miR-142-5p regulates lipopolysaccharide-induced bovine epithelial cell proliferation and apoptosis via targeting BAG5

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Abstract. Bovine mastitis is a threat to the health of the dairy cow. MicroRNAs (miRs) serve an important role in the progression of bovine mastitis, regulating immune and defense responses. The present study aimed to investigate the possible effects and mechanisms of bovine mastitis underlying miR-142-5p and Bcl-2 associated athanogene 5 (BAG5) in *in vitro* lipopolysaccharide (LPS)-induced models. Reverse transcription-quantitative PCR and western blotting were performed to determine mRNA and protein expression levels, respectively. ELISAs were conducted to assess the levels of cytokines and an immunofluorescence assay was performed to determine the expression of BAG5. Cell Counting Kit-8, clone formation and 5-ethynyl-2'-deoxyuridine assays were conducted to determine cell viability and proliferation of bovine mammary epithelial MAC-T cells, respectively. Flow cytometry was performed to measure MAC-T cell cycle distribution and apoptosis, and a luciferase assay was conducted to verify whether BAG5 was a target of miR-142-5p. The results indicated that miR-142-5p was upregulated in MAC-T cells treated with LPS compared with the control group. miR-142-5p mimics transfection significantly activated the cytokines TNF-α, IL-1β, IL-6 and IL-8, and significantly increased the expression levels of NF-κB signaling pathway-related proteins in LPS-treated cells. The luciferase activity of MAC-T cells treated with miR-142-5p mimics and BAG5 3'untranslated region wild type decreased, compared with mutant type. By contrast, BAG5 overexpression significantly downregulated the levels of cytokines, including TNF-α, IL-1β, IL-6 and IL-8, in LPS-treated cells. BAG5 overexpression significantly promoted cell proliferation and viability, decreased apoptosis, and regulated Caspase-3, Caspase-9, Bcl-2 and Bax expression in LPS-treated MAC-T cells, which was significantly reversed by transfection with miR-142-5p mimics. In conclusion, the results of the present study suggested that miR-142-5p may promote the progression of bovine mastitis via targeting BAG5. Therefore, the present study provided the foundations for future investigations.

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Introduction

Bovine mastitis is one of the most prevalent diseases in dairy cows, and reduces milk production and quality (1). The results of bovine mastitis include lowered milk production, increased veterinary drug cost and high culling rate. In 2020, the economic cost of bovine was ~$2 billion in the US (1-3). *Escherichia coli* (*E. coli*) is a crucial factor for bovine mastitis that can result in intramammary infection. Frequent use of antibiotics to treat such infections contributes to the emergence of antibiotic resistance (2,3). Lipopolysaccharide (LPS), the primary cell membrane component of gram-negative bacteria, including *E. coli*, promotes intracellular signaling via Toll-like receptor 4 proteins, activating NF-κB and augmenting the secretion of inflammatory cytokines, which may further promote the progression of bovine mastitis (4-6). Mammary gland epithelial tissue is a crucial barrier against pathogens that serves an important role in milk synthesis and secretion (7). Therefore, investigation of the potential mechanisms employed by bovine epithelial cells against pathogens is of importance to understand and prevent inflammation (8).

MicroRNAs (miRNAs/miRs) are small, endogenous, non-coding RNAs that are 18-22 nucleotides in length (9). miRNAs serve an important role in post-transcriptional control, regulation and modification, for example, repressing gene expression (10). Increasing evidence suggests that miRNAs serve a predominant role in the pathogenesis of numerous diseases, including bovine mastitis, via regulating cell proliferation, apoptosis, and immune and defense responses (11,12). For instance, bovine miR-146a enhances the immune response of mammary epithelial cells (13). Moreover, miR-92a serves as a housekeeping gene to analyze the miRNA involved in bovine mastitis by reverse transcription-quantitative PCR (RT-qPCR) (14). A previous study has also demonstrated that

miR-142-5p is upregulated in bovine mastitis (15). However, the underlying mechanisms have not been fully elucidated.

In the present study, the possible roles of miR-142-5p in bovine mastitis and the mechanisms underlying miR-142-5p-mediated regulation of LPS-treated MAC-T cell proliferation, apoptosis and immune responses were investigated. The present study may provide a potential target for novel treatment of bovine mastitis.

Materials and methods

Cell culture. Bovine mammary epithelial MAC-T cells were obtained from ATCC were incubated in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2 and humidity.

Transfection. miR-142-5p mimics, miR-142-5p mimics negative controls (NC), miR-142-5p inhibitors, miR-142-2-5p inhibitors negative control (NC), pcDNA3.1 and pcDNA3.1-Bcl-2 associated athanogene 5 (BAG5) were purchased from Shanghai GenePharma Co., Ltd. Cells were seeded in a 6-well plate with a density of 10^5 cells/ml and pre-incubated for 12 h at 37˚C, before being transfected with 50 nM miR-142-5p mimics or miR-142-5p mimics NC, 2 μg pc DNA3.1 or pcDNA3.1-BAG5, 50 nM miR-142-5p inhibitors or miR-142-5p inhibitors NC with 20 nmol/l Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) then incubated at 37˚C with 5% CO_2 for 48 h. Cells were harvested 48 h after transfection for subsequent experiments. The sequences of the miRNA constructs were as follows: miR-142-5p mimics NC, 5'-GUGUAACACGUCUAUACGCCCA-3'; miR-142-5p mimic, 5'-CAUAAAGUAGAAAGCACUACU-3'; miR-142-5p inhibitors NC, 5'-UCACAACCUCCUAGAAAGAGU-3'; miR-142-5p inhibitors, 5'-AGUAGUGCUUUCUACUUUAUG-3'.

RT-qPCR. Total RNA was extracted from MAC-T cells using TRIzol® (Invitrogen, Thermo Fisher Scientific, Inc). Reverse transcription was performed using a reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific, Inc.) at 72˚C for 12 min. qPCR was performed using a SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) conducted using the ABI 7900HT real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 5 min, denaturation at 95˚C for 15 sec and annealing and extension at 60˚C for 45 sec, for 40 cycles. Relative miRNA and mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (16) and normalized to the internal reference genes U6 and GAPDH, respectively. The sequences of the primers used were as follows: miR-142-5p forward (F), 5'-GAAGATCTCCAGCCACCTGTTTCACA-3' and reverse (R), 5'-CCGCTCGAGTAGTCCTTCACTTCATG-3'; U6 F, 5'-GCGCGTCGTGAAGCGTTC-3' and R, 5'-GTGCAGGGTCCGAGGT-3'; BAG5 F, 5'-AGGTGTCCCCGGGTTTAG-3' and R, 5'-GATGTTGGTTTCCCATATCCA-3'; GAPDH F, 5'-ATGGAAATCCCATCACCATCTT-3' and R, 5'-CGGCCCACTTGATTTTGG-3'.

