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Original Research Article

Long non-coding RNA reprogramming (LncRNA-ROR) attenuate inflammation induced by LPS through the regulation of miRNA-124-3p and inhibition of C5a and TLR4/Myd88/NF-κB pathways in C28/12 cells

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Abstract

Purpose: *Osteoarthritis (OA), inflammation of joint and articular cartilage, is a serious joint disorder and a major cause of pain and disability. LncRNAs exert their role in OA progression. The purpose of this present research is to investigate the impact and molecular mechanism of lncRNA regulator of reprogramming (LncRNA-ROR) on inflammatory damage induced by lipopolysaccharide (LPS) in C28/12 cells.*
Methods: *The expressions of lncRNA-ROR and miR-124-3p and the mRNA expressions of COX-2, iNOS, IL-8, C5a, and TNF-α were determined in C28/12 cells stimulated with LPS using RT-qPCR. Viability of C28/12 cells was evaluated using CCK-8 assay and C28/12 cell apoptosis was assessed by flow cytometry in LPS-induced C28/12 cells. Moreover, the concentration of IL-8, C5a, and TNF-α and the protein expressions of TLR4/Myd88/NF-κB pathways were examined using Elisa assay.*
Results: *Results revealed that LPS inhibited the proliferation, enhanced rate of cell apoptosis, and promoted mRNA expressions of iNOS, IL-8, C5a, and TNF-α in C28/12 cells. Besides, the expression of lncRNA-ROR was expressively reduced in C28/12 cells stimulated with LPS and the lncRNA-ROR overexpression markedly reduced the inflammatory injury stimulated by LPS in C28/12 cells. Besides, lncRNA-ROR significantly down-regulated the miR-124-3p expression and miR-124-3p exposure inhibited the protecting impact of lncRNA-ROR on LPS-induced C28/12 cells. Furthermore, lncRNA-ROR suppressed the TLR4/Myd88/NF-κB pathways via suppressing the expression of miR-124-3p in LPS-induced C28/12 cells.*
Conclusion: *The finding revealed that lncRNA-ROR protected against LPS-stimulated inflammatory injury in C28/12 cells by suppressing TLR4/Myd88/NF-κB pathways via inhibiting miR-124-3p suggested an efficient therapeutic strategy for OA treatment.*

Keywords: *lncRNA-ROR, miR-124-3p, Osteoarthritis, Inflammation, TLR4/Myd88/NF-κB signaling pathways*

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INTRODUCTION

Osteoarthritis (OA) is a prolonged progressive joint disorder, mainly described by the inflammation of synovial and articular cartilage degradation [1]. Biomechanical, biochemical, and genetic features are involved in the existence and growth of osteoarthritis [2]. It has been previously reported that OA is a main reason of disability in elder people and various symptoms are related with the disease progression such as limitation and swelling of joint motion, transient stiffness, and joint pain [3]. The latest strategies for osteoarthritis treatment focused on the recovery of joint function and control of symptom in the early stage of OA [4,5]. Evidence from the previous study showed that weight management, biomechanical interventions, power training, exercise, and corticosteroid injection were suitable treatments for osteoarthritis [6]. However, all these methods lack the ability to modify the pathogenesis of disease. Therefore, it is urgently needed to explore an efficient and innovative technique for OA treatment.

Long non-coding RNAs (lncRNAs) categorized by nucleotides (> 200), class of non-protein-coding RNAs, exert a vital function in many biological activities by modulating gene expression [7]. Previous researched elucidated that lncRNAs exerts a critical function in various physiological processes including invasion, differentiation, apoptosis, and proliferation [8,9]. Increasing evidences demonstrated that various lncRNAs are involved in progression of osteoarthritis [10,11]. Recently, approximtely 150-4000 types of lncRNAs have been proven to exert essential functions in autophagy, apoptosis, articular cartilage matrix, and neovascularization of cartilage cells [12]. A newly emerging lncRNA regulator of reprogramming (lncRNA-ROR) is found at chromosome 18q21.31, that exert its function in converting segregated cells to multi-potential cells [13,14]. Evidences have revealed that lncRNA-ROR increases the progression of numerous diseases including breast cancer and glioma [15, 16]. Another study demonstrated that lncRNA-ROR augmented myocardial ischemia/ reperfusion injury (MIRI) [17]. However, whether lncRNA-ROR exerts its function in the progression of osteoarthritis still needs to be elucidated.

In the current research, the anti-inflammatory impacts of lncRNA-ROR on chondrocyte C28/12 cells were investigated for the first time. An in vitro study of OA was developed by stimulating C28/12 cells with lipopolysaccharide (LPS) and the role of lncRNA-ROR on the LPS-induced inflammation was investigated in C28/12 cells. Also, the mpact of miR-124-3p on the functions of lncRNA-ROR was studied in LPS-treated C28/12 cells. Also, the effect and the molecular mechanism of lncRNA-ROR on TLR4/Myd88/NF-κB pathways was investigated in LPS-injured C28/12 cells. It was expected that the findings in this work might provide a new insight for osteoarthritis treatment.

