Knockdown of microRNA-203 alleviates LPS-induced injury by targeting MCL-1 in C28/I2 chondrocytes

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\begin{abstract}
Several microRNAs (miRs) are associated with osteoarthritis (OA) and are also functionally implicated in the pathogenesis of the disease. This study was aimed to investigate the potential roles of miR-203 in the development and progression of OA, as well as to illustrate the possible molecular mechanism. OA was simulated in human cartilage C28/I2 cells with treatments of lipopolysaccharide (LPS). LPS-induced cell injury was evaluated according to cell viability, apoptosis and release of pro-inflammatory cytokines. Expression of miR-203 after LPS treatment was assessed. Then, miR-203 was aberrantly expressed, followed by evaluation of LPS-induced cell injury. The target gene of miR-203 was virtually screened by bioinformatics method and verified by luciferase assay. Moreover, the potential associated signaling pathways were also investigated. Results showed that LPS induced decrease of cell viability and increases of cell apoptosis, release of inflammatory cytokines and expression of miR-203. LPS-induced alterations were aggravated by miR-203 overexpression but were alleviated by miR-203 inhibition. Myeloid cell leukemia-1 (MCL-1) was hypothesized and subsequently verified to be a target of miR-203, and miR-203 inhibition affected C28/I2 cells through up-regulating MCL-1. In addition, LPS-induced down-regulations of key kinases as well as phosphorylated kinases were further down-regulated by miR-203 overexpression but were abrogated by miR-203 inhibition. In conclusion, this study suggests that miR-203 suppression may inhibit the progression of OA by targeting MCL-1 and activating the Wnt/β-Catenin and JAK/STAT signal pathways.
\end{abstract}

\section{1. Introduction}
Osteoarthritis (OA), one of the most common degenerative joint diseases, is characterized by degradation of articular cartilage and joint inflammation [1]. Imbalance between repair and degradation of articular cartilage is the most important underlying cause of OA [2]. The risk of OA increases with the age of patient at the time of injury and with time from onset of injury. Obesity, joint mal-alignment and genetic risk factors are additional risk factors which usually lead to more severe outcomes. Approximately 60–80\% of patients with magnetic resonance imaging or arthroscopically documented cartilage injury were found to have cartilage degeneration within 5 years [3,4]. Patients with anterior cruciate ligament (ACL)-deficient knees, with or without a concomitant meniscus injury, are at high risk for post-traumatic OA [5,6]. OA is characterized by inflammation, which is presented as up-regulated levels of pro-inflammatory cytokines in the synovial fluid (SF) suggesting synovitis in the early stages of OA [1].

MicroRNAs (miRNAs) are known to be a group of small non-coding RNAs which bind to target mRNAs and interfere with the translation process of mRNA [7]. miRNAs have diverse functions including regulation of cellular differentiation, proliferation and apoptosis, tumorigenesis, and metastasis [8,9]. Diverse miRNAs are known to show tissue- or developmental stage specific expression pattern, associated with diseases such as cancer, heart disease, diabetes and rheumatoid arthritis [10–14]. Recent studies have shown that miRNAs play an important role in chondrogenesis and OA [15].

Lipopolysaccharide (LPS) is an endotoxin, expressed at the cytoplasmic membrane of Gram-negative bacteria [16]. LPS is considered as a key pro-inflammatory factor that plays a crucial role in the pathogenesis of OA [17]. Currently, mounting literatures have used LPS to simulate the inflammatory response of OA in vitro [18,19]. In our study, LPS was used for the construction of in vitro cell model, mimicking the inflammatory process of OA. The aim of this study was to investigate the potential roles of miRNA-203 (miR-203) in the development and progression of OA, as well as to illustrate the possible molecular mechanism.

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2. Materials and methods

2.1. Cell culture and treatment

Human cartilage C28/I2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in complete RPMI-1640 ( Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) in a humidified incubator containing 5% CO\textsubscript{2} at 37 °C. Cells at fifth to tenth passages after thawing were used for subsequent experiments. For LPS treatments, cells were plated in 12-well plates (5 × 10\textsuperscript{5} cells/cm\textsuperscript{2}) and were cultured at 37 °C for 24 h. Then, cells were exposed to 0, 1, 5 and 10 μg/ml LPS (Sigma, St. Louis, MO, USA) for 5 h.