Western blotting. MAC-T cells were harvested and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was extracted from MAC-T cells. The concentration of total protein was determined using the BCA assay (Abcam). Each protein (20 μg protein/lane) was isolated using a 12% SDS-PAGE gel. The separated proteins were then transferred onto PVDF membranes, which were blocked with 5% skimmed milk overnight at 4˚C. The membranes were incubated at 37˚C for 2 h with primary antibodies targeted against the following: P21 (1:1,000; cat. no. ab109199; Abcam), P27 (1:1,000; cat. no. ab75908; Abcam), Cyclin D1 (1:200; cat. no. ab16663; Abcam), BAG5 (1:1,000; cat. no. ab182658; Abcam), Caspase-3 (1:5,000; cat. no. ab32351; Abcam), cleaved-Caspase-3 (1:500; cat. no. ab13847; Abcam), cleaved-Caspase-9 (1:1,000; cat. no. ab2324; Abcam), Caspase-9 (1:1,000; cat. no. ab184786; Abcam), Bcl-2 (1:1,000; cat. no. ab32124; Abcam), Bax (1:2,000; cat. no. ab32503; Abcam), AKT (1:10,000; cat. no. ab179463; Abcam), phosphorylated (p)-AKT (1:1,000; cat. no. ab38449; Abcam), p65 (1:5,000; cat. no. ab32536; Abcam), p-p65 (1:1,000; cat. no. ab28856; Abcam), GAPDH (1:10,000; cat. no. ab181602; Abcam). Then the membranes were washed with PBS plus 0.1% Tween 20 at room temperature for three times and incubated with HRP-conjugated secondary antibodies (1:5,000; cat. no. ab6721; Abcam) at room temperature for 1 h. Protein bands were visualized using an ECL kit (GE Healthcare) and protein expression was semi-quantified using ImageJ software (version 1.6; National Institutes of Health). GAPDH was used as a loading control.

Dual-luciferase reporter assay. BAG5 was predicted to be a target gene of miR-142-5p based on analysis with TargetScan (http://www.targetscan.org, v7.2), which yielded a context++ score (The context++ score for a specific site is the sum of the contribution of a series of features). The 3'-untranslated region (3'UTR) of BAG5 was amplified via PCR from bovine mammary epithelial MAC-T cell cDNA and then inserted into the multiple cloning site downstream of the luciferase reporter gene in the pMIR-REPORT™ luciferase plasmid (Thermo Fisher Scientific, Inc.) to construct the luciferase reporter plasmid [BAG5 3'UTR wild-type (WT)]. MAC-T cells were transfected with 1 μg 3'UTR WT or mutant (MUT) constructed by chemical synthesis (General Biosystems Co., Ltd.) and 50 nM miR-142-5p mimics or miR-142-5p mimics NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells transfected BAG5 3'UTR WT or MUT plasmid served as blank group. After 24 h incubation at 37˚C, the cells were lysed by lysis buffer as supplied by the Dual-Luciferase Detection kit (Beyotime Institute of Biotechnology) on ice and luciferase activity was measured using a Dual-Lumi II Luciferase Reporter Gene Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Firefly luciferase activities were normalized to *Renilla* luciferase activities.

Cell Counting Kit-8 (CCK-8) assay. At 48 h post-transfection, MAC-T cells were plated into 96 well plates ($2x10^3$ cells/well). MAC-T cells were then treated with 10 μl CCK-8 reagent (Beyotime Institute of Biotechnology) at 37˚C for 2 h and the cell viability detected at 24, 48 and 72 h. The absorbance rate

of each compound was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). Each experiment was performed in triplicate.

Flow cytometry assay. Transfected MAC-T cells were seeded into 24-well plates ($5x10^4$ cells/well) and treated with 500 μl binding buffer, with 5 μl propidium iodide (PI) and Annexin V-FITC from Annexin V-APC/PI Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd.) in the dark for 15 min at room temperature. The stained MAC-T cells were analyzed using flow cytometry (FACScan; BD Biosciences) and the apoptotic rate was determined using FlowJo 7.6.1 software (Tree Star, Inc.).

For cell cycle analysis, cells were fixed with 70% ethanol at 4˚C overnight. The cells were treated with 10 mg/ml RNase A (Beijing Solarbio Science & Technology Co., Ltd.), 400 mg/ml PI (Beyotime Institute of Biotechnology) and 0.1% Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd.) in the dark for 30 min at 37˚C. The results were evaluated via flow cytometry (Cytoflex; Beckman Coulter, Inc.) and quantified by BD CellFIT software (v 7.6.2; BD Biosciences).

Clone formation assay. MAC-T cells were seeded into 6-well plates ($2x10^3$ cells/well) and the culture was terminated when clonal cell cluster became visible to the naked eye and contained >50 cells. Following this, cells were fixed with 10% formaldehyde and 1% crystal violet for 30 min at 25˚C. Colony formation was determined using a light microscope in 10 randomly chosen fields and quantified by ImageJ software v 1.5.3 (National Institutes of Health).

ELISA. MAC-T cells were seeded into a 24-well plate ($5x10^4$ cells/well). The levels of cytokines was determined using TNF-α (TWp024586); IL-1β (TWp023753); IL-6 (TWp023756); IL-8 (TWp023757) and IL-10 (TWp002476) ELISA kits (Shanghai Tongwei Industrial Co., Ltd.) according to the manufacturer's protocol.

5-Ethynyl-2'-deoxyuridine (EdU) assay. An EdU assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to determine MAC-T cell viability. Cells were plated into a 24-well plate ($5x10^4$ cells/well). Each well was supplemented with 50 μM EdU for 2 h at 37˚C. Subsequently, MAC-T cells were stained with DAPI (Beyotime Institute of Biotechnology) for 5 min in the dark at room temperature and then cultured with 1X ApolloR reaction cocktail supplied with EdU assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Cell viability was determined using a fluorescent microscope.