METHODS

Cell Culture

C28/I2 Human chondrocyte cell line was purchased from Sigma Aldrich, Ontario, Canada and cultivated in a DMEM/F12 with 5% FBS in an incubator at 37^0C having CO_2 (5%). The cells were kept in a growth medium and the medium was substituted every three days till cells attained 80-90% confluence. The cells were used after 5 to 10 passages for the further experiments. All the cells were stimulated with different concentrations (0, 2.5, 5, 7.5, 10 µg/ml) of lipopolysaccharide (LPS) for 12 hr.

Cell transfection

The lncRNA-ROR having full length was cloned into pcDNA3.1 and called as pc-ROR. The pcDNA3.1 (Addgene, Teddington, UK) acted as a control. Moreover, miR-124-3p mimic, miR-124 inhibitor, and their consistent NCs were produced by the Life Technologies, NJ, USA. Lipofectamine 2000 (Thermo Fisher, NJ, USA) was used to transfect all the vectors into C28/I2 cells following manufacturer's guidelines. The cells after transfection were gathered at 72 hrs post-transfection for further experiments. All the steps were repeated in triplicate.

CCK-8 assay

96-well plates were used to seed C28/I2 cells at a density 5×10^3 cells/well followed by the incubation in an incubator at 37^0C containing 5% CO_2. After stimulating with LPS at various concentrations (0, 2.5, 5, 7.5, 10 µg/ml) for 12 hrs, CCK-8 solution (10 µl; Abcam, MA, USA) was introduced into each well and cultured again for 1 hr in a 5% CO_2 environment at 37^0C. A MaxSignal microplate reader (Perkin Elmer, MA, USA) was used to detect the optical density at 460 nm. All the steps were repeated in triplicate.

Flow cytometry

Annexin V-FITC/PI apoptosis assay (BD Biosciences, CA, USA) was employed to measure the apoptosis rate following flow cytometry. After treatment with LPS, cells were

rinsed with PBS twice and stained in Annexin V-FITC (5 µl) and propidium iodide (PI; 5 µl; both purchased from Biovision, CA, USA) in the dark at 24^0C for 15 min. FACS (BD Biosciences, NJ, USA) was employed for the evaluation of cell apoptosis followed by the data analysis by using FlowJo software (BD Biosciences, NJ, USA). All the steps were repeated in triplicate.

ELISA assay

C28/12 cells were cultured in 24 well plates followed by the pre-treatment with LPS (5 µg/ml) at 37 ^{o}C for 12 hr. The culture supernatants were gathered and the concentrations of IL-8, C5a, and TNF-α and the protein expressions of MyD88, TLR4, NF-κB p65 were examined by ELISA kit (Abcam, MA, USA) following the manufacturer's guidelines. All the steps were recurrent in triplicate.

RT-qPCR

The total RNA was removed for the C28/12 cells by using Ribozol reagent (VWR, Alberta,Canada). The expression of lncRNA-ROR was detected by One Step SYBR PrimeScript PLUS RT-RNA PCR kit (TakaraBio, CA, USA). The cDNA was constructed using reverse transcription kit (QIAGEN, MD, USA) for the measurement of miR-124-3p followed by detecting miR-124-3p expression using TaqMan MicroRNA Assay containing TaqMan Universal Master Mix II (Biocompare, CA, USA). MultiscribeTM Reverse transcription Kit (Thermo Fisher, Paisley, UK) containing oligo (dT) were used to measure the mRNA expression of COX-2, iNOS, IL-8, C5a, and TNF-α. A classic $2^{-\Delta\Delta CT}$ method was employed for the data analysis. U6 and β-actin were taken as control for stabilizing the expressions.

Statistical analysis

All the results were expressed as the mean ± standard deviation (SD) using SPSS 18.0 (IBM, USA). For the numerical analysis, GraphPad Prism 7.0 (GraphPad Software, LJ, USA) was used. ANOVA (comparison among different groups) and Student's T-test (comparison between two groups) were used to find the P-value. The result was considered as significant when the value of $p < 0.05$. All the trials were done in triplicate.