2.2. Cell Counting Kit-8 (CCK-8) assay

Cell viability of treated cells was measured by CCK-8 assay. In brief, cells were seeded in 96-well plates with 5 × 10\textsuperscript{3} cells/well and cultured at 37 °C. After diverse treatments, the CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was added to the culture medium, and the cultures were incubated for 1 h at 37 °C in humidified 95% air and 5% CO\textsubscript{2}. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.3. Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). Cells with a density of 1 × 10\textsuperscript{5} cells/well were seeded in 6 well-plates and cultured at 37 °C. After diverse treatments, cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in binding buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, Miami, FL, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The released inflammatory cytokines were quantified by ELISA. Cells were seeded onto 24-well plates and cultured at 37 °C. Culture supernatant was collected for ELISA following the protocols supplied by the manufacturer (R & D Systems, Abingdon, UK) to determine the concentrations of inflammatory cytokines.

2.5. Cell transfection

MiR-203 mimic, scramble miRNAs, miR-203 inhibitor, its negative control (NC) and myeloid cell leukemia-1 (MCL-1)-specific small interfering RNA (si-MCL-1) were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's instruction and measured with Flow cytometry (Beckman Coulter, Miami, FL, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

2.6. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA (500 ng) was quantified for synthesis of cDNA. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-203 and U6 (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-203 in cells. MultiScribe RT kit (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems) were used for the reverse transcrip-
sions of IL-1β, IL-6, IL-8 and TNF-α were all elevated markedly when cells were treated with 5 μg/ml LPS compared to control group (P < 0.01 or P < 0.001) (Figs. 1E-1I).

3.2. Effect of LPS stimulation on miR-203 expression

Human cartilage cell-lines upon treatment with LPS revealed miR-203 expression was up-regulated compared to control group (P < 0.05, Fig. 2A). MiR-203 was found to be significantly over-expressed (P < 0.001) in C28/12 cells transfected with miR-203-mimic compared with scramble group. Meantime, miR-203 was significantly down-regulated by transfection with miR-203 inhibitor in C28/12 cells compared with NC group (P < 0.01, Fig. 2B).

Fig. 1. Effects of LPS on cell-viability, apoptosis and levels of various pro-inflammatory cytokines. (A) Effect of LPS on cell-viability. (B-C) Effect of LPS on cell apoptosis. (D) Effect of LPS on expressions of apoptosis-associated proteins. (E) Effect of LPS on mRNA expressions of different pro-inflammatory cytokines. (F-I) Effects of LPS on protein expressions of different pro-inflammatory cytokines. Data represent the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. LPS, lipopolysaccharide; P-, pro; C-, cleaved; IL, interleukin; TNF-α, tumor necrosis factor-α.
Fig. 2. Effect of LPS stimulation on miR-203 expression. (A) Effect of LPS on miR-203 expression. (B) miR-203 expression in transfected cells. Data represent the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. LPS, lipopolysaccharide; miR-203, microRNA-203; NC, negative control of miR-203 inhibitor.

Fig. 3. Effects of aberrantly expressed miR-203 on cell-viability, apoptosis and pro-inflammatory cytokine levels. (A) Cell-viability. (B) Cell apoptosis. (C) Expression of apoptosis-associated proteins. (D) mRNA expressions of different pro-inflammatory cytokines. (E-H) Protein expressions of different pro-inflammatory cytokines. Data represent the mean ± SD of three independent experiments. *P < 0.05; LPS, lipopolysaccharide; miR-203, microRNA-203; NC, negative control of miR-203 inhibitor; P-, pro; C-, cleaved; IL, interleukin; TNF-α, tumor necrosis factor-α.
and protein expressions of these inflammatory factors were all decreased in cells transfected with miR-203 inhibitor compared with LPS+NC group (P < 0.05).

3.4. Effect of abnormally expressed miR-203 on MCL-1 expression

Cells transfected with miR-203 mimic significantly suppressed the mRNA expression of MCL-1 compared to Scramble group (P < 0.05). Cells transfected with miR-203 inhibitor revealed a significant increase in the expression of MCL-1 (P < 0.01) compared to the cells transfected with NC (Fig. 4A). Effects of abnormally expressed miR-203 on protein expression of MCL-1 were consistent with that on mRNA level of MCL-1 (Fig. 4B). Results in Fig. 4C revealed that luciferase activity was significantly decreased by co-transfection with MCL-1-WT and miR-203 mimic compared with co-transfection with MCL-1-WT and scramble miRNAs (P < 0.05), indicating that MCL-1 was a target of miR-203.