Immunofluorescence staining. Cells were seeded into a 24-well plate ($5x10^4$ cells/well) containing slides and incubated for 24 h at 37˚C with 5% CO_2, then the slides were taken out. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, blocked with 5% BSA for 30 min at room temperature and incubated with an anti-BAG5 primary antibody (1:1,000; cat. no. ab182658; Abcam) overnight at 4˚C, followed by incubation with a secondary goat-anti-rabbit antibody (1:5,000; cat. no. ab6721; Abcam) at room temperature for 1 h in the dark. Cells were then stained with DAPI for 5 min at room temperature (Beyotime Institute of Biotechnology). Images were captured using a fluorescent microscope (Nikon Corporation) with Zen imaging software 2.3 (Carl Zeiss AG).

Statistical analysis. Data are presented as the mean ± SD of at least three independent experiments. Statistical analyses were performed using SPSS 19.0 (IBM Corp.). Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-142-5p. The expression of miR-142-5p was significantly upregulated or downregulated following transfection with miR-142-5p mimics or miR-142-5p inhibitors compared with other treatments, respectively (Fig. 1A). Additionally, compared with the blank groups, the fluorescence intensity of GFP notably increased or decreased following transfection with miR-145-5p mimics or miR-142-5p inhibitors, respectively (Fig. 1B). Furthermore, the expression of miR-142-5p in cells treated with LPS was significantly increased, which was significantly increased following transfection with miR-142-5p mimics. By contrast, compared with the LPS group, miR-142-5p expression was significantly lower following transfection with miR-142-5p inhibitors (Fig. 1C).

miR-142-5p regulates the expression of cytokines and NF-κB signaling pathway-related proteins. Cytokines have previously been shown to contribute to the progression of bovine mastitis. Sun *et al* reported that blocking NF-κB pathway significantly relieves bovine mastitis (17). Wang *et al* reported that TNF-α, IL-1β and IL-6 and IL-8 levels are imported indicators in bovine mastitis (18). As shown in Fig. 2A, compared with the LPS group, miR-142-5p mimics significantly increased TNF-α, IL-1β and IL-6 and IL-8 levels, whereas the opposite effect was observed in cells transfected with miR-142-5p inhibitors. Meanwhile, compared with the LPS + miR-142-5p mimics NC group, the protein expression levels of AKT and p65 in cells treated with miR-142-5p mimics + LPS were significantly upregulated, but miR-142-5p inhibitors transfection significantly downregulated LPS-induced protein expression levels, compared to LPS + miR-142-5p inhibitor NC group (Fig. 2B). These results were consistent with the western blotting results for p-AKT and p-p65 protein expression levels (Fig. 2B).

miR-142-5p regulates the progression of LPS-induced MAC-T cells. To further investigate the potential roles of miR-142-5p in bovine mastitis, CCK-8, clone formation and EdU assays were performed to evaluate the effects of miR-142-5p on the proliferation and cell viability of LPS-treated MAC-T cells. As shown in Fig. 3A, LPS significantly suppressed MAC-T cell viability, which was significantly enhanced in the LPS + miR-142-5p mimics group. By contrast, compared with the LPS group, MAC-T cell viability was significantly increased following transfection with miR-142-5p inhibitor. These results were in line with those from clone formation; compared with blank group, LPS-treatment suppressed the