RESULTS

LPS stimulated inflammation in C28/12 cells

The C28/12 cells Human chondrocyte cells were stimulated with different doses (0, 2.5, 5, 7.5, 10 µg/ml) of LPS in order to establish an inflammatory injury model of osteoarthritis in vitro. CCK-8 examined the change in cell viability in C28/12 cells and our results showed that with enhance in the concentration of LPS, the cell viability markedly reduced in C28/12 cells indicating that increase in the concentration of LPS inhibited the viability in C28/12 cells. (Figure 1A). Consequently, optimum concentration of LPS **(5** µg/ml) was used throughout our study. Then, the rate of cell apoptosis was determined in C28/12 cells using flow cytometry and our findings revealed that the rate of cell apoptosis was expressively enhanced in LPS-induced C28/12 cells compared to control group. (Figure 1B). Furthermore, the mRNA expressions of COX-2, iNOS, IL-8, C5a, and TNF-α were significantly enhanced in C28/12 cells induced with LPS compared to that of control group. (Figure 1C). Furthermore, results from ELISA assay indicated that the protein concentration of IL-8, C5a, and TNF-α were also promoted by the treatment of LPS in C28/12 cells. (Figure 1D). The findings elucidated that LPS could induce inflammatory injury in C28/12 cells.

LncRNA-ROR suppressed miR-124-3p expression in C28/12 cells

Further, we investigated, whether, lncRNA-ROR exert critical function in the modulation of LPS-stimulated inflammatory damage in C28/12 cells. The lncRNA-ROR expression was assessed by RT-qPCR and our data elucidated that the lncRNA-ROR expression considerably improved with rise in the concentration of LPS in C28/12 cells. Also, pc-ROR and pcDNA3.1 were transfected into C28/12 cells and our results demonstrated that lncRNA-ROR expression was markedly augmented in pc-ROR compared with pcDNA3.1 group. (Figure 2A-B). These findings indicated that the lncRNA-ROR expression was regulated by LPS. Further, we measured the C28/12 cell viability by using CCK-8 assay and our results revealed that the C28/12 cells viability was significantly enhanced by lncRNA-ROR overexpression. (Figure 2C). Moreover, the results of flow cytometry indicated that lncRNA-ROR overexpression markedly suppressed the rate of cell apoptosis in LPS-stimulated C28/12 cells. (Figure 2D). Also, the mRNA expressions of COX-2, iNOS, IL-8, C5a, and TNF-α were determined using RT-qPCR and our findings elucidated that overexpression of lncRNA-ROR expressively reduced the mRNA expression of these inflammatory mediators in LPS injured C28/12 cells. (Figure 2E). Furthermore, the stimulating impact of LPS on IL-8, TNF-α, and C5a levels were remarkably reserved by the

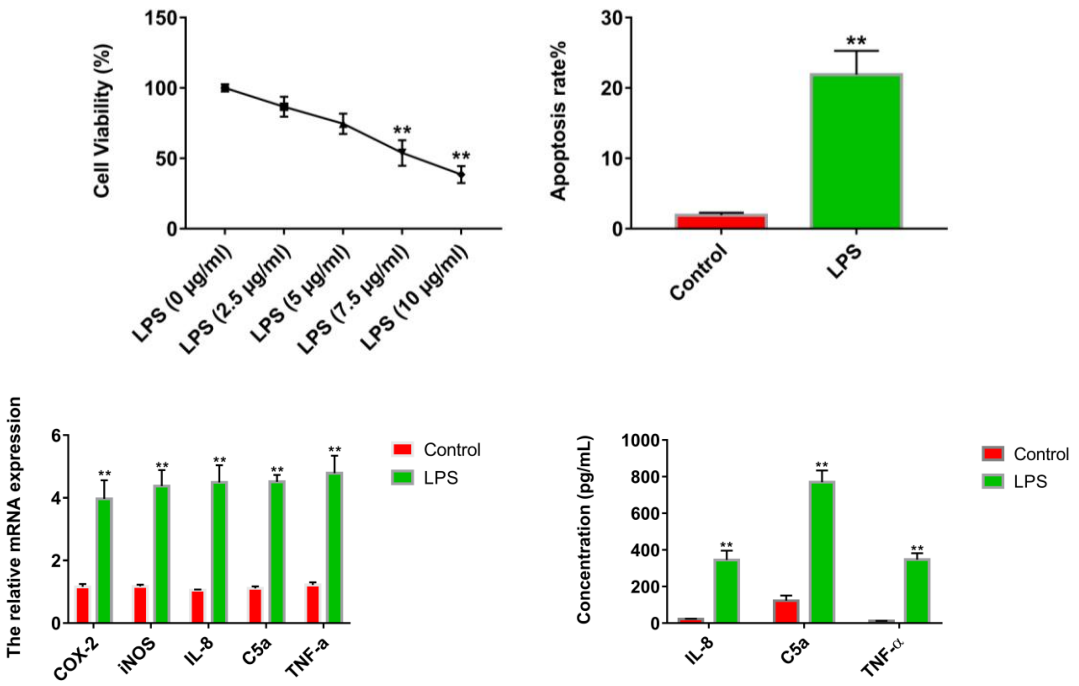

Figure 1: LPS stimulated inflammation in C28/12 cells: CCK-8 was employed to test the cell proliferation in C28/12 cells after LPS (5 μg/ml) stimulation. B) C28/12 cell apoptosis was detected using FCM apoptosis assay after LPS (5 μg/ml) stimulation. C) The mRNA level of COX-2, iNOS, IL-8, C5a, and TNF-α in C28/12 cells after LPS (5 μg/ml) stimulation were assessed by RT-qPCR. D) The protein level of IL-8, TNF-α and C5a were assessed using ELISA assay. **P < 0.05

