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RESEARCH ARTICLE

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miR-145 eliminates lipopolysaccharides-induced inflammatory injury in human fibroblast-like synoviocyte MH7A cells

Feng Zhong | **Jian Xu** | **Xirui Yang** | **Qi Zhang** | **Zhaomeng Gao** | **Yao Deng** | **Lei Zhang** | **Chunyan Yu**

Department of Rheumatology and Immunology, Shengli Oilfield Central Hospital, Dongying, Shandong, China

Correspondence
Chunyan Yu, Department of Rheumatology and Immunology, Shengli Oilfield Central Hospital, No. 31, Jinan Road, Dongying 257034, Shandong, China.
Email: fengzhong0195@sina.com

Abstract

Recently, it has been accepted that miR-based therapy may be beneficial for rheumatoid arthritis (RA). This study aimed to evaluate the potential involvement of miR-145 in RA in vitro. The expression of miR-145 in the human fibroblast-like synoviocyte line MH7A was overexpressed by miR-mimic transfection, after which cells were subjected to lipopolysaccharides (LPS). Cell viability, apoptosis, and the release of pro-inflammatory cytokines were measured. The result showed that the apoptosis and the release of IL-1β, IL-6, IL-8, and TNF-α were significantly induced by LPS. Meanwhile, LPS treatment led to downregulation of miR-145. miR-145 overexpression in LPS-untreated MH7A cells had no impacts on cell apoptosis and inflammation. But, restoring miR-145 expression in LPS-stimulated cells by supplementation of a miR-145 mimic protected MH7A cells against LPS-induced apoptosis and inflammation. Furthermore, miR-145 overexpression in LPS-untreated MH7A cells slightly blocked the PI3K/ATK and mTOR pathways, whereas miR-145 overexpression in LPS-stimulated cells notably repressed the LPS-induced activation of PI3K/ATK and MAPK/mTOR pathways. Our study suggested that miR-145 protected MH7A cells against LPS-induced apoptosis and inflammation by inhibiting the PI3K/AKT and MAPK/mTOR pathways.

KEYWORDS

inflammation, lipopolysaccharides (LPS), MH7A cell, miR-145, rheumatoid arthritis (RA)

1 | INTRODUCTION

Rheumatoid arthritis (RA) is an antoimmune connective tissue disease characterized by systemic inflammation and synovial inflammation.[1] Joint synovial chronic inflammation leads to the formation of pannus, which destroys cartilage bone and surrounding tissues. Approximately 1% of the world population suffers from this disease, and it puts a tremendous economic burden on the society.[2] The management of RA is limited to retarding the progression of the disease, improving functional capacity, and relieving pain. Besides, over time, patients with severe cases have to receive surgical interventions, such as total hip arthroplasty.[3]

miRNAs are a class of endogenous, single-stranded, non-coding RNAs with about 22 nucleotides. miRNAs were initially considered as "junk," because they do not code protein directly. But in recent years, it has been estimated

Feng Zhong and Jian Xu contributed equally to this study.

that more than 60% of human protein-coding genes are regulated by miRNAs.[4] Also, the importance of miRNAs in physiological and pathological processes has been gradually recognized. With regard to RA, miRNAs may serve as potential biomarkers for both diagnosis and prognosis for RA,[5] with, in particular, evidence for miR-146a/b upregulation in RA synovial tissue,[6] and miR-125 upregulation in peripheral blood mononuclear cells and plasma post-treatment.[7] Besides, miRNAs have been regarded as potential pharmacological candidates for therapeutic treatment in RA. For instance, exosomes-delivered miR-548a-3p repressed inflammatory response via the TLR4/NF-κB signaling pathway in RA.[8] Downregulation of miR-10a-5p contributed in joint inflammation by targeting TBX5.[9]

miR-145, a miRNA transcribed from the cluster at chromosome 5q32, has been demonstrated as a tumor suppressor in a wide range of cancers, including non-small cell lung cancer,[10] endometrial cancer,[11] head and neck squamous cell carcinoma,[12] and gastric cancer.[13] The importance of miR-145 in the pathogenesis of RA has been mentioned by several researchers. Semaphorin-3A, an immune modulator that has been proposed as a potential therapeutic tool, can be suppressed by miR-145.[14] Besides, it has been pointed out that miR-145 contributes to VEGF165-induced anti-apoptosis and cell survival in fibroblast-like synoviocytes by targeting Semaphorin-3A.[15] However, the function of miR-145 in RA still needs to be further investigated to enhance our understanding of RA.