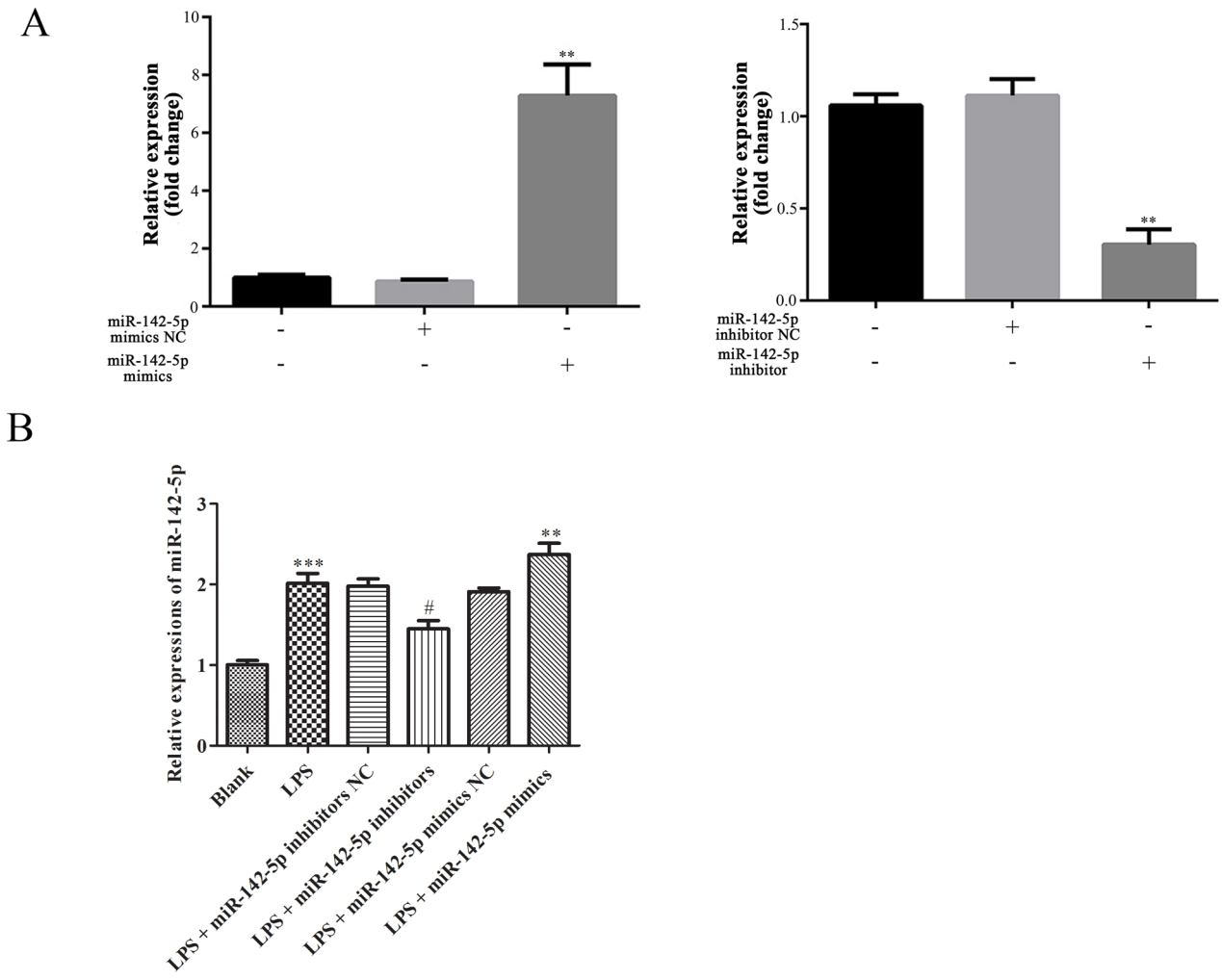

Figure 1. Expression of miR-142-5p. (A) Expression level of miR-142-5p following transfection. (B) Expression level of miR-142-5p. **P<0.01 vs. LPS + miR-142-5p mimics NC, ***P<0.001 vs. Blank, $^{\#}$P<0.05 vs. LPS + miR-142-5p inhibitors NC. miR, microRNA; LPS, lipopolysaccharide; NC, negative control; p, phosphorylated.

clone forming ability of cells, by contrast, compared with the LPS group, the clone forming ability of cells was increased with miR-142-5p inhibitor transfection. Similar results were shown by EdU assay (Fig. 3B and C).

miR-142-5p regulates the cell cycle and apoptosis of MAC-T cells. Compared with the blank group, a marked increase in cells arrested in the G_0/G_1 phase of the cell cycle was observed in the LPS group, which was notably enhanced in the LPS + miR-142-5p mimics group, but obviously reversed in the LPS + miR-142-5p inhibitors group (Fig. 4A). Furthermore, the protein expression levels of P21 and P27 were significantly increased, whereas Cyclin D1 protein expression levels were significantly decreased in the LPS + miR-142-5p mimics group compared with the LPS group (Fig. 4B). By contrast, miR-142-5p inhibitors significantly reversed LPS-mediated effects on protein expression.

To further verify the possible roles of miR-142-5p in regulating MAC-T cells, the rate of apoptosis was also examined. As shown in Fig. 4C, miR-142-5p mimics significantly increased the apoptotic rate in LPS-induced MAC-T cells, whereas miR-142-5p inhibitors significantly reversed LPS-induced MAC-T cell apoptosis. Additionally, miR-142-5p mimics significantly increased the protein expression levels of cleaved-Caspase-3/Caspase-3, cleaved-Caspase-9/Caspase-9 and Bax, and significantly decreased Bcl-2 protein expression levels in LPS-treated MAC-T cells, whereas the opposite effect was observed following miR-142-5p inhibitors transfection (Fig. 4D).

BAG5 is a target of miR-142-5p. Bioinformatics analysis demonstrated BAG5 was a target of miR-142-5p. TargetScan (http://www.targetscan.org/vert_72/) predicted that BAG5 was a target of miR-142-5p, possessing a relatively high Context++ score. The binding sites of miR-142-5p on BAG5 are presented in Fig. 5A. The luciferase reporter assay results suggested that, compared with the miR-142-5p mimics NC group, the luciferase activity of MAC-T cells was significantly reduced after co-transfection with miR-142-5p mimics and BAG5 3'UTR WT, there was no significant difference between the blank group (transfected BAG5 3'UTR WT or MUT plasmid) and miR-142-5p mimics NC groups, but following

