

Protective effects of miR-155-5p silencing on IFN- γ -induced apoptosis and inflammation in salivary gland epithelial cells

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Abstract. Previous studies have demonstrated that microRNAs (miRNAs/miRs) serve a vital role in the pathogenesis of Sjögren's syndrome (SS). The present study aimed to investigate the role of miR-155-5p in SS and determine its underlying molecular mechanism. An inflammatory lesion model was established by stimulating salivary gland epithelial cells (SGECs) with interferon- γ (IFN- γ). The apoptosis of SGECs was measured by using flow cytometry. Levels of proinflammatory factors were detected by reverse transcription-quantitative PCR and ELISA, respectively. Immunofluorescence was used for p65 staining. Dual-luciferase reporter assay was performed to verify the interaction between miR-155-5p and arrestin β 2 (ARRB2). The protein levels in the NF- κ B signaling pathway were assessed by western blotting. The results of the present study demonstrated that treatment with IFN- γ increased miR-155-5p expression, in addition to inducing apoptosis and inflammation in SGECs. Furthermore, overexpression of miR-155-5p promoted IFN- γ -induced apoptosis and inflammation in SGECs. Overexpression of miR-155-5p also increased Bax protein expression, enzyme activities of caspase 3 and caspase 9, release of inflammatory cytokines interleukin-6 and tumor necrosis factor- α , and decreased Bcl-2 protein expression in IFN- γ -treated SGECs. By contrast, all of the effects aforementioned were reversed following miR-155-5p knockdown. These results demonstrated that miR-155-5p activated the NF- κ B signaling pathway, where treatment with the NF- κ B inhibitor, pyrrolidinedithiocarbamate, reversed the effects of miR-155-5p overexpression on the inflammatory factors in IFN- γ -induced SGECs. miR-155-5p was demonstrated to target

ARRB2 and negatively regulated its expression levels, such that overexpression of ARRB2 reversed the effects of miR-155-5p overexpression on the inflammatory response, apoptosis and the NF- κ B signaling pathway in IFN- γ -treated SGECs. Collectively, results from the present study suggest that miR-155-5p may activate the NF- κ B signaling pathway by negatively regulating ARRB2 to promote salivary gland damage during SS pathogenesis. This suggests that miR-155-5p may serve to be a potential target for the treatment of SS.

Introduction

Sjögren's syndrome (SS) is a common systemic autoimmune disease of exocrine glands, particularly the salivary glands (1). Dry mouth caused by salivary gland dysfunction is one of the typical features of SS (2). The pathogenesis and etiology of SS remain unclear, since complex elements, including genes and the environment, have been reported to contribute to the development of this disease (3,4). The inflammatory process of salivary glands is a common feature in patients with SS (5), where inflammation is associated with the persistence of interferon (IFN) signaling (6).

MicroRNAs (miRNAs/miRs) bind to target transcripts to suppress translation (7). Previous studies have demonstrated that miRNAs can regulate several biological processes, such as the innate immune response (8,9). miR-155-5p has multiple functions, including the regulation of tumor development (10), immune regulation (11) and oxidative stress (12). Furthermore, due to its notable regulatory effect on the immune system, miR-155-5p is closely associated with a variety of immune-related diseases (13,14), including during rheumatoid arthritis (15), systemic lupus erythematosus (16) and SS (2,17,18). It has been previously reported that miR-155-5p expression is markedly elevated in the peripheral mononuclear cells of patients with primary SS (19). However, the function of miR-155-5p in SS remains unclear, where its potential effect on salivary glands damaged by SS has not been reported previously.

Arrestin β 2 (ARRB2) is a scaffolding protein of the arrestin family, which exerts multiple functions, including promoting angiogenesis, alleviating neuropathic pain and modulating the sensitivity of cancer cells to chemotherapy drugs (20-22). Previous studies have demonstrated that ARRB2 also exhibits

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anti-inflammatory effects in some inflammatory diseases, such as colitis and sepsis (23-25). In addition, ARRB2 has been reported to inhibit NF- κ B signaling in septic and lipopolysaccharide-treated mice (23,26). Previous studies have demonstrated that NF- κ B serve a promoting role in SS and its complications (27,28). Therefore, based on these previous findings aforementioned, the present study hypothesized that miR-155-5p may participate in SS-induced salivary gland damage by targeting ARRB2.

Materials and methods

Isolation, transfection and treatment of salivary gland epithelial cells (SGECs). The present study was approved by the Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University (Mudanjiang, China) and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (29).

BALB/c mice, aged 7-8 weeks, weighted 20 ± 2 g ($n=30$; 15 male and female) were purchased from Beijing Huafukang Biotechnology Co., Ltd. (<http://www.hfkbio.com/>). The housing conditions of the mice were: 12-h light/dark cycle, $25 \pm 1^\circ\text{C}$, and 45-55% humidity. All the mice were free access to food and water. Mice were euthanized by an intraperitoneal injection of sodium pentobarbital (200 mg/kg). In total, five mice were randomly selected from the 30 mice and the salivary glands were collected from the parotid, submandibular and sublingual glands. In accordance with previous research, SGECs were extracted from the salivary glands of five mice and pooled (30). Briefly, the salivary glands were washed 2-3 times with PBS. Tissue samples were minced into fragments ($1-2\text{ mm}^3$) and cultured in a petri dish. Following incubation for 2 h at 37°C , DMEM/F12 complete medium (Procell Life Science & Technology Co., Ltd.) was added to the petri dish and the fragments were further incubated for 72 h at 37°C in 5% CO_2 and saturated humidity. The culture medium was replaced every 3 days until the cell density reached $\sim 80\%$. The isolated SGECs were cultured in DMEM/nutrient mixture F-12 medium (Procell Life Science & Technology Co., Ltd.) supplemented with streptomycin (100 $\mu\text{g/ml}$), epidermal growth factor (10 ng/ml; Sino Biological), insulin (0.5 mg/ml; Shenyang Bying Biotechnology Co., Ltd.), hydrocortisone (0.4 mg/ml; Shanghai Aladdin Biochemical Technology Co., Ltd.) and FBS (3%; Biological Industries), at 37°C in 5% CO_2 . SGECs were identified via immunocytochemistry staining of cytokeratin (CK) 7, CK8 and CK19 (31,32).