In the current study, the human fibroblast-like synoviocyte line MH7A was subjected to lipopolysaccharides (LPS) to mimic an in vitro model of RA. Before this, the expression of miR-145 in MH7A cells was overexpressed by mimic transfection. The functional impacts of miR-145 on LPS-stimulated MH7A cells were assessed, and the underlying mechanism of the action was explored. The findings in this study will offer us a new possibility with regard to miR-based therapy for RA.

2 | MATERIALS AND METHODS

2.1 | Cell and LPS treatment

The human fibroblast-like synoviocyte line MH7A was obtained from Riken cell bank (Ibaraki, Japan). The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). The cells were maintained at 37°C in a humidified incubator with 5% CO_2. Culture medium was replaced every other day. Subculture was obtained by using trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) when 80% to 90% confluence was reached.

LPS (Sigma-Aldrich) from *Escherichia coli* O111:B4 at various doses was used to treat MH7A cells for 5 hours.

2.2 | miRNAs transfection

miR-145 mimic (sense, 5′-GUC CAG UUU UCC CAG GAA UCC CU-3′, anti-sense 5′-GGA UUC CUG GGA AAA CUG GAC UU-3′) was synthetized by GenePharma (Shanghai, China). The synthesized cel-miR-67-3p with the sequence 5′-UCA CAA CCU CCU AGA AAG AGU AGA-3′ (GenePharma) was transfected as a negative control (mimic-control). Transfection was performed in six-well plates for 48 hours by using Lipofectamine 3000 reagent (Invitrogen). Transfection efficiency was verified by quantitative real-time polymerase chain reaction (qRT-PCR).

2.3 | qRT-PCR

Total RNAs were extracted by using TRIzol reagent-phenol chloroform (Invitrogen). For the test of IL-1β, IL-6, IL-8, and TNF-α, the extracted RNAs were subjected to reverse transcription by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT-PCR was performed on ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA), after the synthesized cDNA was mixed with SYBR Green Master (Roche). For the test of miR-145, a PrimeScript RT reagent kit (TakaRa, Dalian, China) was used for cDNA synthesis. qRT-PCR was performed by using Taqman Universal Master Mix II (Applied Biosystems). β-actin and U6 were used as internal controls for mRNAs and miRNAs, respectively. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.4 | CCK-8 assay

The miR-transfected MH7A cells were seeded in 96-well plates with a density of 5000 cells/well for adherence. After 5 hours of LPS stimulation and another 48 hours of incubation under normal conditions, the cells were washed twice with phosphate buffer saline (PBS), and then 10 μL CCK-8 solution (Dojindo Molecular Technologies, Kyushu, Japan) was added into each well. The samples were incubated at 37°C for another 1 hour, after which the absorbance of each well was detected by a Microplate Reader (Bio-Rad, Hercules, CA) at 450 nm.

2.5 | Assessment of apoptosis

An Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) was utilized in this study to determine the percentage of apoptotic cells. After being transfected in six-well plates, cells were treated with 5 μg/mL LPS for 5 hours. The cells from each sample were collected by trypsin digestion and centrifugation, and they were resuspended in 200 μL Binding Buffer containing 10 μL Annexin V-FITC and 5 μL PI. The mixed samples were

incubated in the dark over ice for 30 minutes. Before assessment in a flow cytometer (Beckman Coulter, Fullerton, CA), 300 μL ice-cold PBS was added. Early apoptotic cells were recognized by Annexin V-FITC positive and PI negative and were calculated by FlowJo software (Tree Star, San Carlos, California).

2.6 | Enzyme-linked immunosorbent assay

After transfection and LPS treatment, the cell culture supernatant was collected from each sample to detect the concentrations of IL-1β, IL-6, IL-8, and TNF-α. Human enzyme-linked immunosorbent assay kits specific for IL-1β (ab100562), IL-16 (ab100555), IL-18 (ab215539), and TNF-α (ab181421; Abcam, Cambridge, MA) were used according to the manufacturers' protocol.