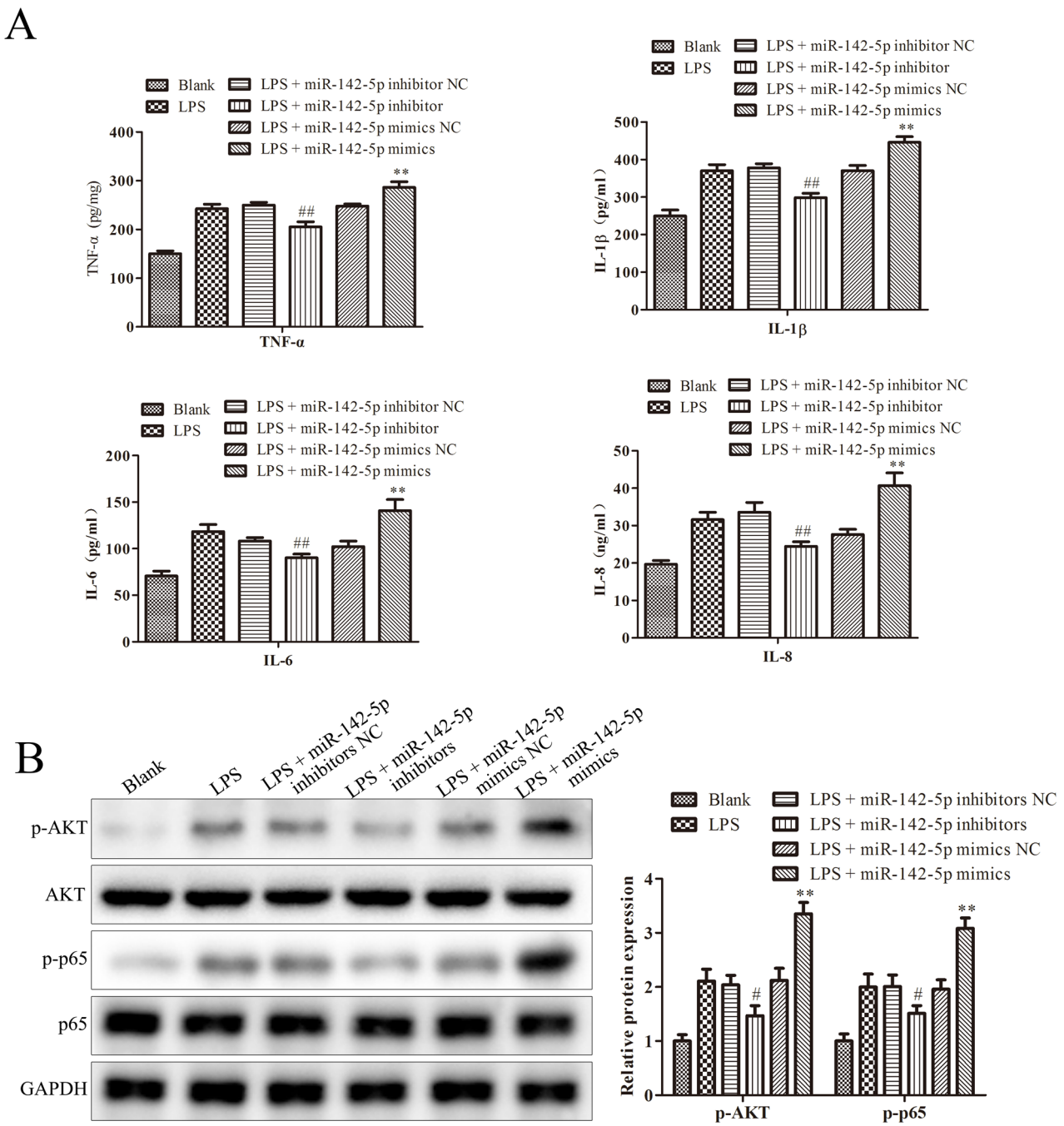

Figure 2. miR-142-5p regulates the expression of cytokines and NF-κB signaling pathway-related proteins. (A) Levels of cytokines. (B) Expression levels of NF-κB signaling pathway-related proteins. **P<0.01 vs. LPS + miR-142-5p mimics NC, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. LPS + miR-142-5p inhibitors NC. LPS, lipopolysaccharide; NC, negative control; miR, microRNA; p, phosphorylated.

co-transfection with miR-142-5p mimics and BAG5 3'UTR MUT, the levels of luciferase activities did not change significantly.

RT-qPCR and western blotting were performed to determine the mRNA and protein expression levels of BAG5, respectively. BAG5 mRNA expression was significantly increased by transfection with Lv BAG5 compared with BAG5 NC (Fig. 5B). As shown in Fig. 5C, the mRNA expression level of BAG5 and, in 5D, the protein expression levels of BAG5 were significantly decreased by miR-142-5p mimics compared with miR-142-5p mimics NC. The immunofluorescence assay demonstrated that the signal density of BAG5 was markedly reduced by miR-142-5p mimics transfection compared with miR-142-5p mimics NC transfection (Fig. 5E).

BAG5 regulates the levels of cytokines. ELISAs were performed to determine the levels of cytokines in the cell culture medium. The results indicated that the level of BAG5 was increased by LPS-induced activation of cytokines, such as TNF-α, IL-1β and IL-6 and IL-8, which was significantly reversed by transfection with miR-142-5p mimics (Fig. 6).