Transfection. Negative control (NC)/miR-155-5p agomir (25 nmol/l; Shanghai GenePharma Co., Ltd.) or NC/miR-155-5p antagomir (25 nmol/l; Shanghai GenePharma Co., Ltd.) were transfected into SGECs for 24 h at 37°C using Lipofectamine[®] 3000 reagent (5 μl ; Invitrogen; Thermo Fisher Scientific, Inc.). SGECs were treated with IFN- γ (10 ng/ml, Sino Biological <https://www.sinobiological.com/>) for 12 h at 37°C to induce inflammation as previously described (30). For drug inhibition, SGECs were transfected with miR-155-5p agomir/agomir NC for 24 h at 37°C , followed by treatment with IFN- γ (10 ng/ml) and pyrrolidine dithiocarbamate (PDTC, 100 μM ; Shanghai Aladdin Biochemical Technology Co., Ltd.) for 12 h. For rescue experiments, ARRB2 overexpression vector (pcDNA3.1;

121.3 ng/ μl , Genscript) or pcDNA3.1 vector (154.6 ng/ μl , Genscript) and miR-155-5p agomir were co-transfected into the SGECs. These concentrations of ARRB2 overexpression vector and its NC were used for co-transfection. Following transfection for 24 h at 37°C , the cells were treated with IFN- γ (10 ng/ml) for 12 h. The sequences of the miR-155-5p agomir/NC agomir and miR-155-5p antagomir/NC antagomir were: miR-155-5p agomir sense, 5'-UUA AUGCUAAUUGUG AUAGGGGU-3' and antisense, 5'-CCCUAUCACAAUAG CAUUA AUU-3'; NC agomir sense, 5'-UUCUCCGAACGU GUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGG AGAATT-3'; miR-155-5p antagomir, 5'-ACCCCUAUCACA AUUAGCAUUA-3' and NC antagomir, 5'-CAGUACUUU UGUGUAGUACAA-3'.

Immunocytochemistry. SGECs (3×10^4 cells each well) in 12-well plate were fixed in 4% paraformaldehyde for 15 min at room temperature and subsequently incubated with 3% H_2O_2 for 15 min at room temperature to inhibit endogenous peroxidase activity. No antigen retrieval was performed before this. Cells were blocked in 100% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at room temperature and incubated with primary antibodies against CK7 (1:200, cat. no. A4765; ABclonal Biotech Co., Ltd.), CK8 (1:200, cat. no. 17514-1-AP; ProteinTech Group, Inc.) and CK19 (1:200, cat. no. A19040; ABclonal Biotech Co., Ltd.) overnight at 4°C . Following the primary antibody incubation, cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:500, cat. no. 31460; Thermo Fisher Scientific, Inc.) for 2 h at 37°C (Thermo Fisher Scientific, Inc.). DAB and hematoxylin (both purchased from Beijing Solarbio Science & Technology Co., Ltd.) were used for color development (5 min at room temperature) and counterstaining (5 min at room temperature), respectively. The slides were observed under a light microscope (magnification, $\times 400$; Olympus Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from IFN- γ -treated SGECs using TRIpure reagent (cat. no. RP1001; BioTeke Corporation,) and reverse transcribed into cDNA using M-MLV reverse transcriptase (cat. no. PR6502; BioTeke Corporation.), dNTPs (Beijing Solarbio Science & Technology Co., Ltd.), and primers (Genscript, random hexamers and poly-A were used). The temperature protocol was used for reverse transcription for miR-155-5p: 37°C for 30 min, 42°C for 30 min and 70°C for 10 min. For interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), ARRB2 and β -actin, the temperature protocol was: 25°C for 10 min, 42°C for 50 min and 80°C for 10 min. The qPCR was performed using SYBR[®] Green I nucleic acid gel stain (cat. no. S9430; Sigma-Aldrich; Merck KGaA) and 2X Power Taq PCR Master Mix (cat. no. PR1702, BioTeke Corporation). The temperature protocol was used for qPCR for miR-155-5p was the following: Initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 20 sec and 72°C for 15 sec. For IL-6, TNF- α , ARRB2 and β -actin, the thermocycling conditions were the following: Initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 25 sec and 72°C for 30 sec. The following primer sequences were used for qPCR: miR-155-5p forward, 5'-TTA ATGCTAATTGTGATAGGGGT-3' and reverse, 5'-GCAGG

TCCGAGGTATTC-3'; 5S rRNA forward, 5'-CTAAAGATT TCCGTGGAGAG-3' and reverse, 5'-TGGTGCAGGGTCCGA GGTAT-3'; IL-6 forward, 5'-ATGGCAATTCTGATT GTATG-3' and reverse, 5'-GACTCTGGCTTTGTC TTTCT-3'; TNF- α forward, 5'-CAGGCGGTGCCTATG TCTCA-3' and reverse, 5'-GCTCTCCACTTGGTGGTTT-3'; ARRB2 forward, 5'-CCATTGTGAAGGAGGGAG-3' and reverse, 5'-GCATTAGGACGAAGGGTAG-3' and β -actin forward, 5'-CTGTGCCCATCTACGAGGGCTAT-3' and reverse, 5'-TTTGATGTACGCACGATTTCC-3'. Relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (33) and normalized to the internal reference gene β -actin. 5S rRNA served as the internal control for miRNA expression.

Cell Counting Kit-8 (CCK-8) assay. CCK-8 assay was performed to assess cell viability. SGEs were seeded into 96-well plates (3×10^3 cells each well). Following transfection for 24 h at 37°C, cells were incubated with CCK-8 solution for 1 h following the manufacturer's protocols at 37°C (10 μ l each well; Sigma-Aldrich, Merck KGaA) before viability was subsequently analyzed at a wavelength of 450 nm, using a microplate reader.

Apoptosis analysis. Early and late apoptotic cells were assessed using flow cytometry. Cells were seeded into six-well plates at a density of 1×10^5 cells/well. Following transfection and treatment, apoptotic cells were analyzed. Briefly, cells in each group were collected and resuspended with 195 μ l Annexin V-FITC (Beyotime Institute of Biotechnology). Cells (1×10^5) were subsequently treated with 5 μ l Annexin V-FITC and 10 μ l propidium iodide (both purchased from Beyotime Institute of Biotechnology) for 15 min at room temperature. Apoptotic cells were subsequently detected by flow cytometer (NovoCyte; ACEA Bioscience, Inc.) and analyzed by NovoExpress (version 1.2.5; ACEA Biosciences, Inc.).