2.7 | Western blot analysis

Protein was isolated from cells by using RIPA lysis buffer (Beyotime) supplemented with 10 mg/mL phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich). The extracted protein (0.1 mg) was resolved over SDS-PAGE and was transferred onto PVDF membranes by using a Trans-Blot®Turbo™ transfer system (Bio-Rad Laboratories; 100 mA for 40 minutes). The membranes were blocked in 5% (w/v) non-fat milk for 1 hour at

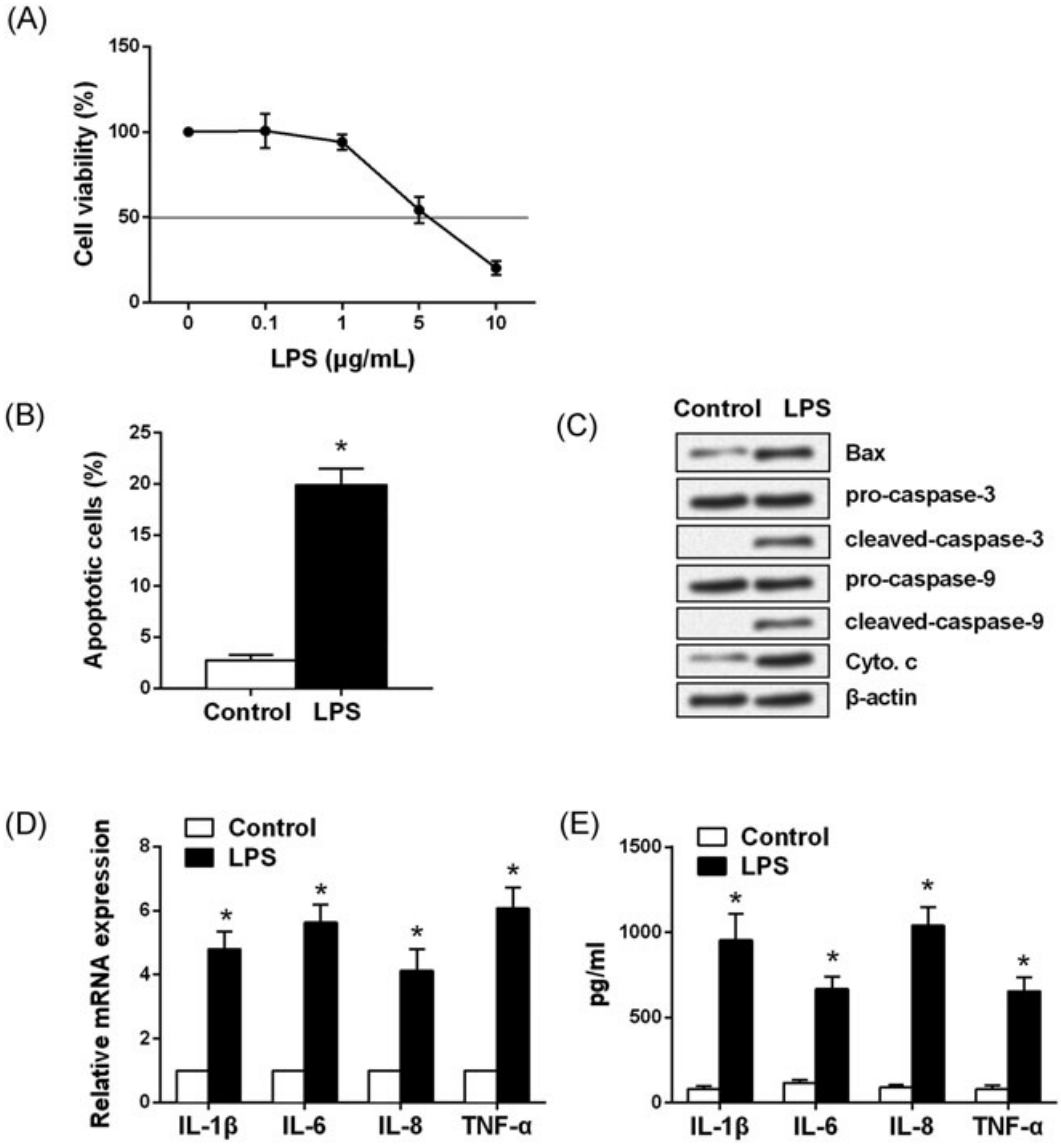

FIGURE 1 Effect of LPS on MH7A cells. A, The viability of MH7A cells after treated with various doses of LPS for 5 hours. B, Percentage of apoptotic cell; C, protein expressions of apoptosis-related factors; D, mRNA level expressions of pro-inflammatory cytokines; and E, concentrations of pro-inflammatory cytokines in the culture supernatant were respectively measured, after MH7A cells were treated with 5 μg/mL for 5 hours. $^*P < 0.05$. LPS, lipopolysaccharides