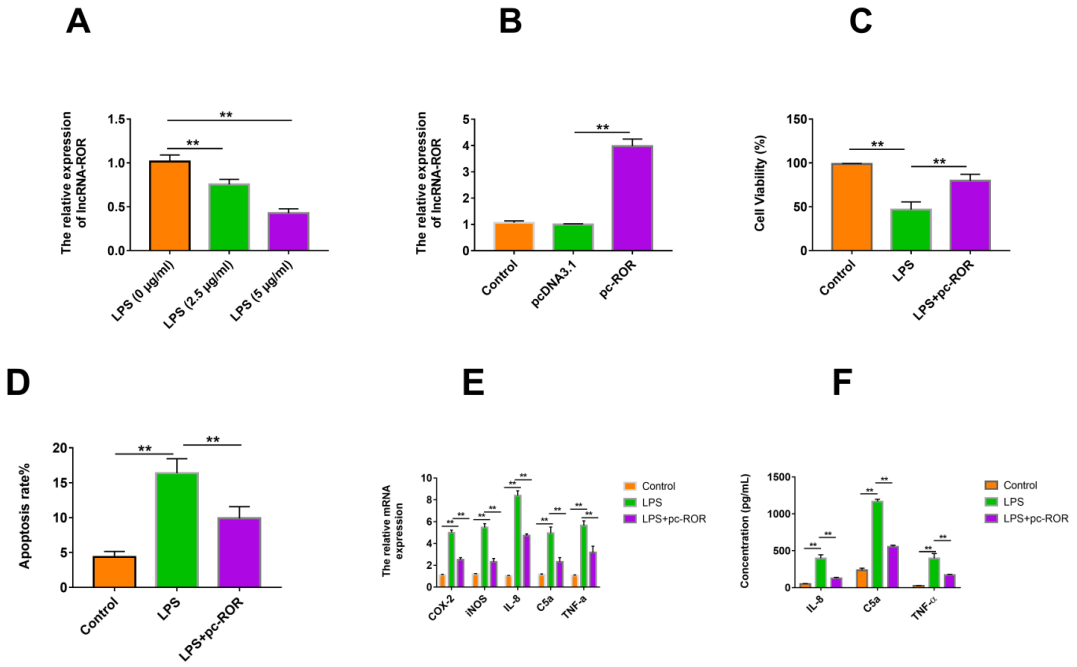

Figure 2: LncRNA-ROR suppressed miR-124-3p expression in C28/12 cells: A) The C28/12 cells were stimulated with disparate concentrations of LPS and lncRNA-ROR expression was examined by RT-qPCR. B) The transfection efficacy was detected by RT-qPCR after transfecting pc-ROR and pcDNA3.1 in C28/12 cells. C) CCK-8 was employed to test the C28/12 cell viability after transfecting pc-ROR and pcDNA3.1 in C28/12 cells. D) C28/12 cell apoptosis was examined using FCM apoptosis assay after transfecting pc-ROR and pcDNA3.1 in C28/12 cells. E) The mRNA level of iNOS, COX-2, IL-8, TNF-α, and C5a after transfecting pc-ROR and pcDNA3.1 in C28/12 cells were assessed using RT-qPCR. F) The protein level of IL-8, C5a, and TNF-α were assessed using ELISA assay. **P < 0.05

overexpression of lncRNA-ROR (Figure 2F). The findings elucidated that lncRNA can inhibit the LPS stimulated inflammatory injury in C28/12 cells.

MiR-124-3p mediated inflammatory injury stimulated by LPS in C28/12 cells

RT-qPCR was performed to measure miR-124-3p expression in LPS-treated C28/12 cells and our findings demonstrated that the expression of miR-124-3p was expressively reduced by the overexpression of lncRNA-ROR in C28/12 cells compared to control group. Also, the miR-124-3p was regulated in C28/12 cells by transfecting miR-124-3p mimic and our findings elucidated the miR-124-3p expression was considerably enhanced in miR-124-3p mimic transfected group compared to that of NC group. Moreover, with increase in the concentration of LPS, the miR-124-3p expression was markedly enhanced. (Figure 3A-C) Furthermore, the miR-124-3p overexpression noticeably decreased the viability and enhanced apoptosis rate in C28/12 cells treated with LPS. (Figure 3D-E) Also, exposure of miR-124-3p considerably enhanced the mRNA level of iNOS, COX-2, IL-8, TNF-α, and C5a in LPS-stimulated C28/12 cells. (Figure 3F) Besides, the miR-124-3p overexpression evidently enhanced the protein level of IL-8, TNF-α, and C5a in C28/12 cells treated with LPS, while, miR-124-3p inhibitor indicated the opposite results in Fig. 3B-G. (Figure 3G) Our work demonstrated that miR-124-3p was related with LPS-stimulated inflammatory damage in C28/12 cells.