Immunofluorescence staining. Cell slides were fixed with 4% paraformaldehyde for 15 min at room temperature and then incubated with 0.1% Triton X-100 for 30 min at room temperature. After blocking with 100% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at room temperature, the cell slides were incubated with the anti-p65 antibody (1:200; total non-phosphorylated version; cat. no. 10745-1-AP; ProteinTech Group, Inc.) overnight at 4°C. After the primary antibody incubation, cells were treated with Cy3-labeled goat anti-rabbit IgG (1:200, cat. no. A0516, Beyotime Institute of Biotechnology) for 60 min at room temperature. After staining the nucleus with DAPI (cat. no. C1002; Beyotime Institute of Biotechnology) at room temperature, slides were analyzed with a fluorescence microscope (magnification, x400; Olympus Corporation).

Western blotting. Total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology). Cytoplasmic protein and nuclear protein were extracted using a nuclear protein extraction kit (cat. no. P0027; Beyotime Institute of Biotechnology). Bicinchoninic acid protein assay kit was used to measure the protein concentration. Equal amounts of protein (20–40 μ g) were separated via SDS-PAGE (10 and 12% gel). The separated proteins were subsequently

transferred onto PVDF membranes (EMD Millipore) and blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with primary antibodies against Bcl-2 (1:1,000; cat. no. A19693; ABclonal Biotech Co., Ltd.), Bax (1:1,000; cat. no. A19684; ABclonal Biotech Co., Ltd.), Inhibitor of NF- κ B (I- κ B; 1:1,000; cat. no. A11397; ABclonal Biotech Co., Ltd.), phosphorylated (p)-I- κ B (1:1,000; cat. no. AP0707; ABclonal Biotech Co., Ltd.), NF- κ B p65 (1:1,000; cat. no. AF5006; Affinity Biosciences), ARRB2 (1:1,000; cat. no. A1171; ABclonal Biotech Co., Ltd.), Histone H3 (1:2,000; cat. no. AM8433; Abgent Inc.) and β -actin (1:1,000; cat. no. sc-47778; Santa Cruz Technology, Inc.) overnight at 4°C. Following primary antibody incubation, membranes were incubated with HRP-conjugated anti-rabbit/mouse IgG (1:5,000; cat. nos. A0208 and A0216; Beyotime Institute of Biotechnology) at room temperature for 30 min. Proteins bands were visualized using an enhanced chemiluminescence reagent solution (Beyotime Institute of Biotechnology) and analyzed using a Gel-Pro-Analyzer (version 4.0; Beijing Liuyi Biotechnology, Inc.).

Caspase-3 and -9 activities. After the cells were harvested and lysed, caspase-3 detection kit (cat. no. C1116; Beyotime Institute of Biotechnology) was used to detect caspase-3 activity in the lysates, whilst caspase-9 activity was measured using a caspase-9 detection kit (cat. no. BC3890; Beijing Solarbio Science & Technology Co., Ltd.). Caspase-3 and -9 activities were subsequently analyzed at a wavelength of 405 nm detected using a microplate reader (BioTek Instruments, Inc.).

ELISA. The ELISA kits (Wuhan Boster Biological Technology, Ltd.), were used to detect the expression levels of IL-6 (cat. no. EK0411) and TNF- α (cat. no. EK0527) in cell culture supernatant, according to the manufacturer's protocols. Tetramethylbenzidine (TMB) substrate solution (Wuhan Boster Biological Technology, Ltd.) was used to incubate the samples for 20 min at 37°C in the dark. TMB stop solution (Wuhan Boster Biological Technology, Ltd.) was then used to suspend the color reaction. A microplate reader (BioTek Instruments, Inc.) was used to obtain the optical density value at a wavelength of 450 nm. The lower detection limit of the ELISA kits was 15.6 pg/ml.

Dual-luciferase reporter assay. The binding sites between miR-155-5p and ARRB2 were predicted using TargetScan 7.2 (http://www.targetscan.org/vert_72). Briefly, the species was 'Human', following which the microRNA name 'miR-155-5p' was typed in and the 'submit' button was clicked. A number of genes potentially targeted by miR-155-5p can then be obtained. After searching for ARRB2, the 'Sites in UTR' button was clicked to obtain the targeted binding sequence between miR-155-5p and ARRB2. The association between miR-155-5p and ARRB2 was detected using dual-luciferase reporter assay. The mirGLO-ARRB2-3'-UTR-WT and pmirGLO-ARRB2-3'-UTR-MT plasmids were synthesized by GenScript. The plasmid (0.5 μ g) and miR-155-5p mimic or its NC (25 pmol) were co-transfected into 293T cells at ~70%. The co-transfection was mediated by Lipofectamine® 3000 reagent (9 μ l; Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation for 48 h at 37°C, luciferase

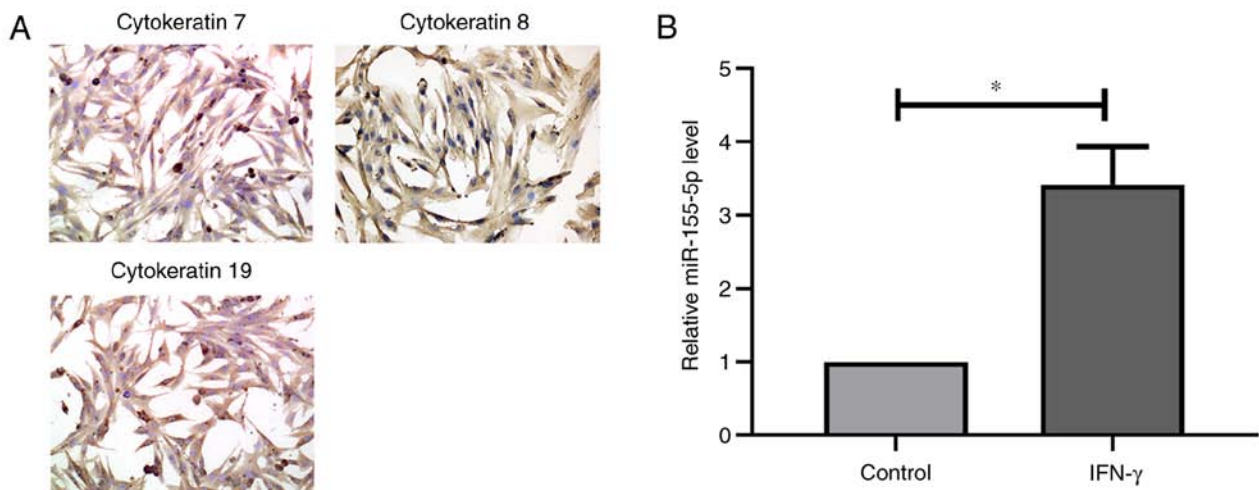


Figure 1. Expression of miR-155-5p in interferon- γ -treated SGEs. (A) Immunohistochemical staining of cytokeratin 7, cytokeratin 8 and cytokeratin 19 in SGEs (magnification, x200). (B) miR-155-5p expression was analyzed using reverse transcription-quantitative PCR. All data are presented as mean $\pm$ SD, $n=3$. * $P<0.05$. miR, microRNA; IFN- γ , interferon- γ ; SGEs, salivary gland epithelial cells.