room temperature, after which primary antibodies were applied to incubate the membranes for 12 hours at 4°C. Antibodies for the test of Bax (AF820), cleaved-caspase-3 (MAB835-SP), cytochrome c (MAB897), PTEN (AF847), and β-actin (MAB8929) were purchased from R&D Systems (Minneapolis, MN), and antibodies for the test of pro-caspase-3 (ab32150), pro-caspase-9 (ab2013), cleaved-caspase-9 (ab2324), PI3K (ab86714), p-PI3K (ab182651), AKT (ab8805), p-AKT (ab38449), p38MAPK (ab197348), p-p38MAPK (ab47363), mTOR (ab32028), p-mTOR (ab137133), S6K (ab32529), and p-S6K (ab2571) were purchased from Abcam. The membranes were then incubated with the secondary antibodies for 1 hour at room temperature. An enhanced chemiluminescence technique was used to visualize the positive bands in membranes. The intensity of the films was qualified by using ImageQuant LAS 4000 (GE Healthcare, Chicago, IL) and Image Lab™ software (Bio-Rad Laboratories).

2.8 | Statistics

Data were represented as means ± standard deviation. Statistics were performed in SPSS version 19.0 software (SPSS Inc, Chicago, IL). Significant differences between groups were calculated by one-way analysis of variance with Duncan post-hoc. A P value of <0.05 was considered to indicate a significant difference.

3 | RESULTS

3.1 | Establishment of an in vitro model of RA

To start with, MH7A cells were subjected to various doses of LPS for 5 hours, after which cell viability was detected to assess the cytotoxicity of LPS. The data in Figure 1A show that the viability of MH7A cells was remarkably reduced by LPS in a dose-dependent manner; the IC50 value was about 5.71 μg/mL. LPS with the dose of 5 μg/mL was utilized in the following experiments. Figure 1B showed that LPS induced a significant increase in apoptosis ($P < 0.05$). The apoptosis induced by LPS was also confirmed by performing Western blot analysis, as Bax and cytochrome c proteins were upregulated, and caspase-3 and caspase-9 were cleaved in response to LPS (Figure 1C). Besides, the release of pro-inflammatory cytokines was observed in the LPS-treated cells. As shown in Figure 1D,E, the mRNA levels of IL-1β, IL-6, IL-8, and TNF-α and the concentrations of these cytokines in the culture supernatant were all significantly increased ($P < 0.05$) by LPS treatment.

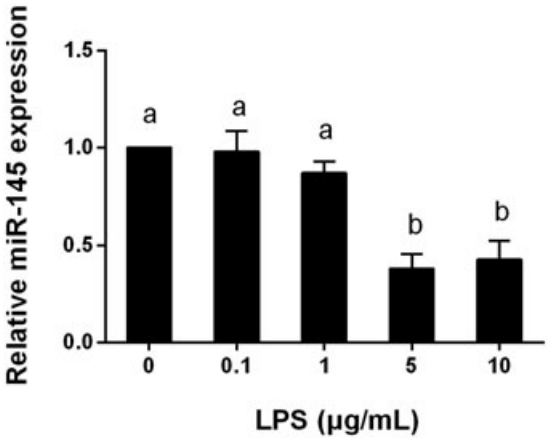

FIGURE 2 Effect of LPS on the expression of miR-145. The expression of miR-145 in MH7A cells after treated with 5 μg/mL of LPS for 5 hours. Different letters above the columns indicate that the means of different groups were significantly different ($P < 0.05$). LPS, lipopolysaccharides

3.2 | Down-regulations of miR-145 in LPS-stimulated MH7A cells

The expression changes of miR-145 in MH7A cells after LPS treatment were monitored by qRT-PCR. The results shown in Figure 2 revealed that the expression levels of miR-145 were significantly downregulated by LPS treatment ($P < 0.05$) in a dose-dependent manner.

