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The LncRNA ZBED3-AS1 induces chondrogenesis of human synovial fluid mesenchymal stem cells

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ABSTRACT

Human synovial fluid-derived mesenchymal stem cells (SFMSCs) have great potential for cartilage induction and are promising for cell-based strategies for articular cartilage repair. Many long non-coding RNAs (lncRNAs) regulate chondrogenesis of MSCs. We hypothesized that the divergent lncRNA ZBED3-AS1, which binds locally to chromatin, could promote the expression of zbed3, a novel Axin-interacting protein that activates Wnt/β-catenin signaling, involved in chondrogenesis. However, the function of ZBED3-AS1 in SFMSCs is unclear. In this study, the expression, biological function, and roles of ZBED3-AS1 in SFMSC chondrogenesis were examined by multilineage differentiation, flow cytometry, and gain-of-function studies. We found that ZBED3-AS1 promotes chondrogenesis. Furthermore, ZBED3-AS1 could directly increase zbed3 expression. Finally, the wnt-inhibitor DKK1 could reverse the stimulatory effect of ZBED3-AS1 on chondrogenesis. These findings demonstrate the role of a new lncRNA, ZBED3-AS1, in SFMSC chondrogenesis and may improve osteoarthritis treatment.

1. Introduction

Osteoarthritis is a degenerative joint disease accompanied by progressive reductions in the extracellular matrix of joint cartilage and bone, eventually leading to joint dysfunction [1]. Stress-bearing joints, such as the knee, hip, and temporomandibular joint (TMJ), are commonly affected. Cartilage tissues have a limited capacity for self-repair and remodeling. Current treatment strategies are generally aimed at relieving symptoms, instead of curing the disease. However, one potential treatment strategy for articular cartilage repair is cell-based therapy. Human mesenchymal stem cells (hMSCs), which have the capacity for self-renewal and multipotential differentiation, especially chondrogenic differentiation, are promising for cell-based strategies for articular cartilage repair [2,3].

MSCs can be obtained from a variety of tissues, including the bone marrow, adipose tissue, synovium, and synovial fluid. They are particularly easy to obtain from TMJ synovial fluid [4–7]. Human synovial fluid-derived mesenchymal stem cells (SFMSCs) have greater potential for cartilage induction than other tissues [8]. Accordingly, SFMSCs derived from patients with TMJ disorders could be used for therapeutic approaches.

Long non-coding RNAs (lncRNAs) are a large class of non-protein-coding transcripts of > 200 bases involved in numerous physiological and pathological processes [9]. A number of lncRNAs regulate stem cell chondrogenesis, including ZBED3-AS1 [10], H19 [11], HOTAIR [12], and HOTTIP [13]. ZBED3-AS1 is one of the most highly expressed lncRNAs in the chondrogenic differentiation of human bone marrow MSCs [14]. Based on genomic and functional analyses of pluripotent cells, Luo et al. [15] suggest that a major class of lncRNAs arranged divergently to nearby genes play pivotal roles in transcriptional regulation to fine-tune gene expression and lineage differentiation. We hypothesize that as a divergent lncRNA, ZBED3-AS1, which binds locally to zbed3, promotes zbed3 transcription in cis. There is convincing evidence that Zbed3, a novel Axin-interacting protein, activates Wnt/β-catenin signaling [16–18], which promotes chondrogenesis [19,20]. Accordingly, ZBED3-AS1 could promote chondrogenesis of SFMSCs.

We examined the ZBED3-AS1 and zbed3 expression in induced SFMSCs, overexpressed ZBED3-AS1 to detect its function in chondrogenesis, and examined its relationship with zbed3 and Wnt signaling. These analyses clarified the roles of ZBED3-AS1 in SFMSC chondrogenesis.

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2. Materials and methods

2.1. SFMSC culture

This study was performed in accordance with the relevant polices of the Institutional Review Board of Guanghua School of Stomatology, Sun Yat-sen University, and complies with the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects. Synovial fluid samples were collected from ten TMD patients (nine women and one man, ranging in age from 20 to 35 years) during arthrocentesis of the TMJ cavity. Cells of individual samples were seeded on 6-cm culture dishes with 3 mL of complete culture medium (10% fetal bovine serum (FBS) + αMEM) and incubated at 37 °C in 5% CO_2. After 72 h, the medium was withdrawn to remove non-adherent cells and fresh medium was added. Adherent cells were expanded in a monolayer culture. The medium was refreshed every 3 days; after reaching confluence, SFMSCs were trypsinized and plated at 1000/cm^2 in 10-cm dishes. SFMSC experiments were carried out at passages 4 to 6. One volunteer with a mandibular tumor was subjected to mandibular resection, and the normal condylar process was removed for research.

2.2. Multilineage differentiation of SFMSCs

2.2.1. Osteogenic differentiation

Cells were plated in 6-well plates and incubated in complete culture medium at 37 °C in 5% CO_2. When cells reached ~ 80% confluence, the medium was replaced with osteogenic induction medium consisting of α-MEM (Gibco, Waltham, MA, USA) with 10% FBS (Gibco), 10 mM sodium-glycerophosphate (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 100 nM dexamethasone (MP Biomedicals, Santa Ana, CA, USA), and 50 mM ascorbic acid-2-phosphate (Wako, Osaka, Japan). The medium was replaced every 3 days for 14 days. Osteogenesis was assessed by Alizarin red staining. Cells were examined under an inverted phase contrast microscope (Axiovert 40; Zeiss, Oberkochen, Germany).