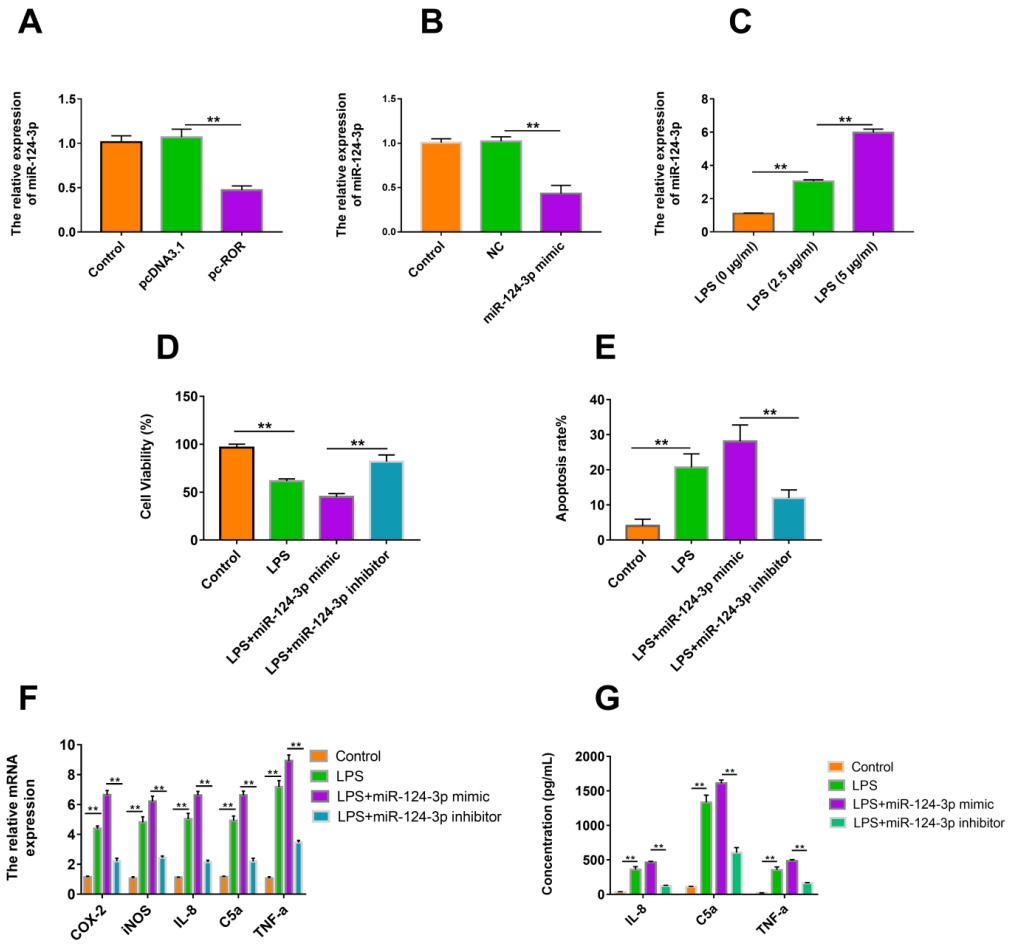

Figure 3: MiR-124-3p mediated inflammatory damage stimulated by LPS in C28/12 cells: A) The miR-124-3p expression was examined using RT-qPCR after transfecting pc-ROR and pcDNA3.1 in C28/12 cells. B) The miR-124-3p expression was examined by RT-qPCR after transfecting miR-124-3p mimic and NC in C28/12 cells. C) The C28/12 cells were stimulated with disparate concentrations of LPS and the expression of miR-124-3p was examined by RT-qPCR. D) CCK-8 was employed to test the cell viability after transfecting miR-124-3p mimic and miR-124-3p inhibitor in LPS-stimulated C28/12 cells. E) C28/12 cell apoptosis was examined using FCM apoptosis assay after transfecting miR-124-3p mimic and miR-124-3p inhibitor in LPS-stimulated C28/12 cells. F) The mRNA level of COX-2, iNOS, IL-8, C5a, and TNF-α after transfecting miR-124-3p mimic and miR-124-3p inhibitor in LPS-stimulated C28/12 cells was assessed using RT-qPCR. G) The protein level of IL-8, C5a, and TNF-α were assessed using ELISA assay. **P < 0.05