3.3 | Anti-apoptotic effects of miR-145 on LPS-stimulated MH7A cells

The involvement of miR-145 in LPS-induced apoptosis in MH7A cells was evaluated to see the importance of miR-145 expression in RA. To do this, a miR-145 mimic and a mimic-control were respectively transfected into MH7A cells. The qRT-PCR data showed that transfection of cells with the miR-145 mimic significantly increased the miR-145 expression level, as compared with NC transfection (Figure 3A). Figure 3B shows that miR-145 mimic transfection did not impact ($P > 0.05$) the viability of LPS-untreated MH7A cells, but could significantly increase the viability of MH7A cells in the presence of LPS ($P < 0.05$). Also, apoptosis was unaffected by the miR-145 mimic in LPS-untreated MH7A cells ($P > 0.05$), but the apoptosis-induced by LPS was significantly attenuated by the miR-145 mimic ($P < 0.05$; Figure 3C). The same trend was observed in Figure 3D, as the protein levels of Bax, caspase-3, caspase-9 and cytochrome c were unaffected by the miR-145 mimic in LPS-untreated MH7A cells. But, the upregulations of Bax and cytochrome c, as well as

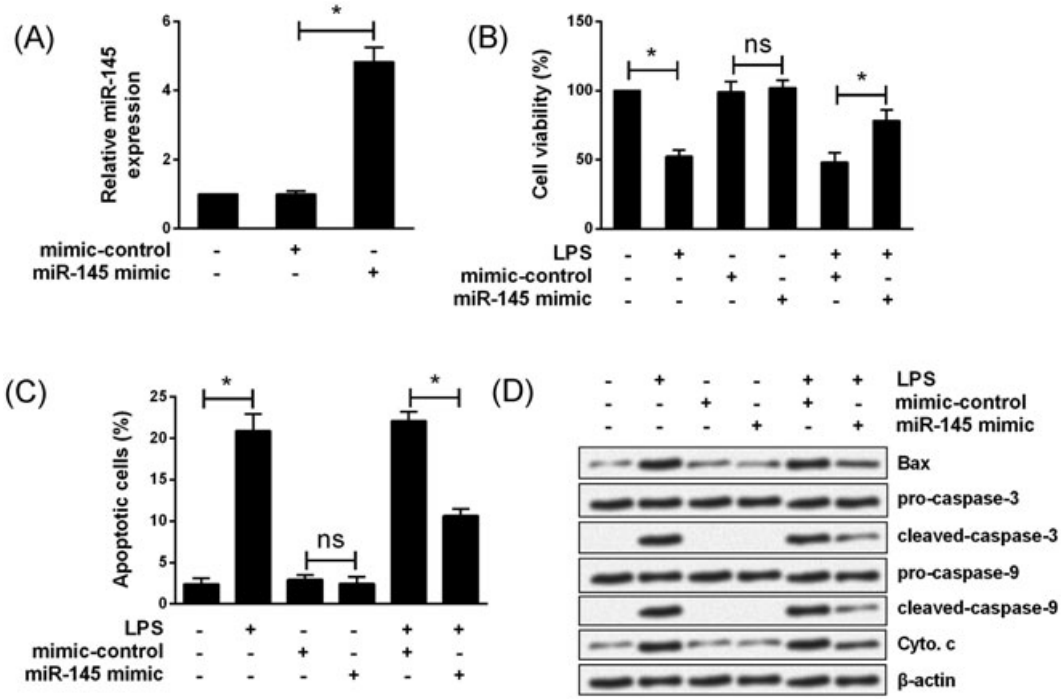

FIGURE 3 Effect of miR-145 overexpression on LPS-induced apoptosis in MH7A cells. A, The expression of miR-145 in MH7A cells after transfection with miR-145 mimic or the mimic-control. B, The viability; C, percentage of apoptotic cell; and D, the protein expressions of apoptosis-related factors were respectively measured, after MH7A cells were transfected with miR-145 mimic or mimic-control, and subsequently treated with 5 μg/mL of LPS for 5 hours. $^*P < 0.05$. LPS, lipopolysaccharides; ns, not significant

the cleavage of caspase-3 and caspase-9 induced by LPS were partially eliminated by the miR-145 mimic.

3.4 | Anti-inflammatory effects of miR-145 on LPS-stimulated MH7A cells

The impact of miR-145 on LPS-induced the release of inflammatory cytokines was also detected. As expected, the miR-145 mimic had no impact on the expression and release of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) in LPS-untreated MH7A cells ($P > 0.05$), but it could significantly attenuate the LPS-induced increases in these cytokines ($P < 0.05$; Figure 4A,B).