2.2.2. Adipogenic differentiation

Cells were plated in 6-well plates and cultured in complete culture medium. After cells reached ~80% confluence, the medium was replaced with adipogenic induction medium that consisted of α-MEM (Gibco) containing 10% FBS (Gibco), 200 mM indomethacin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM isobutyl methylxanthine (MP Biomedicals), 1 mM dexamethasone (MP Biomedicals), and 10 mg/mL insulin (MP Biomedicals). The medium was replaced every 3 days for 14 days. After 14 days, adipogenic induction was assessed by Oil Red O staining and visualization under an inverted phase contrast microscope (Axiovert 40; Zeiss).

2.2.3. Chondrogenic differentiation

Approximately 3×10^5 cells were transferred to a 15-mL centrifuge tube and centrifuged at 450×*g* for 10 min. Then, 450 μL of chondrocyte differentiation induction medium consisting of α-MEM (Gibco), 1 × ITS-A (Gibco), 100 nM dexamethasone (MP Biomedicals), 50 mM ascorbic acid (Sigma-Aldrich), 40 mg/mL proline (Sigma-Aldrich), and 10 ng/mL transforming growth factor (TGF)-β1 (Life Technologies, Carlsbad, CA, USA) was added. The medium was added with or without 250 ng/mL wnt3a (R&D Systems, Minneapolis, MN, USA) or 200 ng/mL rhDKK1 (R&D Systems) and was refreshed every 3 days. The cell mass formed a pellet automatically. Experiments were carried out using the pellets. Chondrogenic differentiation was assessed by immunochemical staining for col II, Safranin O, and Alcian blue as well as an sGAG analysis.

2.3. Flow cytometric analysis of SFMSCs

Surface markers, including CD90 (1:20; cat. no. 562245; BD Biosciences, San Jose, CA, USA), CD105 (1:11; cat. no. 130-094-941; Miltenyi Biotec, Bergisch Gladbach, Germany), CD73 (1:20), CD44 (1:20), and CD45/CD34/CD11b/CD19/HLA-DR (1:20) (cat. no. 562245; all from BD Biosciences), were detected using the FC500 flow cytometer and MXP software and data were analyzed using CXP software (both from Beckman Coulter, Brea, CA, USA).

2.4. Western blotting

Proteins were isolated from SFMSCs and pellets were ground using liquid nitrogen. Equal amounts of protein were extracted in RIPA Lysis Buffer (Thermo Scientific, Waltham, MA, USA) with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Danvers, MA, USA). Proteins were separated on a Protein Gel (1.0 mm), transferred to a PVDC membrane (Bio-Rad, Hercules, CA, USA), blocked with 5% BSA, and detected with anti-sox9 (1:1000; Cell Signaling Technology), anti-col II (1:1000; Abcam, Cambridge, UK), anti-zbed3 (1:1000; Abcam), anti-β-catenin (1:1000; Cell Signaling Technology), anti-GSK-3beta (1:1000; Cell Signaling Technology), and anti-axin1 (1:1000, Cell Signaling Technology) antibodies. Images were developed with ECL using an HRP-secondary goat anti-rabbit IgG antibody.

2.5. Immunofluorescent staining for zbed3 and β-catenin

Cells were plated in 35-mm glass bottom dishes and incubated in complete culture medium or chondrogenic medium for 2 weeks. The cells were fixed in 4% paraformaldehyde for 30 min before treatment with 0.3%-Triton-X100 for 15 min. Cells were blocked for 2 h with 5% bovine serum albumin (BSA). Cells were washed with 1 × PBS and stained with rabbit anti-human zbed3 (Abcam) or β-catenin (CST) diluted 1:200 for 18 h at 4 °C. After they were washed with 1 × PBS, the cells were incubated with the secondary antibody, DyLight 488-TFP ester-conjugated goat anti-rabbit IgG antibody (EarthOx, Millbrae, CA, USA) diluted 1:100 in 1% BSA for 60 min at 37 °C. After washing with 1 × PBS, the cells were incubated with 1 mg/mL DAPI (Cell Signaling Technology) for 5 min. The cells were then washed before viewing under a confocal microscope (Zeiss).

2.6. Immunochemical staining for zbed3 and col II

The condylar process was decalcified for 7 days, and the condylar process and pellet were then fixed with 4% formalin for 24 h and placed in embedding cassettes. Samples were embedded in paraffin blocks and cut at 5 mm. Sections were deparaffinized, and treated with 3% H_2O_2 for 10 min. Sections were incubated in 0.01 M citrate buffer for 20 min at 94–98 °C, cooled at room temperature for 30 min, immersed in blocking buffer (5% BSA in PBS), and incubated at 37 °C for 1 h. The tissue sections were then incubated in a solution of rabbit anti-human zbed3 (Abcam) diluted 1:100 and rabbit anti-human col II (Abcam) diluted 1:100 in blocking buffer for 16 h at 4 °C. After three 5-min washes in PBS, slides were incubated in a solution of biotinylated goat anti-rabbit IgG (Boster, Pleasanton, CA, USA) for 30 min at 37 °C. Slides were washed in PBS and streptavidin-biotin complex reagent (Boster) was applied for 30 min at 37 °C. After three 3-min washes in PBS, 3,3′-diaminobenzidine (DAB; Boster) was applied for visualization. Slides were viewed under a light microscope (Axioskop 40; Zeiss).

2.7. Gene expression analysis by RT-PCR

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA,

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