosis may be of great clinical value for OA treatment.

During the past decades, abrogation of epigenetic regulation is evident in OA. Epigenetics enables tight control at the transcriptional level via gene expression resulting in changes to chromatin 3D structure, and the translational level (microRNAs (miRNAs), long non-coding RNAs (lncRNAs), mRNA editing and mRNA stability) affecting protein expression [17]. The deregulation and dysfunction of mRNAs [18–20], lncRNAs [21,22] and miRNAs [23–25] in OA have been reported. Previously, Fu et al. [21] demonstrated a total of 3007 upregulated lncRNAs and 1707 downregulated lncRNAs in OA cartilage compared with normal

* Corresponding author.
E-mail address: lei.kuang@csu.edu.cn (L. Kuang).

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samples (Fold change >/ = 2.0); of these differentially-expressed lncRNAs, XIST was the top 1 upregulated in OA cartilage knee tissues [21]. Although XIST has been regarded as an oncogene in many cancers, its role in OA has not been identified.

To investigate the detailed functions and mechanism, differentially-expressed miRNAs related to CXCR4 and XIST were selected and validated for their function on chondrocyte proliferation and apoptosis, as well as their interactions. The involvement of downstream MAPK signaling was examined. Taken together, we provided a novel mechanism by which chondrocyte apoptosis can be regulated in OA pathological progression from the perspective of lncRNA-miRNA-mRNA regulation.

2. Materials and methods

2.1. Tissue specimens

Eight normal cartilage tissues were obtained from non-OA traumatic amputees and twenty-two cartilage tissues were obtained from OA patients who had undergone a total knee replacement under the approval of the institutional review board and ethics committee of The Second Xiangya Hospital, Central South University according to the approved guidelines. Written informed consents were obtained from all subjects. The cartilage tissues were assessed following the methods described in previous studies [26–28].

2.2. Primary OA chondrocytes isolation, identification, culture and transfection

Primary OA chondrocytes were isolated, identified and cultured following the methods described in previous studies [26–28].

XIST expression was achieved by transfection of si-XIST (GeneCopoecia, Guangzhou, China). The expression of miR-211 was achieved by transfection of miRNA mimics or miRNA inhibitor (Genepharma, Shanghai, China) with the help of Lipofectamine 2000 (Invitrogen).

2.3. Hematoxylin and eosin (H&E)/Alcian-Blue staining

Normal and OA cartilage tissues were fixed in 10% zinc-buffered formalin overnight and then processed by paraffin embedding and sectioning. Sections of 4 μm were deparaffinized, rehydrated, and then stained using H&E/Alcian-Blue staining kit (Beyotime, Shanghai, China) following the protocols.

2.4. Real-time PCR

Total RNA was extracted from targeted tissues or cells using Trizol reagent (Invitrogen, CA, USA) and then treated with DNase I (Invitrogen, USA) according to the manufacturer's instructions. Synthesis of the first strand (cDNA) was performed using oligo (dT) 20 and Superscript II reverse transcriptase (Invitrogen, USA). The expression of mRNA was detected by SYBR green PCR Master Mix (Qiagen) using GAPDH as an endogenous control. The expression of miRNA was examined by a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China) using RNU6B as an endogenous control. The data were processed using a $2^{-\Delta\Delta CT}$ method.

2.5. MTT assay

MTT assay was performed to evaluate cell viability. 24 h after seeding into 96-well plates (5×10^3 cells/well), cells were transfected and/or treated as described. 48 h after transfection, 20 μl MTT (at a concentration of 5 mg/ml; Sigma-Aldrich) was added, and the cells were incubated for an additional 4 h in a humidified incubator. 200 μl DMSO was added after the supernatant discarded to dissolve the formazan. $OD_{490\ nm}$ value was measured. The viability of the non-treated cells (control) was defined as 100%, and the viability of cells from all other groups was calculated separately from that of the control group.

2.6. BrdU incorporation assay

By measuring 5-Bromo-2-deoxyUridine (BrdU) incorporation, the DNA synthesis in proliferating cells was determined. BrdU assays were conducted at 24 h and 48 h after transfection. Cells were seeded in 96-well culture plates at a density of 2×10^3 cells/well, cultured for 24 h or 48 h, then incubated with a final concentration of 10 μM BrdU (BD Pharmingen, San Diego, CA, USA) for 2 h. When the incubation period ended, the medium was removed, the cells were fixed for 30 min at RT, incubated with peroxidase-coupled *anti*-BrdU-antibody (Sigma-Aldrich) for 60 min at RT, washed three times with PBS, incubated with peroxidase substrate (tetramethylbenzidine) for 30 min, and the 450 nm absorbance values were measured for each well. Background BrdU immunofluorescence was determined in cells not exposed to BrdU but stained with the BrdU antibody.

2.7. Flow cytometer assay

Quantification of apoptotic cells was performed with Annexin V-FITC apoptosis detection kit (Keygen, China). Briefly, the cell samples were harvested with 0.25% trypsin without EDTA 48 h after transfection and then washed twice with ice-cold PBS and resuspended in 500 μl binding buffer. Then cells were incubated with 5 μl Annexin V-FITC specific antibodies and 5 μl propidium iodide (PI) then incubated for 15–20 min in dark and detected by BD Accuri C6 flow cytometer (BD, USA) with the excitation wavelength of Ex = 488 nm and emission wavelength of Em = 530 nm. Each experiment was repeated three times in triplicate.

2.8. Immunoblotting

The protein levels of CXCR4, p-MARK, MARK, *p*-JNK, JNK, p-*c*-Jun and c-Jun were examined by immunoblotting. Target cells in lysed using RIPA buffer with 1% PMSF; the proteins were extracted and analyzed for protein concentration using the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, China). Proteins were then loaded onto an SDS-PAGE minigel and transferred onto PVDF membrane. Thereafter, the membrane was probed with the antibodies listed below: *anti*-CXCR4 (ab124824, Abcam, Cambridge, CA, USA), *p*-MAPK (p-38, ab197348, Abcam), *anti*-MAPK (p38, ab4822, Abcam), *anti-p*-JNK (phospho T183 + T183 + T221, ab124956, Abcam), *anti*-JNK (ab179461, Abcam), *anti*-p-*c*-Jun (ab30620, Abcam) and *anti-c*-Jun (ab31419, Abcam) and *anti*-GAPDH (ab8245, Abcam) at 4 °C overnight. Thereafter, the blots were incubated with HRP-conjugated secondary antibody (1:5000). Signals visualization was conducted by ECL Substrates (Millipore, MA, USA) using GAPDH as an endogenous protein for normalization. The gray intensity analysis was performed using ImageJ software (NIH).

2.9. Luciferase reporter assay

To validate the predicted binding of miR-211 to XIST and CXCR4, respectively, the fragment of CXCR4 3′-UTR or XIST was cloned to the downstream of the Renilla psiCHECK2 vector (Promega, Madison, WI, USA), named wt-CXCR4 3′-UTR or wt-XIST. To generate the

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