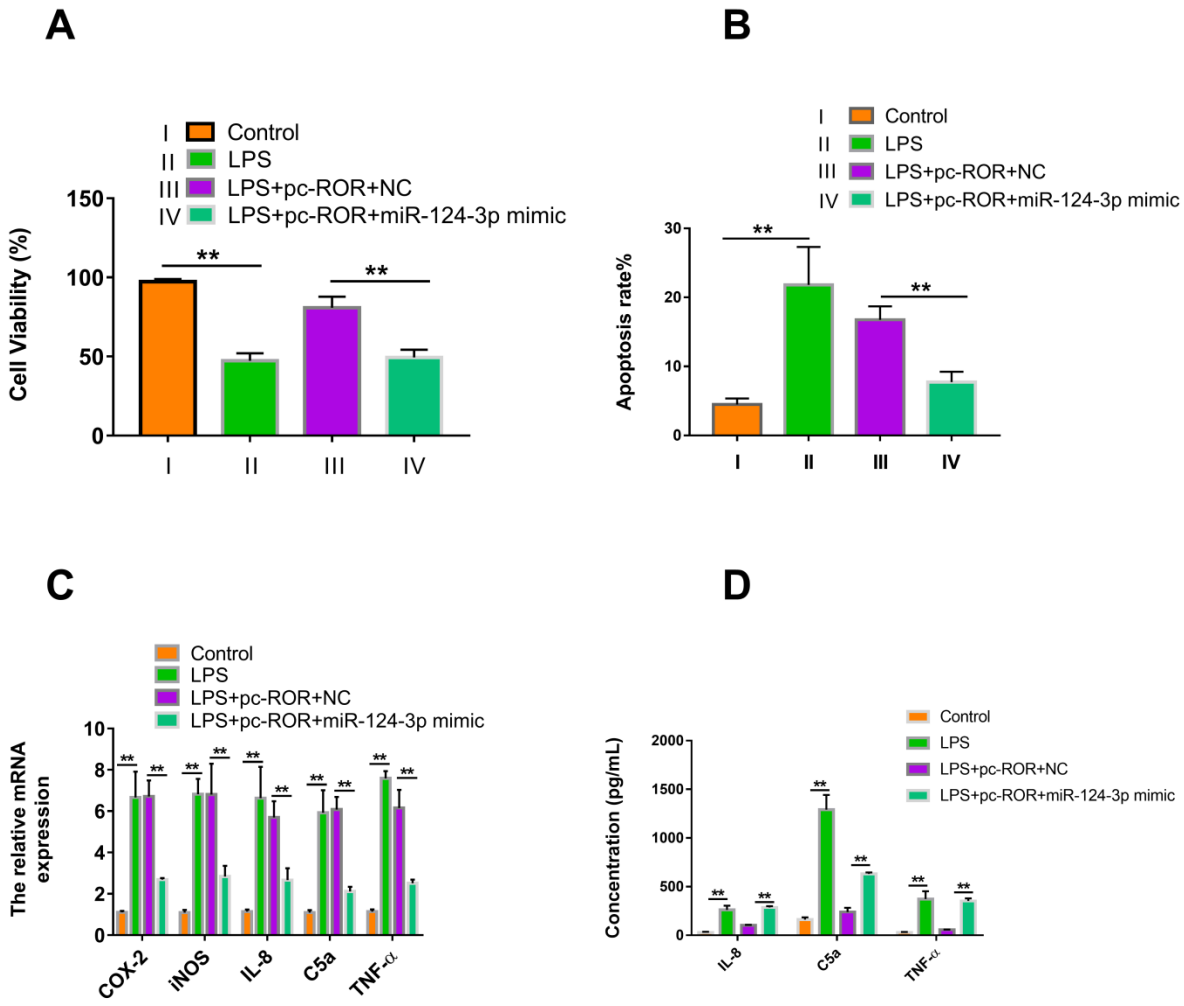

Figure 4: LncRNA-ROR inhibited the inflammation in C28/12 cells via reducing miR-124-3p expression: A) CCK-8 was employed to test the C28/12 cell viability after transfecting miR-124-3p mimic or pc-lncRNA-ROR in LPS-stimulated C28/12 cells. B) C28/12 cell apoptosis was examined using FCM apoptosis assay after transfecting miR-124-3p mimic or pc-lncRNA-ROR in LPS-stimulated C28/12 cells. C) The mRNA level of COX-2, iNOS, IL-8, C5a, and TNF-α after transfecting pc-lncRNA-ROR or miR-124-3p mimic was assessed by RT-qPCR. D) The protein level of IL-8, TNF-α and C5a were assessed using ELISA assay. **P < 0.05