3.5. Effect of miR-203 inhibition on cell-viability, apoptosis and pro-inflammatory cytokines was abrogated by MCL-1 knockdown

Significant increase (P < 0.05) and significant decrease (P < 0.05) in cell-viability and apoptosis, respectively, were observed in LPS+ miR-203 inhibitor group compared to LPS+NC group (Figs. 5A-5B). However, the alterations of cell-viability and apoptosis, induced by miR-203 inhibition, were markedly reversed by MCL-1 knockdown compared with LPS+miR-203 inhibitor group (P < 0.01). As expected, ratios of Bax/Bcl-2, cleaved/pro caspase-3 and cleaved/pro caspase-9 were decreased by miR-203 inhibition but the decreases were reversed by MCL-1 knockdown (Fig. 5C). Again, mRNA and protein expressions of IL-1β, IL-6, IL-8 and TNF-α were all significantly reduced by miR-203 inhibition compared with LPS+NC group (P < 0.05 or P < 0.01), whereas these reductions were all abrogated by MCL-1 knockdown compared with LPS+miR-203 inhibitor group (P < 0.05 or P < 0.01, Figs. 5D-5H).

3.6. Potential signal pathways for miR-203

Western blot analysis of the proteins associated with the Wnt/β-Catenin and the JAK/STAT pathways revealed that expression levels of Wnt3a, Wnt5a and β-Catenin as well as phosphorylated levels of JAK1, STAT1 and STAT3 were all decreased by LPS treatments. Then, the LPS-induced decreases were further reduced by miR-203 overexpression but were reversed by miR-203 inhibition (Figs. 6A-6B). Results illustrated that the miR-203-associated modulation of LPS-induced alterations in C28/I2 cells are related to Wnt/β-Catenin and JAK/STAT pathways.

4. Discussion

OA is known to be a complex, multifactorial inflammatory disease of the bony joints of the body [22]. There are scientific reports which show that more than 25 miRNAs are associated with OA and are also functionally implicated in the pathogenesis of the disease [15]. Loss of miR-140 leads to development of age-related OA-like changes. MiR-140 is expressed in cartilage and regulates cartilage development and homeostasis [23]. MiR-21 has been reported to be up-regulated in OA patients, and over-expression of miR-21 attenuates the process of chondrogenesis [24]. Previous reports also suggested that miR-210 might be associated with OA [25,26]. Thus, we focused on the potential regulatory effects of miRNAs on OA in our study.

To simulate OA at cell levels, LPS was used to induce cell injury in C28/I2 cells. In this study, we observed that LPS treatment of the C28/I2 cells led to significant decrease in cell viability and significant increase in apoptosis in a dose-dependent manner. In addition, anti-apoptotic Bcl-2 was down-regulated while pro-apoptotic Bax was up-

### Figures

**Fig. 4.** MicroRNA (miR)–203 negatively regulated expression of myeloid cell leukemia-1 (MCL-1) and MCL-1 was a target of miR-203. (A) mRNA expression of MCL-1 in transfected cells. (B) Protein expression of MCL-1 in transfected cells. (C) Luciferase activity assay. Data represent the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01. NC, negative control of miR-203 inhibitor; MCL-1-WT, pMiR-report vector carrying the 3’UTR of wild-type MCL-1 containing the binding site of miR-203; MCL-1-Mut, mutant MCL-1-WT.
regulated after LPS treatments, resulting in obvious up-regulation of active caspase-9 and active caspase-3, which may facilitate degradation of cellular structural proteins. Thus, LPS affected C28/I2 cells through mitochondrial- and caspase-dependent pathways, providing a rational explanation for the altered cell viability and apoptosis. Also, mRNA and protein expression levels of the different inflammatory markers, namely IL-1β, IL-6, IL-8 and TNF-α, were all increased following treatment with LPS, indicating that inflammation was induced by LPS in C28/I2 cells.

MiR-203 has been reported to act as a tumor suppressor or oncogenic factor in diverse cancer types, along with dramatic effects on cell proliferation and apoptosis [27,28]. Another study also reported the regulatory effects of miR-203 on inflammatory response [29]. Therefore, we hypothesized that miR-203 might affect the progression of OA, which has not been investigated currently. Accordingly, we then identified LPS treatment of C28/I2 cells significantly increased miR-203 expression, which provides a support for the possible role of miR-203 in OA. Then, LPS-induced changes of C28/I2 cells were exacerbated by miR-203 overexpression but were alleviated by miR-203 inhibition, consolidating the functional roles of miR-203 in OA. The elevated release of pro-inflammatory cytokines by miR-203 overexpression was consistent with a literature described previously [30].