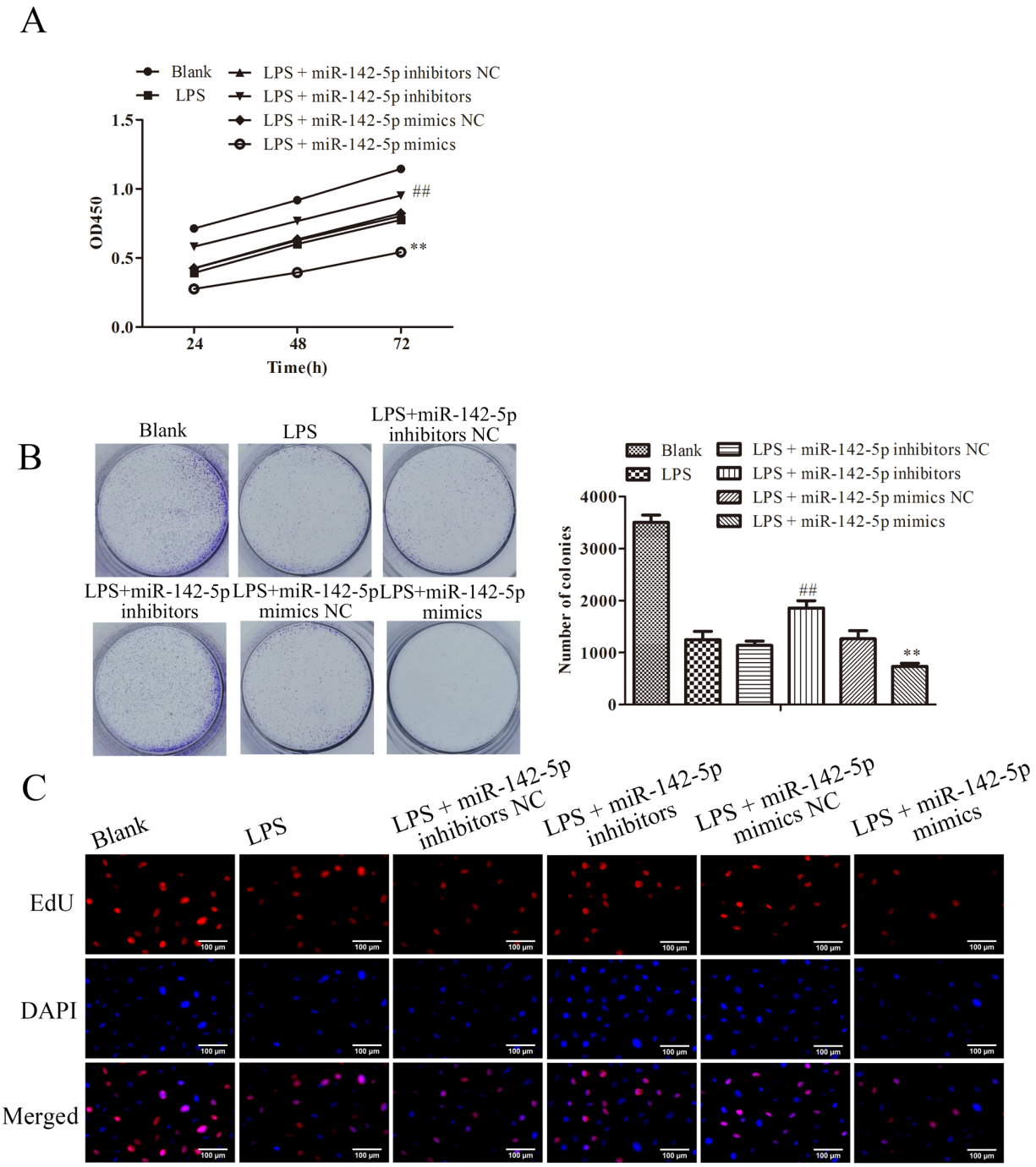

Figure 3. miR-142-5p regulates the proliferation of LPS induced MAC-T cells. (A) Cell viability of MAC-T cells. (B) Proliferation of MAC-T cells was determined by performing the clone formation assay. (C) Proliferation of MAC-T cells was examined by performing the EdU assay. **P<0.01 vs. LPS + miR-142-5p mimics NC, $^{\#\#}$P<0.01 vs. LPS + miR-142-5p inhibitors NC. LPS, lipopolysaccharide; NC, negative control; miR, microRNA; EdU, 5-Ethynyl-2'-deoxyuridine; OD, optical density.

BAG5 regulates the cell viability of LPS-treated MAC-T cells. As shown in Fig. 7A and B, the mRNA and protein expression levels of BAG5 were decreased in cells transfected with LPS treatment, which was reversed by co-transfection with pc-BAG5. The immunofluorescence assay results were consistent with the RT-qPCR and western blotting results (Fig. 7C). The results suggested that Lv BAG5 markedly increased BAG5 expression levels, which was notably reversed by co-transfection with miR-142-5p mimics (Fig. 7D).

BAG5 regulates the proliferation and apoptosis of LPS-treated MAC-T cells. BAG5 overexpression increased cell proliferation in LPS-treated MAC-T cells, which was alleviated by miR-142-5p mimics transfection (Fig. 8A). As shown in Fig. 8B, BAG5 overexpression significantly decreased the apoptotic rate of LPS-treated MAC-T cells, which was significantly reversed by miR-142-5p mimics transfection. Moreover, compared with the LPS group, BAG5 overexpression significantly decreased the protein expression levels of cleaved-Caspase-3/Caspase-3, cleaved-Caspase-9/Caspase-9

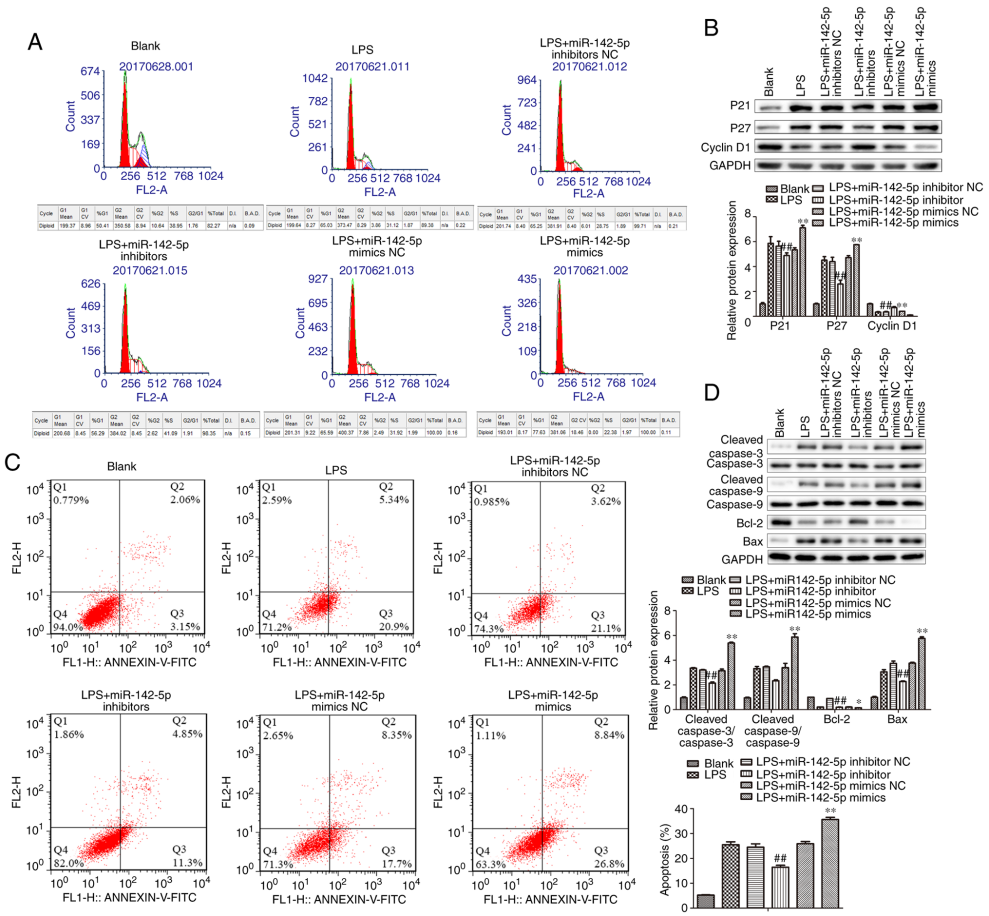

Figure 4. miR-142-5p regulates the apoptosis of LPS-induced MAC-T cells. (A) Cell cycle distribution of MAC-T cells. (B) Expression levels of P21, P27 and Cyclin D1. (C) Apoptotic rate of MAC-T cells. (D) Expression levels of Caspase-3, Caspase-9, Bcl-2 and Bax. *P<0.05, **P<0.01 vs. LPS + miR-142-5p mimics NC, $^{\#\#}$P<0.01 vs. LPS + miR-142-5p inhibitors NC. LPS, lipopolysaccharide; NC, negative control; miR, microRNA.