LncRNA-ROR inhibited the inflammation in C28/12 cells via reducing miR-124-3p expression

The function of miR-124-3p on LPS-stimulated inflammatory injury was investigated by transfecting miR-124-3p mimic in C28/12 cells. Results elucidated that the stimulating impact of lncRNA-ROR on C28/12 cell viability was altered by the miR-124-3p overexpression. (Figure 4A) The suppressing impact of lncRNA-ROR overexpression on C28/12 cell apoptosis was reversed by the overexpression of miR-124-3p. (Figure 4B) Also, miR-124-3p exposure enhanced the mRNA expression of COX-2, iNOS, IL-8, C5a, and TNF-α after stimulation with lncRNA-ROR and LPS in C28/12 cells. (Figure 4C) Besides, our work revealed that miR-124-3p overexpression altered the suppressing impact of lncRNA-ROR on the concentration of IL-8, TNF-α, and C5a in C28/12 cells treated with LPS. (Figure 4D). These data demonstrated that miR-124-3p suppressed the protective impact of lncRNA-ROR on LPS-treated C28/12 cells.

LncRNA-ROR inhibited TLR4/Myd88/NF-κB pathways via regulating miR-124-3p

Next, we investigated the impact and mechanism of lncRNA-ROR on TLR4/Myd88/NF-κB pathways by using ELISA assay. Our results exposed that the protein expressions of NF-κB p65, Myd88, and TLR4 were considerably enhanced in LPS-treated C28/12 cells compared to control, while, the overexpression of lncRNA-ROR expressively repressed the protein expressions of NF-κB p65, Myd88, and TLR4 signaling pathways. Moreover, the inhibitory

effect of lncRNA-ROR on NF-κB p65, Myd88, and TLR4 protein expressions was altered by the overexpression of miR-124-3p in LPS-treated C28/12 cells. (Figure 5). These data elucidated that overexpression of lncRNA-ROR inhibited the NF-κB p65/Myd88/TLR4 signaling pathways via regulating the expression of miR-124-3p.

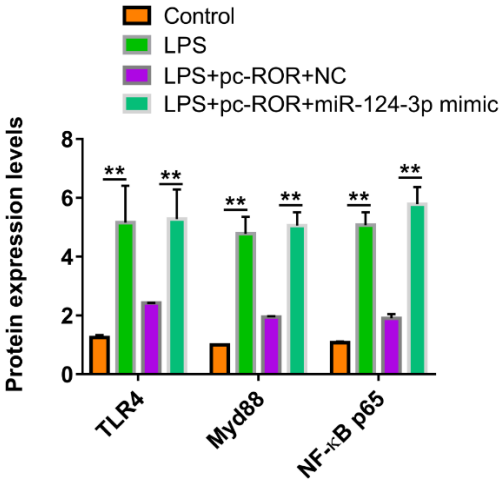

Figure 5: LncRNA-ROR inhibited TLR4/Myd88/NF-κB pathways through the regulation of miR-124-3p: Elisa assay was used to assess the protein expressions of NF-κB, Myd88, and TLR4, and after transfecting pc-lncRNA-ROR or miR-124-3p mimic in LPS-stimulated C28/12 cells. **P < 0.05

DISCUSSION

The findings from this study demonstrated that lncRNA-ROR was expressively restrained in C28/12 cells treated with LPS. LncRNA-ROR overexpression significantly reduced the LPS-stimulated inflammatory damage in C28/12 cells. Besides, our work elucidated that the miR-124-3p expression was suppressed by lncRNA-ROR in LPS-treated C28/12 cells, whereas, miR-124-3p overexpression altered the therapeutic impact of lncRNA-ROR on LPS-treated C28/12 cells. Furthermore, our data demonstrated that lncRNA-ROR suppressed TLR4/Myd88/NF-κB protein expressions via regulating the miR-124-3p expression in LPS-treated C28/12 cells.

It has been previously reported that inflammation acts as an essential factor for the cartilage pathology of OA [18]. Numerous factors related to the inflammation that exert important role in OA development, and are related to cartilage degradation [19]. A number of evidences showed that LPS play an essential function in the cell apoptosis and viability and increase the release of inflammatory mediators in various inflammatory diseases such as OA [20]. Another study revealed that LPS could enhance the expression of iNOS in osteoarthritic synoviocytes [21]. Moreover, the expression of COX-2, IL-6, IL-8, and IL-1β were enhanced in OA patients [22]. A recent study indicated that LPS can be used to establish osteoarthritis inflammatory injury model [23]. C5a is generated from the activation of complement C5 system through the alternative or classic pathway [24]. C5a is considered as an essential pro-inflammatory element that exerts a critical function in allergic response, membrane attack complex formation, complement stimulation, and immune cell chemotaxis [25]. In the current study, we examined the C28/12 cell viability, apoptosis rate, and the level of iNOS, COX-2, IL-8, C5a, and TNF-α in C28/12 cells stimulated with LPS. Our results demonstrated that LPS markedly reduced the C28/12 cell viability, enhanced apoptosis and significantly enhanced the mRNA level of iNOS, COX-2, IL-8, TNF-α, and C5a in C28/12 cells.