3.5 | Inhibitory effects of miR-145 on PI3K/AKT and MAPK/mTOR pathways

To get insights into the molecular mechanism by which miR-145 attenuated LPS-induced cell damage in MH7A cells, the phosphorylation patterns of components of the PI3K/AKT and MAPK pathways were evaluated. As the results show in Figure 5A,B, PI3K, AKT, p38MAPK, mTOR and S6K were remarkably phosphorylated by LPS, and the protein level of PTEN was remarkably reduced by LPS. The miR-145 mimic reduced the phosphorylation of

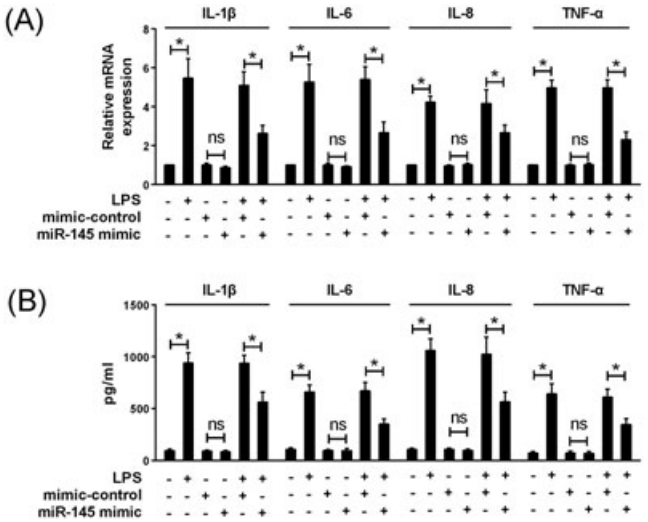

FIGURE 4 Effect of miR-145 overexpression on LPS-induced inflammation in MH7A cells. A, mRNA level expressions of pro-inflammatory cytokines, and (B), concentrations of pro-inflammatory cytokines in the culture supernatant were respectively measured, after MH7A cells were transfected with miR-145 mimic or mimic-control, and subsequently treated with 5 μg/mL of LPS for 5 hours. $^*P < 0.05$. LPS, lipopolysaccharides; ns, not significant

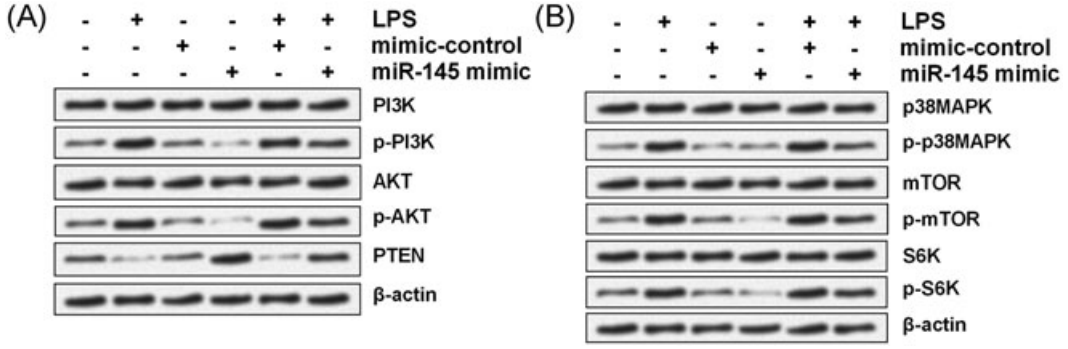

FIGURE 5 Effect of miR-145 overexpression on PI3K/AKT and MAPK/mTOR pathways. Protein expressions of core components of (A), PI3K/AKT, and (B), MAPK pathways were evaluated, after MH7A cells were transfected with miR-145 mimic or mimic-control, and subsequently treated with 5 μg/mL of LPS for 5 hours. LPS, lipopolysaccharides

PI3K, AKT, p38MAPK, mTOR, and S6K, and increased the protein expression of PTEN in both LPS-untreated and LPS-treated cells.