detection kit (cat. no. E1910; Promega Corporation) was used to detect luciferase activity. The changes in luciferase activities were measured using a microplate reader (Tecan Group Ltd.). Firefly luciferase activity was normalized to that of *Renilla* luciferase activity.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc.). Data are presented as the mean $\pm$ standard deviation. Student's unpaired t-test was used to compare differences between two groups, whilst one-way ANOVA with Tukey's post hoc test was used to compare differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least in triplicate.

Results

miR-155-5p expression in IFN- γ -treated SGEs. SGEs were phenotyped by immunocytochemistry staining (Fig. 1A). The results demonstrated that the isolated cells exhibited strong cytokeratin expression, including that of epithelial markers CK7, CK8 and CK19, suggesting that the isolated cells were SGEs. RT-qPCR analysis demonstrated that miR-155-5p expression was significantly increased in SGEs following treatment with IFN- γ (Fig. 1B). Taken together, these results suggest that IFN- γ treatment increases miR-155-5p expression.

miR-155-5p promotes IFN- γ -induced apoptosis in SGEs. The effects of miR-155-5p knockdown and overexpression on the apoptosis of IFN- γ -treated SGEs were next assessed. RT-qPCR analysis demonstrated that miR-155-5p expression was significantly upregulated following transfection with miR-155-5p agomir, but was significantly downregulated following transfection with miR-155-5p antagomir compared with that in their corresponding NCs (Fig. 2A). Transfected SGEs were subsequently treated with IFN- γ (10 ng/ml) for 12 h. Treatment with IFN- γ significantly reduced cell viability and promoted apoptosis in SGEs (Fig. 2B and C). Overexpression of miR-155-5p significantly decreased cell

viability and induced apoptosis in IFN- γ -treated SGEs, whereas miR-155-5p knockdown significantly increased cell viability and inhibited apoptosis compared with those in their corresponding NCs (Fig. 2B and C). Furthermore, treatment with IFN- γ significantly decreased Bcl-2 protein expression, but significantly increased Bax protein expression and the enzyme activity of caspase 3 and 9 in SGEs (Fig. 2D and E). Compared with those in their corresponding NCs, overexpression of miR-155-5p significantly potentiated the effect of IFN- γ on apoptotic protein expression and caspase 3 and 9 enzyme activity, whilst opposite effects were observed following the downregulation of miR-155-5p (Fig. 2D and E). Collectively, these results suggest that the overexpression of miR-155-5p aggravates IFN- γ -induced apoptosis, whereas miR-155-5p knockdown reversed IFN- γ -induced apoptosis in SGEs.

miR-155-5p promotes IFN- γ -induced inflammation in SGEs. The association between miR-155-5p and inflammation in IFN- γ -treated SGEs was assessed. Compared with those in control, treatment with IFN- γ significantly increased the mRNA expression levels of IL-6 and TNF- α in SGEs, which was significantly enhanced further following overexpression of miR-155-5p (Fig. 3A). By contrast, this phenomenon was reversed by miR-155-5p knockdown (Fig. 3A). Similar results were obtained according to results from ELISA (Fig. 3B). Taken together, these results suggest that overexpression of miR-155-5p may promote IFN- γ -induced inflammation, whilst miR-155-5p knockdown may alleviate IFN- γ -induced inflammation in SGEs.

miR-155-5p activates the NF- κ B signaling pathway in IFN- γ -treated SGEs. The effects of miR-155-5p knockdown and overexpression on the NF- κ B signaling pathway in IFN- γ -treated SGEs were next assessed. Western blot analysis demonstrated that the p-I- κ B/I- κ B ratio and the nuclear/cytoplasmic ratio of p65 were significantly increased following treatment with IFN- γ , which was significantly potentiated following the transfection with miR-155-5p