Evidence from previous study reported that lncRNAs perform a vital function in improving the inflammatory injury in OA chondrocytes [26]. LncRNA GAS5 inhibited the rate of cell apoptosis and LPS-stimulated inflammatory damage in ATDC5 cells by regulating the KLF2 expression [27]. Another study showed that lncRNA H19 enhanced C28/12 cell viability, reduced apoptosis, and inhibited the level of inflammatory modulators in LPS-induced C28/12 cells [28]. LncRNA-ROR is an emerging non-coding RNA which exerts its critical function in the growth of several tumors [29]. A recent research revealed that lncRNA-ROR is involved in the cell apoptosis and cell proliferation of nasopharyngeal carcinoma [30]. LncRNA-ROR exerts an essential function in the metastasis and tumorigenesis of breast cancer and could induce EMT [31]. Also, lncRNA-ROR plays function in regulating metastasis, migration, proliferation, and invasion in pancreatic cancer via enhancing EMT and ZEB1 expression [14]. Previous research demonstrated that lncRNA-ROR regulates autophagy and apoptosis in human osteoarthritis chondrocytes [29]. Conversely, the molecular mechanism of lncRNA-ROR in OA pathogenesis still remains unclear. Our data demonstrated that expression of lncRNA-ROR was reduced in C28/12 cells treated with LPS. Moreover, overexpression of lncRNA-ROR considerably increased the C28/12 cell viability, suppressed apoptosis, and attenuated the level of inflammatory factors in LPS-stimulated C28/12 cells. These data elucidated that lncRNA-ROR can suppress the LPS-stimulated inflammatory damage in C28/12 cells.

MicroRNAs are a class of non-coding RNAs having 20-23 nucleotides that play a vital function in gene regulation associated with immune system and in the growth and progression of osteoarthritis [32]. Previous study has reported that numerous miRNAs including miR-602, miR-27, miR-146a, miR9, miR-140, miR-558, miR-34a have been abnormally expressed in osteoarthritis [33]. Another study revealed that miR-203 inhibition reduced the inflammatory injury stimulated by LPS in C28/12 cells through targeting MCL-1 [34]. miR-124-3p play an essential function in the pathogenesis of numerous cancers via regulating tumor cell differentiation, apoptosis, and proliferation [35]. Moreover, miR-124-3p suppressed the neuronal inflammation in microglial exosomes through mTOR signaling pathway [36]. miR-124-3p inhibited the inflammation via down-regulating NF-κB pathway in OA chondrocytes [37]. However, the effect of miR-124-3p on inflammatory injury induced by LPS in C28/12 cells needs to be elucidated. Our data elucidated that miR-124-3p expression was restrained by lncRNA-ROR. Moreover, overexpression of miR-124-3p altered the impact of lncRNA-ROR on C28/12 cells treated with LPS. LncRNA-ROR down-regulated the expression of miR-124-3p signifying that miR-124-3p exhibited a critical function on inflammatory injury stimulated by LPS in C28/12 cells. TLR4 has been recently gained attention for its capability to identify host derived or microbial ligands in osteoarthritis [38]. Another study showed that suppressing the TLR4 expression in cartilage reduced the severity of osteoarthritis in mouse model [39]. MyD88 is an essential adaptor protein of TLR4 that stimulate the transduction of inflammatory mediators [40]. NF-κB exerts its critical function in release of pro-inflammatory mediators which essential in the pathogenesis of osteoarthritis [41]. Consequently, we dedicated our work to eplore the effect of lncRNA-ROR on these pathways. Our findings revealed that lncRNA-ROR suppressed the activation of TLR4/MyD88/NF-κB pathways via targeting miR-124-3p in LPS-treated C28/12 cells.

CONCLUSION

This work elucidated that lncRNA-ROR reduced inflammatory injury stimulated by LPS in C28/12 cells via inhibiting TLR4/MyD88/NF-κB signaling through regulating miR-124-3p suggested a new therapeutic technique for OA treatment.

DECLARATIONS

List of Abbreviations

OA: Osteoarthritis; ROR: Regulator of reprogramming; C5a: Complement 5a; COX-2: Cyclooxygenase 2; iNOS: Inducible Nitric Oxide synthase; TLR4: Toll like receptor 4; LPS: Lipopolysaccharide.

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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