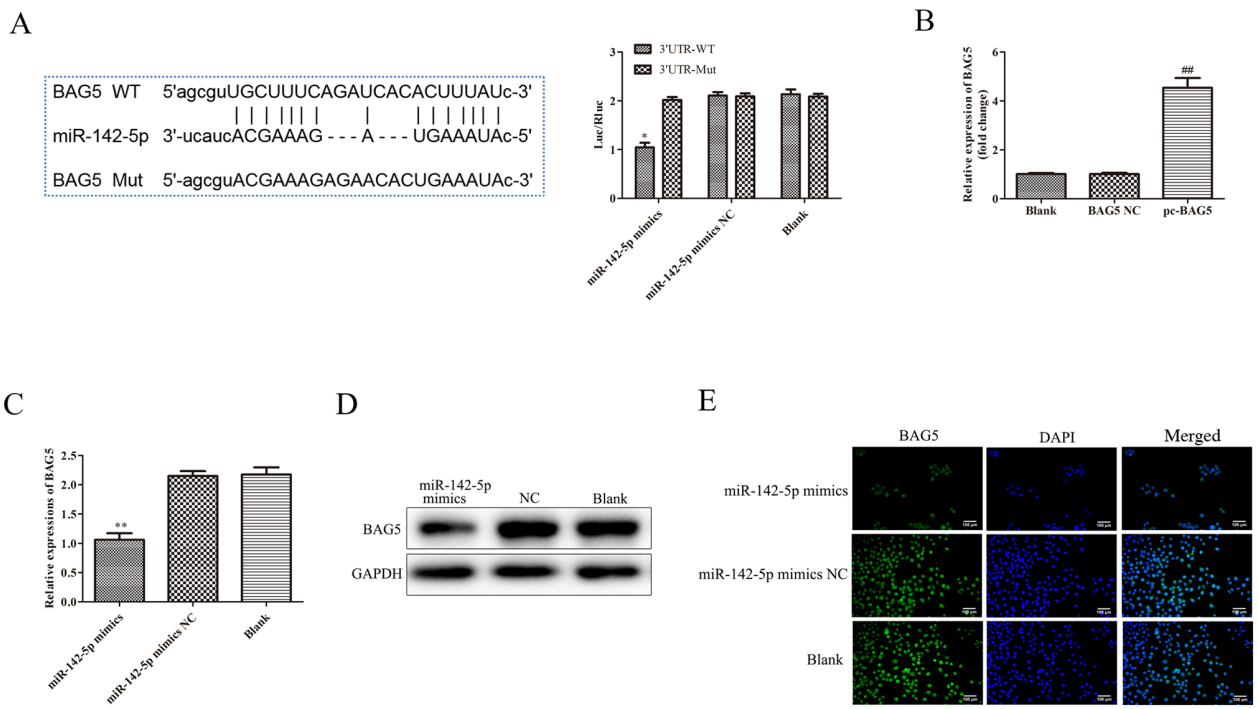

Figure 5. BAG5 is a target of miR-142-5p. (A) BAG5 targeted by miR-142-5p. (B) Expression of BAG5 in cells transfected with pc-BAG5 was significantly upregulated compared with BAG5 NC. (C) mRNA expression level of BAG5. (D) Protein expression level of BAG5. (E) Signal density of BAG5 was reduced after treatment of miR-142-5p mimics, compared with miR-142-5p mimics NC group. *P<0.05, **P<0.01 vs. miR-142-5p mimics NC, $^{\#\#}$P<0.01 vs. BAG5 NC. NC, negative control; miR, microRNA; UTR, untranslated region; MUT, mutant; WT, wild-type; BAG5, Bcl-2 associated athanogene 5.

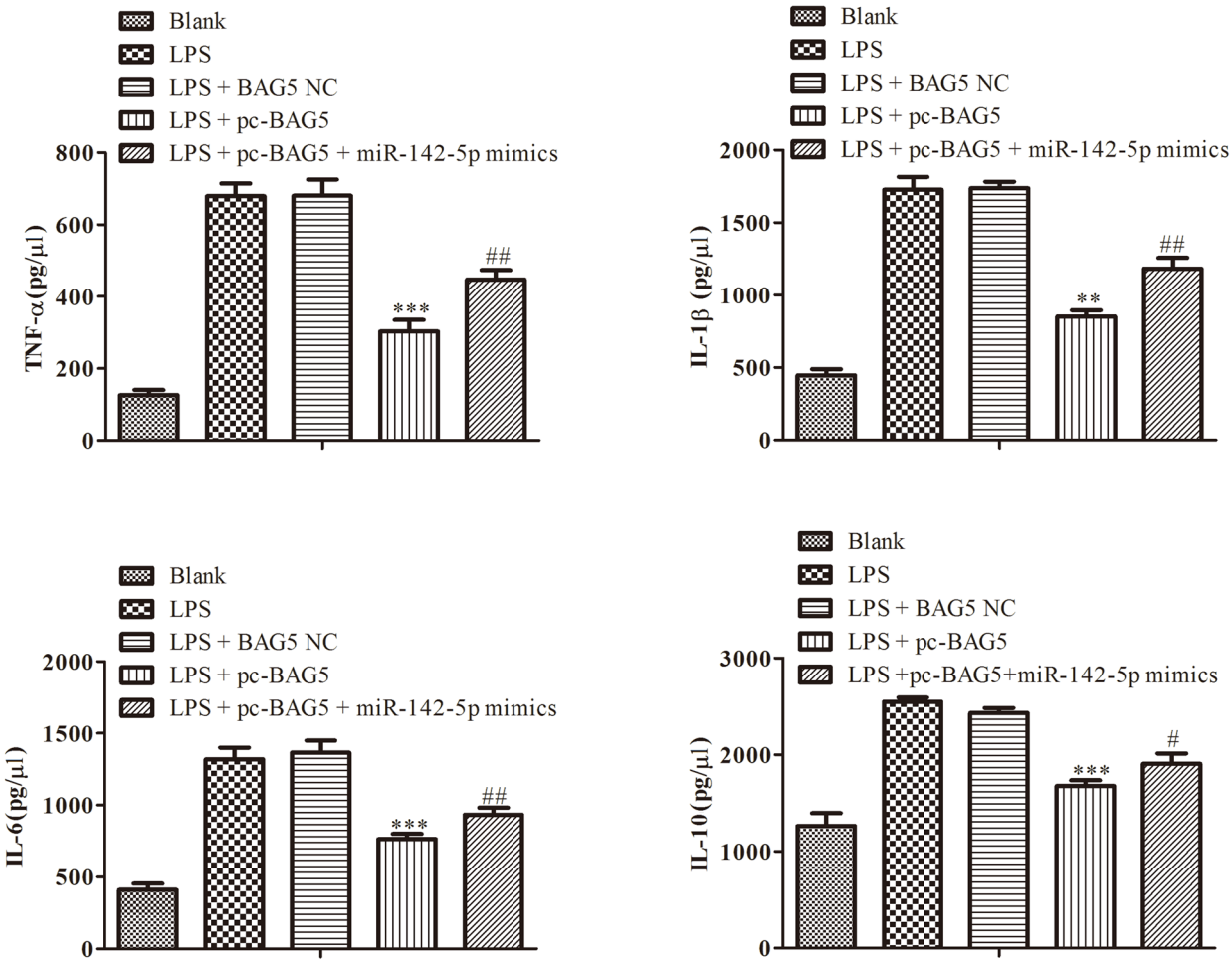

Figure 6. BAG5 regulates the cytokines. Levels of TNF-α, IL-1β, IL-6 and IL-8. **P<0.01 and ***P<0.001 vs. LPS. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS + pc-BAG5 group. LPS, lipopolysaccharide; NC, negative control; miR, microRNA; BAG5, Bcl-2 associated athanogene 5; ns, not significant.