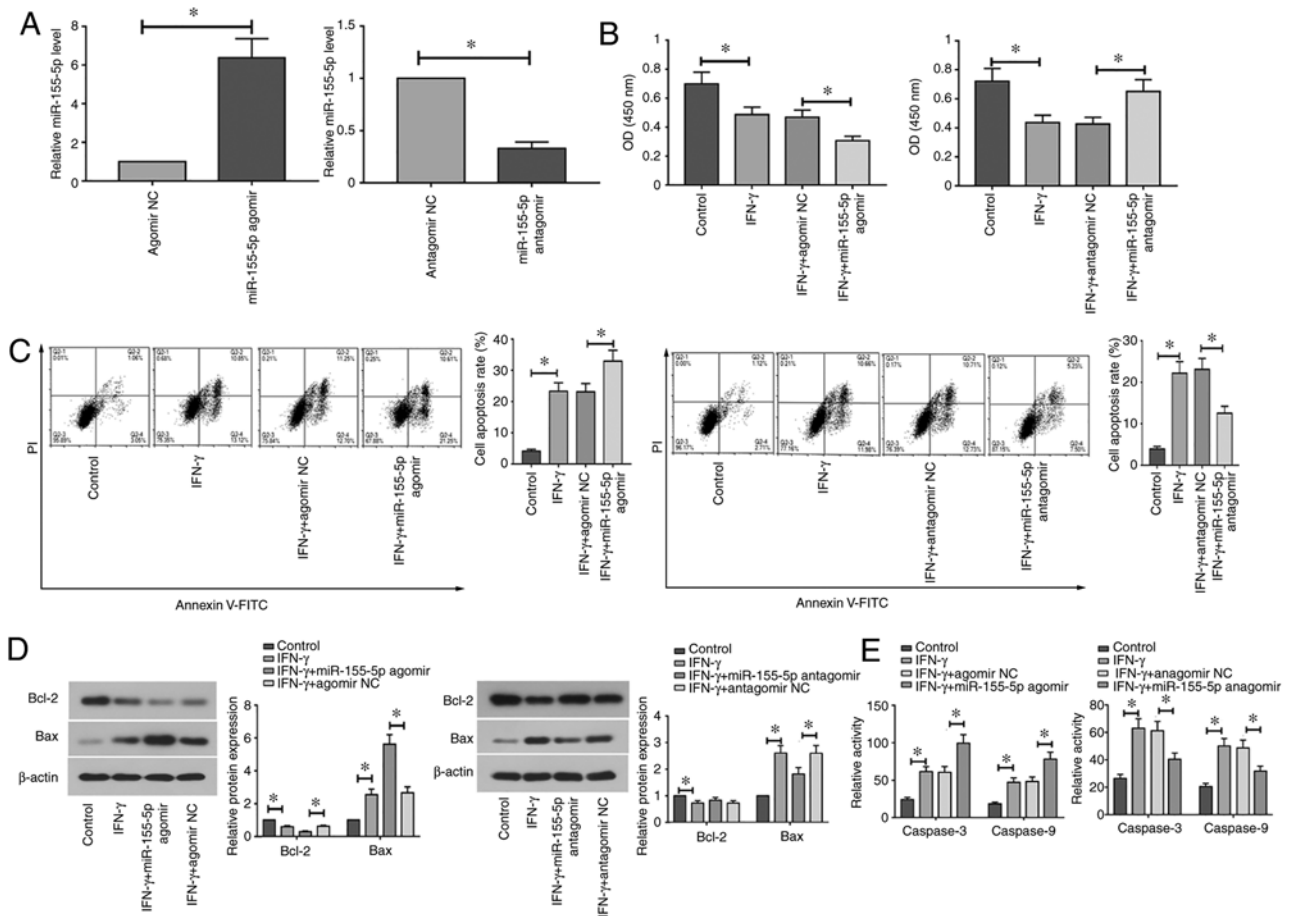


Figure 2. miR-155-5p overexpression promotes IFN- γ -induced apoptosis in SGECs. (A-E) SGECs were transfected with miR-155-5p agomir/agomir NC or miR-155-5p antagomir/antagomir NC for 24 h, before the transfected SGECs were treated with IFN- γ at a concentration of 10 ng/ml for 12 h. (A) The efficiency of miR-155-5p agomir or antagomir transfection was detected by reverse transcription-quantitative PCR. (B) Cell viability of SGECs was detected by Cell Counting Kit-8 assay. (C) Early and late apoptotic cells were assessed by flow cytometry. The regions of Q2-2 + Q2-4 represent the apoptotic cells. (D) The expression of Bcl-2 and Bax was detected by western blotting. (E) Caspase-3 activity and caspase-9 activity were measured. All data were presented as mean $\pm$ SD, n=3. *P<0.05. SGECs, salivary gland epithelial cells; NC, negative control; IFN- γ , interferon- γ ; miR, microRNA; OD, optical density.

agomir (Fig. 4A and B). By contrast, they were significantly reversed following transfection with miR-155-5p antagomir (Fig. 4A and B). Immunofluorescence staining demonstrated that the overexpression of miR-155-5p promoted the nuclear translocation of NF- κ B p65 (total non-phosphorylated version) in IFN- γ -treated cells, whilst miR-155-5p knock-down resulted in the opposite effect being observed (Fig. 4C). Following the transfection with miR-155-5p agomir/agomir NC, SGECs were treated with IFN- γ (10 ng/ml) and PDTC (100 μ M). The results demonstrated that the blockade of NF- κ B signaling by PDTC significantly decreased the expression levels of IL-6 and TNF- α in miR-155-5p-overexpressed SGECs (Fig. 4D). Collectively, these results suggest that miR-155-5p overexpression aggravates IFN- γ -induced NF- κ B signaling in SGECs.

ARRB2 is a downstream target gene of miR-155-5p. The binding sites of miR-155-5p on *ARRB2* were predicted using TargetScan 7.2 (http://www.targetscan.org/vert_72), where miR-155-5p was predicted to target *ARRB2* directly (Fig. 5A). Dual-luciferase reporter assay results showed that luciferase activity in miR-155-5p agomir + WT 3'UTR group was significantly decreased compared with that in the miR-155-5p

agomir + MT 3'UTR and Agomir NC + WT 3'UTR groups (Fig. 5A). The association between miR-155-5p expression and *ARRB2* was subsequently assessed. The results demonstrated that *ARRB2* mRNA and protein expression levels were significantly inhibited in IFN- γ -treated SGECs following transfection with miR-155-5p agomir compared with those in IFN- γ -treated cells transfected with NC agomir (Fig. 5B and C). Conversely, *ARRB2* mRNA and protein expression levels were significantly elevated in IFN- γ -treated SGECs following transfection with miR-155-5p antagomir compared with those in IFN- γ -treated cells transfected with NC antagomir (Fig. 5B and C). Subsequently, the *ARRB2* overexpression plasmid and miR-155-5p agomir were co-transfected into SGECs before IFN- γ (10 ng/ml) was used to treat the transfected cells for 12 h. The transfection efficiency of the *ARRB2* plasmid into SGECs was first verified by western blotting (Fig. 5D). In the presence of both IFN- γ and miR-155-5p mimics, overexpression of *ARRB2* significantly reduced the expression levels of IL-6 and TNF- α (Fig. 5E). In addition, the overexpression of *ARRB2* significantly suppressed the miR-155-5p overexpression-induced apoptosis in IFN- γ -treated SGECs (Fig. 5G). Translocation of NF- κ B p65 from the cytoplasm to the nucleus, which was