miRNAs are widely accepted to function through binding to the 3′UTR of target genes [31]. MCL-1 belongs to the prosurvival Bcl-2 subfamily that also includes Bcl-xl, Bcl-2, and A1 [32]. A study by Liu et al. showed that MCL-1, which was over-expressed in rheumatoid arthritis synovial fibroblasts, contributes to their resistance to apoptosis [33]. The study also suggested that MCL-1 might be a powerful therapeutic target to suppress the chronic inflammation observed in the rheumatoid arthritis joint. In continuation with the same context, in our study, we evaluated the effect of miR-203 on MCL-1 among numerous putative targets using bioinformatics method. We observed that MCL-1 was negatively correlated with miR-203 level. The luciferase assay verified that miR-203 could directly bind to the 3′UTR of MCL-1. Moreover, the effects of miR-203 inhibitor on LPS-treated C28/I2 cells were abrogated by MCL-1 knockdown, suggesting that miR-203 inhibitor affected C28/I2 cells through up-regulating MCL-1 expression. All the results described above illustrated that MCL-1 was a target of miR-203 in LPS-treated C28/I2 cells.

Wnt/β-Catenin signaling pathway is reported to participate in OA through triggering catabolism chondrocytes and activation of matrix metalloproteinase 13 (MMP-13) [34]. Overexpression of miR-1 was reported to control development of OA through Wnt/β-Catenin signaling pathway [35]. In addition, JAK/STAT is a signaling pathway that is closely associated with inflammatory response [36]. In order to uncover the underlying mechanisms about the miR-203-associated modulations, effects of aberrantly expressed miR-203 on expressions of key kinases in Wnt/β-Catenin signaling pathway and phosphorylated
levels of key kinases in JAK/STAT pathways were explored. Data in our study showed the inhibition of these two pathways, induced by LPS, was abrogated by miR-203 inhibition but was further inhibited by miR-203 overexpression, indicating the possible involvement of these two pathways in miR-203-associated modulation in LPS-treated C28/I2 cells.

Taken together, LPS induced decrease of cell-viability and increases of cell apoptosis, release of pro-inflammatory cytokines and miR-203 level. The LPS-induced alterations were alleviated by miR-203 inhibition through targeting MCL-1, involving in Wnt/β-Catenin and JAK/STAT signaling pathways. The study provides a potential therapeutic strategy for treatment of OA, which needs more verified experiments in animals for clinical application.

Authors’ contributions

Conceived and designed the experiments: C.F. Zhao and T. Yu; Performed the experiment and analyzed the data: C.F. Zhao, Y. Wang and H. Jin; Wrote the manuscript: C.F. Zhao, Y. Wang and H. Jin; Revised the manuscript: T. Yu.

Conflict of interest

Authors declare that there is no conflict of interest.

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References

[28] L. Tian, M. Li, J. Ge, Y. Guo, Y. Sun, M. Liu, H. Xiao, MiR-203 is downregulated in laryngeal squamous cell carcinoma and can suppress proliferation and induce

[29] Z. Yang, L. Zhong, S. Zhong, R. Xian, B. Yuan, miR-203 protects microglia
mediated brain injury by regulating inflammatory responses via feedback to MyD88

C.D. Buckley, P.P. Tak, S. Gay, D. Kyburz, Altered expression of microRNA-203 in
rheumatoid arthritis synovial fibroblasts and its role in fibroblast activation,
Arthritis Rheum. 63 (2011) 373–381.

MicroRNAs are involved in the regulation of ovary development in the pathogenic


[33] H. Liu, P. Eksarko, V. Temkin, G.K. Haines 3rd, H. Perlman, A.E. Koch,
B. Thimmapayya, R.M. Pope, Mcl-1 is essential for the survival of synovial
8337–8345.

[34] W. Bouaziz, J. Sigaux, D. Modrowski, C. Marty, S. Provot, H.K. Ea, M. Cohensolal,
E. Hay, OP0255 MMP13 is transcriptionally repressed by the HIF1α/β-Catenin
Interaction in Chondrocytes and Osteoarthritis in Mice, Ann. Rheum. Dis. 74 (168)

[35] D. Xing, B. Wang, Y. Xu, K. Tao, J. Lin, Overexpression of microRNA-1 controls the
development of osteoarthritis via targeting FZD7 of Wnt/β-catenin signaling,

[36] C.J. Malemud, Negative regulators of JAK/STAT signaling in rheumatoid arthritis