4 | DISCUSSION

In recent years, it has been well-documented that gene-based therapy may be beneficial for numerous diseases, including RA.[16] Gene-based therapy has been reported to be highly therapeutic in many animal models and to be safe in several clinical trials.[16,17] miRNAs are pivotal regulators in many pathological processes. This study aimed to reveal the therapeutic potential of miR-based therapy for RA. Herein, human fibroblast-like synoviocyte line MH7A was subjected to LPS. As a result, apoptosis and release of pro-inflammatory cytokines were induced significantly by LPS. Meanwhile, LPS treatment led to a downregulation of miR-145. Restoring miR-145 expression by supplementation of a miR-145 mimic protected MH7A cells against LPS-induced injury. Our further investigations indicated that, the PI3K/ATK and MAPK/mTOR pathways might be implicated in the protective actions of miR-145.

Because primary RA fibroblast-like synoviocytes gradually lose the characteristics of RA during expanding and maintaining, we selected MH7A, a usefulness cell line for investigating the regulation of RA fibroblast-like synoviocytes,[18] to explore the function of miR-145 in RA in vitro. We found that LPS significantly injured MH7A cells by inducing a mitochondria-dependent apoptosis and by releasing pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α). This observation was consistence with several previous studies,[19,20] indicating that RA conditions were successfully established in this study.

Apoptosis in synovial fibroblasts is a critical feature for the pathogenesis of RA.[21] Several miRNAs have been recognized as key regulators of apoptosis in RA. And the regulatory role of miRNAs in synovial fibroblasts is stimulus-dependent. For example, miR-34a sheltered RA synovial fibroblasts from FasL-mediated apoptosis but had no effect on TRAIL-induced cell death.[22] miR-155 overexpression repressed spontaneous apoptosis in untreated CD14+cells, whereas it could not affect Fas-induced cell death in CD14+cells.[23] miR-145 has been reported to be an RA-related miRNA, which elevates expression of miR-145, contributing to the anti-apoptotic activity of VEGF165 in synovial fibroblasts.[15] In the current study, our data demonstrated that miR-145 overexpression had no impact on the LPS-untreated MH7A cells but could protect MH7A cells against LPS-induced apoptosis.

In addition to apoptosis, immune response is another critical event in the pathogenesis of RA. The importance of miR-145 in the immune system has been sporadically studied. Loss of miR-145 contributes to the induction of immune-related diseases, such as colitis,[24] peri-implantitis,[25] ischemic stroke,[26] atherosclerosis,[27] and chronic glomerulonephritis.[28] Moreover, miR-145 was reported to exert anti-inflammatory functions via inhibiting the expression of pro-inflammatory cytokines IL-1α, IL-2, IL-6, and TNF-α.[28] We demonstrated that miR-145 overexpression had no impact on the release of IL-1β, IL-6, IL-8, and TNF-α in the LPS-untreated MH7A cells, but it could reduce the release of these pro-inflammatory cytokines in LPS-stimulated MH7A cells. Collectively, our finding suggested that miR-145 could protect MH7A cells against LPS-induced inflammatory injury.

PI3K/ATK and MAPK/mTOR are two signaling pathways involved in the apoptosis and inflammation of RA fibroblast-like synoviocytes.[29-32] LPS increases the secretion of pro-inflammatory cytokines in fibroblast-like synoviocytes, which leads to the activation of the PI3K/AKT and MAPK/mTOR pathways, thereby inducing apoptotic and inflammatory injury in these cells.[31,33] Additionally, a common thread that appears to link

miR-145 to apoptosis and inflammation is its capacity to block the PI3K/AKT and MAPK/mTOR pathways.[34-37] We demonstrated that miR-145 could partially eliminate the LPS-induced activation of PI3K/AKT and MAPK/mTOR pathways in MH7A cells, implying that the protective activities of miR-145 might be realized by blocking the PI3K/AKT and MAPK/mTOR pathways. We additionally found that miR-145 could block PI3K/AKT and mTOR pathways in the LPS-untreated MH7A cells, but the blockage failed to modulate cell apoptosis and inflammation. One of the possible reasons is that the apoptosis and inflammation levels in the LPS-untreated MH7A cells are already very low.

In conclusion, our study provided evidence that miR-145 protected MH7A cells against LPS-induced apoptosis and inflammation by inhibiting the PI3K/AKT and MAPK/mTOR pathways. These findings suggested that miR-145 might be a potential candidate for miR-based therapy in RA. More effort is required to validate the anti-apoptotic and anti-inflammatory role of miR-145 in primary RA fibroblast-like synoviocytes and in animal model.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ORCID

Feng Zhong http://orcid.org/0000-0002-3148-279X

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