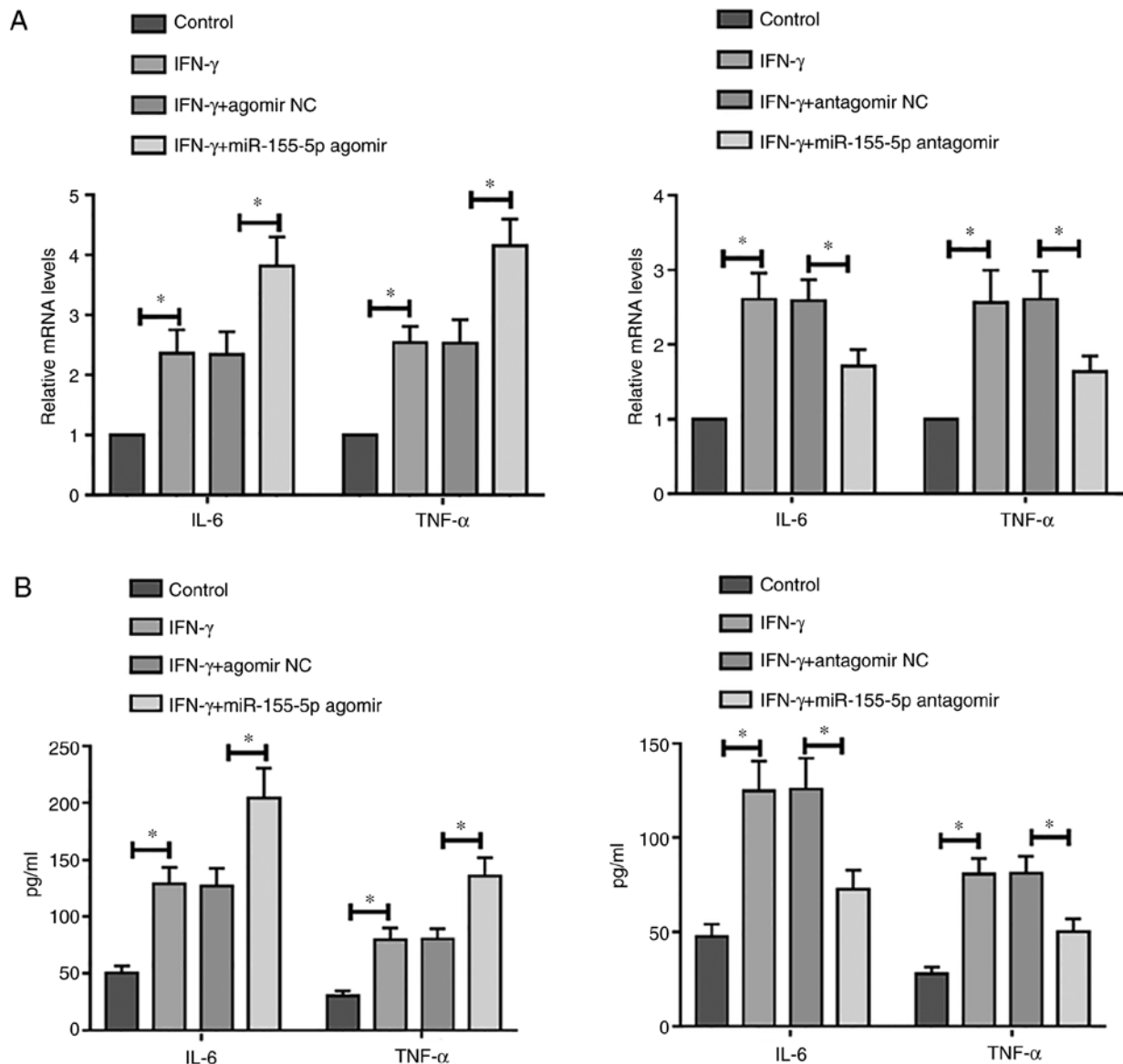


Figure 3. miR-155-5p promotes IFN- γ -induced inflammation in SGEs. (A and B) SGEs were transfected with miR-155-5p agomir/agomir NC or miR-155-5p antagomir/antagomir NC for 24 h, before the transfected SGEs were treated with IFN- γ at a concentration of 10 ng/ml for 12 h. (A) IL-6 and TNF- α mRNA expression was assessed using reverse transcription. (B) Levels of IL-6 and TNF- α in cell culture supernatant were quantified using ELISA. All data were presented as mean $\pm$ SD, $n=3$. * $P<0.05$. SGEs, salivary gland epithelial cells; IFN- γ , interferon- γ ; NC, negative control; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α .

observed to be induced by the overexpression of miR-155-5p, was also significantly abrogated following the overexpression of ARRB2 in IFN- γ -treated SGEs (Fig. 5F). Taken together, these results suggest that ARRB2 may partially or completely mediate the effects of miR-155-5p on inflammation and apoptosis in IFN- γ -treated SGEs.

Discussion

The results of the present study demonstrated that treatment with IFN- γ increased miR-155-5p expression, such that apoptosis and inflammation in IFN- γ -treated SGEs may be induced by this increased miR-155-5p expression. Furthermore, it was demonstrated that miR-155-5p may activate NF- κ B signaling by negatively regulating ARRB2, thereby promoting salivary gland damage in SS.

SS is an autoimmune disease, particularly in the exocrine glands, such as salivary and lacrimal glands (34). However, the pathogenesis of SS remains unclear. Therefore, it is necessary to study the pathogenesis of SS and its potential therapeutic targets. It has been previously demonstrated that miRNAs can regulate in immune responses, including infection and autoimmunity (35,36). Previous studies have also reported that miR-155-5p exerts an important regulatory role in the generation of humoral and cellular immune responses during infection and autoimmunity (37,38).

Salivary gland damage is a common clinical symptom of SS (39). Previous studies have demonstrated that patients with SS and animal models of SS exhibit secretory dysfunction, particularly in the salivary gland epithelium (40,41). In addition, inflammation and apoptosis of SGEs have also been reported to be a possible mechanism for impaired secretory function (42).

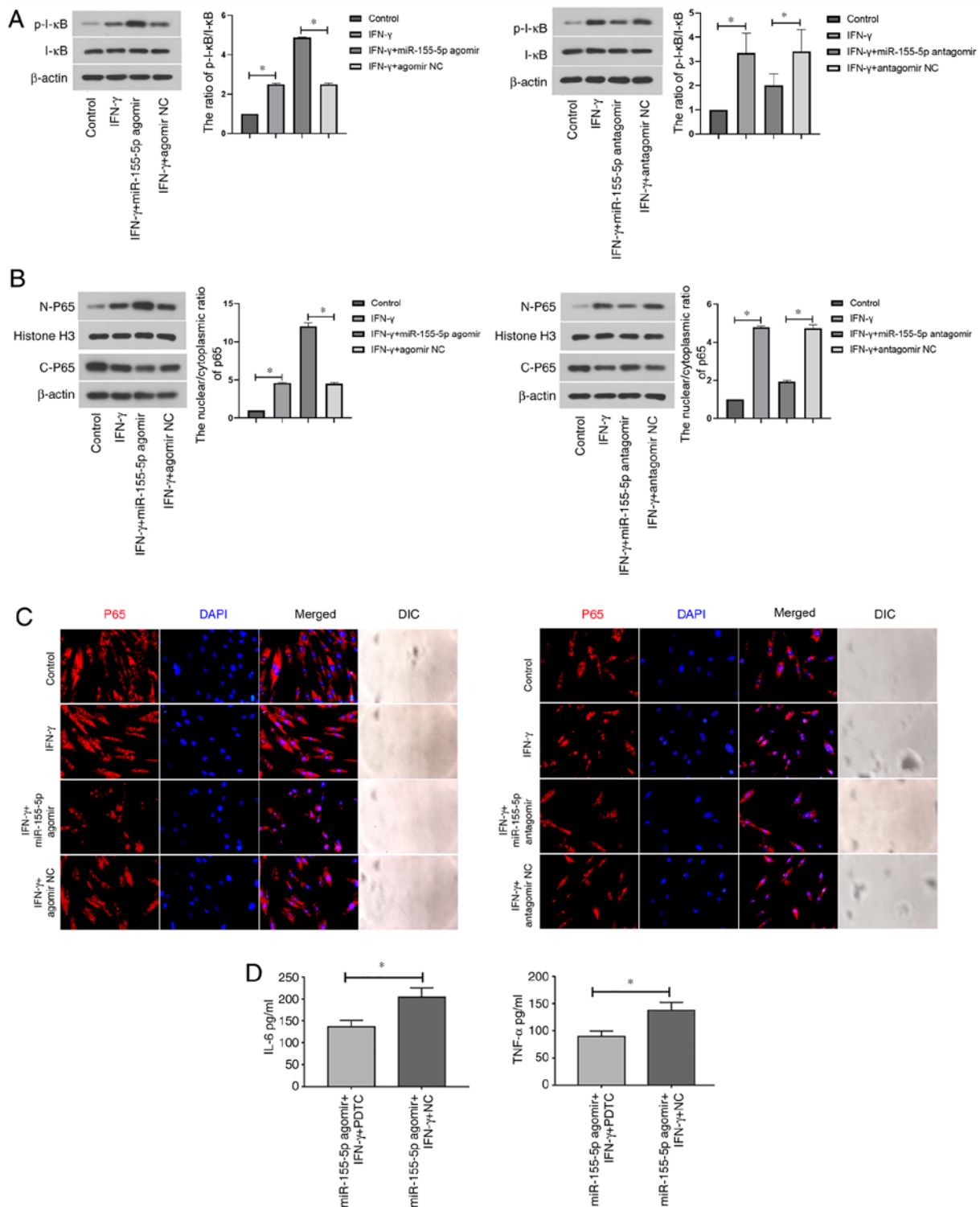


Figure 4. miR-155-5p activates the NF-κB signaling pathway in IFN-γ-treated SGEs. (A-C) SGEs were transfected with miR-155-5p agomir/agomir NC or miR-155-5p antagonist/antagonist NC for 24 h, before the transfected SGEs were treated with IFN-γ at a concentration of 10 ng/ml for 12 h. Expression of (A) p-I-κB, I-κB and (B) activation of NF-κB p65 was measured by western blotting. (C) Immunofluorescence staining and DIC images of p65 in each group were shown (magnification, x400). (D) miR-155-5p agomir/agomir NC-transfected SGEs were treated with IFN-γ (10 ng/ml) and PDTC (100 μM) for 12 h, before the levels of IL-6 and TNF-α were detected by ELISA. All data were presented as mean ± SD, n=3. *P<0.05. SGEs, salivary gland epithelial cells; n-, nuclear; c-, cytoplasmic; NC, negative control; IFN-γ, interferon-gamma; PDTC, pyrrolidine dithiocarbamate; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; p-, phosphorylated; I-κB, inhibitor of NF-κB; DIC, differential interference contrast.

Release of proinflammatory cytokines, including TNF-α and IFN-γ, in the exocrine glands of patients with SS and apoptosis of SGEs significantly increases (43,44). The present study investigated the effects of miR-155-5p on the apoptosis and

inflammation in SGEs. Previous studies have demonstrated that miR-155-5p expression is positively associated with primary SS (19). High levels of miR-155-5p have also been reported in inflammatory lesion models, such as cerebral

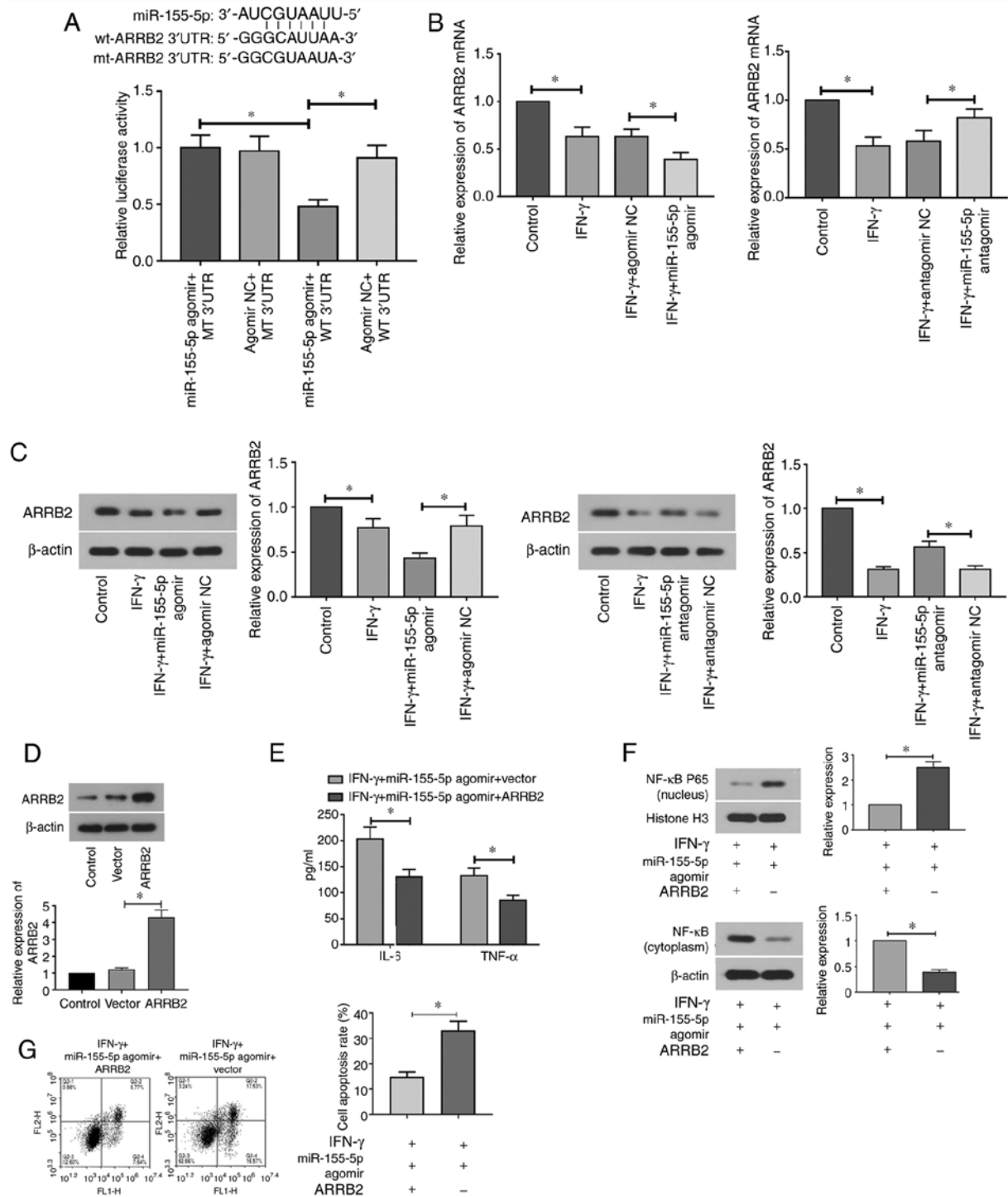


Figure 5. ARR2 is a downstream target gene of miR-155-5p. (A) The specific binding site of miR-155-5p on ARR2 is shown, where the interaction between miR-155-5p and ARR2 was assessed using the dual-luciferase activity assay. (B) mRNA levels of ARR2 in IFN- γ -treated SGEs after miR-155-5p agomir or antagomir transfection were measured using reverse transcription-quantitative PCR. (C) Protein levels of ARR2 in IFN- γ -treated SGEs after miR-155-5p agomir or antagomir transfection were determined by western blotting. (D) Overexpression efficiency of ARR2 in salivary gland epithelial cells was measured by western blotting. (E-G) SGEs were co-transfected with ARR2 vector and miR-155-5p agomir before they were treated with IFN- γ (10 ng/ml) for 12 h. (E) The levels of IL-6 and TNF- α were detected by ELISA. (F) The expression and activation of NF- κ B p65 was measured by western blotting. (G) Both early and late apoptotic cells were assessed by flow cytometry. The regions of Q2-2 + Q2-4 represent the apoptotic cells. All data were presented as mean $\pm$ SD, $n=3$. * $P<0.05$. ARR2, β -arrestin 2; IFN- γ , interferon- γ ; SGEs, salivary gland epithelial cells; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; miR, microRNA; WT, wild type; mut, mutant; UTR, untranslated region.

ischemia-reperfusion injury (45). As previously reported, an inflammatory lesion model was established in SGEs by treatment with IFN- γ , where IFN- γ -treatment increased apoptosis and IL-6 and TNF- α mRNA expression (30).

Results from the present study demonstrated that miR-155-5p overexpression promoted IFN- γ -induced apoptosis in SGEs, since cell viability was decreased and the apoptotic rate was increased, in addition to the increased expression levels of

apoptosis-related proteins in miR-155-5p overexpressing cells. These results also demonstrated that miR-155-5p overexpression promoted IFN- γ -induced inflammation, which was evidenced by the increased IL-6 and TNF- α levels in miR-155-5p overexpressing cells. Overall, these results suggest that miR-155-5p may exert a role in salivary gland damage during SS by promoting the inflammatory response and apoptosis of SGEs.

NF- κ B is chronically active in several inflammatory autoimmune diseases, including inflammatory bowel disease (46), rheumatoid arthritis (47) and SS (48). Sisto *et al* (49) demonstrated that the NF- κ B signaling pathway is activated in human SGEs derived from active primary patients with SS. Lisi *et al* (50) reported that activation of NF- κ B signaling is a potentially important mechanism for SS development. Furthermore, it has been demonstrated that dysregulation of NF- κ B in glandular epithelial cells results in Sjogren's-like features (51). Activation of NF- κ B signaling promotes inflammation and induces apoptosis of human SGEs in primary SS (52). Proinflammatory cytokines, such as IFN- γ , activate the I κ B kinase complex, which phosphorylates I κ B and targets it for proteasomal degradation (53). This releases NF- κ B which, after phosphorylation, allows it to translocate into the nucleus (53). NF- κ B either acts alone in the nucleus or with other transcription factors to induce target gene expression (53). The results of the present study demonstrated that the phosphorylation levels of I κ B and the nuclear translocation of p65 were increased, suggesting that miR-155-5p activates NF- κ B signaling. Taken together, these results suggest that miR-155-5p may promote salivary gland damage in SS by regulating the NF- κ B signaling pathway.

ARRB2 is a downstream target gene of miR-155-5p (54). The results of the present study verified this association. Li *et al* (55) demonstrated that the overexpression of ARRB2 may inhibit the release of proinflammatory cytokines and decrease experimental arthritis severity. In addition, ARRB2 has exhibited antiapoptotic effects in human endometrial cancer heterotransplants in nude mice (56,57). The results of the present study demonstrated that overexpression of ARRB2 reversed the effects of miR-155-5p overexpression on the inflammatory response, apoptosis and the NF- κ B signaling pathway in this inflammatory lesion model. ARRB2 has been previously reported to inhibit the NF- κ B signaling pathway in a sepsis mouse model and LPS-induced liver injury (23,26). Collectively, these results suggest that miR-155-5p may promote salivary gland damage in SS by negatively regulating ARRB2. Notably, miR-155-5p and IFN- γ can influence each other, whereby the overexpression of miR-155-5p increased the production of IFN- γ (58). IFN- γ has been demonstrated to induce miR-155-5p expression in human dermal lymphatic endothelial cells (58-60). Results of the present study demonstrated that the treatment with IFN- γ induced miR-155-5p expression, indicating that IFN- γ may in part induce the apoptosis and inflammation of SGEs by regulating miR-155-5p expression.

In conclusion, functional studies in the present study demonstrated that miR-155-5p overexpression can promote IFN- γ -induced apoptosis and inflammation in SGEs. Mechanistic studies have indicated that miR-155-5p activates NF- κ B signaling by negatively regulating ARRB2, thereby

promoting salivary gland damage of SS. The results of the present study verified the role and the potential molecular mechanism of miR-155-5p in salivary gland damage in SS, suggesting that miR-155-5p may serve to be a potential target for SS treatment.

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Availability of data and materials

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

Authors' contributions

JLZ and HZZ designed the study and wrote the manuscript. LLZ and HS performed the experiments, confirmed the authenticity of all the raw data and conducted statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University (Mudanjiang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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