and Bax, and significantly increased Bcl-2 protein expression levels in LPS-treated MAC-T cells, which was significantly reversed by miR-142-5p mimics transfection (Fig. 8C).

Discussion

Dysregulated miR-142 expression induced by inflammatory stimuli such as LPS has been revealed in organs, such as human bones and lungs (19,20). However, the expression of miR-142 differs under varying conditions (19,20). For instance, miR-142-3p is downregulated in human MH7A cells induced by LPS, but upregulated in LPS-induced acute kidney injury rat mesangial cells (21). As a crucial element of the *E. coli* cell membrane, LPS induces an inflammatory response in the host (4-6). Increasing evidence has suggested that various miRNAs participate in inflammatory responses to LPS (11,12). In the present study, the expression of miR-142-5p was significantly upregulated in bovine mammary epithelial MAC-T cells treated with LPS compared with the blank group. In LPS-treated cells, miR-142-5p mimics transfection significantly increased the expression of cytokines, which function as prestigious regulators of inflammation, serve an important role in the pathogenesis of various diseases, such as, acute lung injury in human, myocarditis in human and bovine mastitis, and promote the activation of NF-κB signaling pathways (22-24). Previous studies have also demonstrated that miR-142-5p upregulated the expression of cytokines through activation of NF-κB signaling pathways (25,26). In the present study, the results indicated that miR-142-5p activated NF-κB signaling pathways, further upregulating the expression of cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, in LPS-treated cells. These results demonstrated that miR-142-5p may have a proinflammatory role in bovine mastitis, consistent with Sun *et al* (15). Furthermore, a 2017 study revealed that the degradation of epithelial cells induces the secretion of toxic and proinflammatory endogenous substances, promoting the progression of bovine mastitis (27). For example, miR-142-5p overexpression contributes to the proliferation of epithelial cells in colorectal cancer (28). However, the potential mechanisms responsible for these outcomes have not been fully elucidated.

In the present study, to investigate the possible roles of miR-142-5p in bovine mastitis, MAC-T cells were exposed to LPS. Subsequently, cell cycle progression and the inflammatory response, which was assessed by the differential expression of cytokines, were investigated. An increased rate of apoptosis, G_0/G_1 cell cycle phase arrest, and suppressed cell proliferation

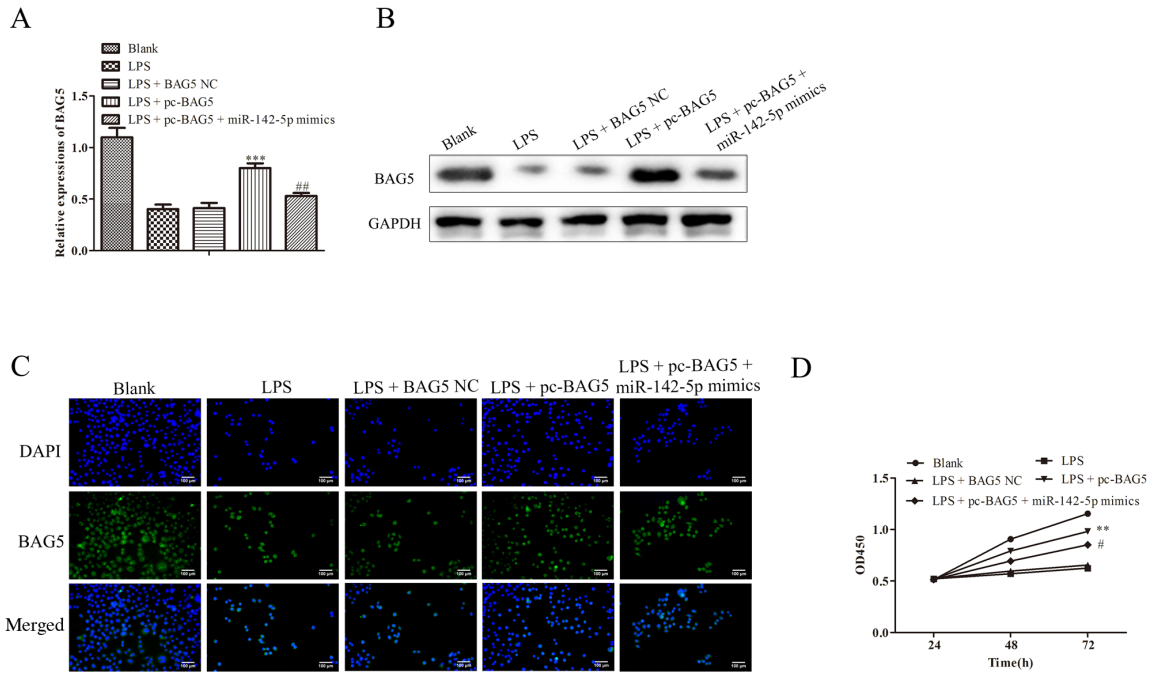

Figure 7. BAG5 regulates the cell viability of LPS-treated MAC-T cells. (A) mRNA expression levels of BAG5. (B) Protein expression levels of BAG5. (C) Signal density of BAG5 (magnification, x200). (D) Cell viability of MAC-T cells. **P<0.01 and ***P<0.001 vs. LPS. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS + pc-BAG5 group. LPS, lipopolysaccharide; NC, negative control; miR, microRNA; BAG5, Bcl-2 associated athanogene 5; OD, optical density.

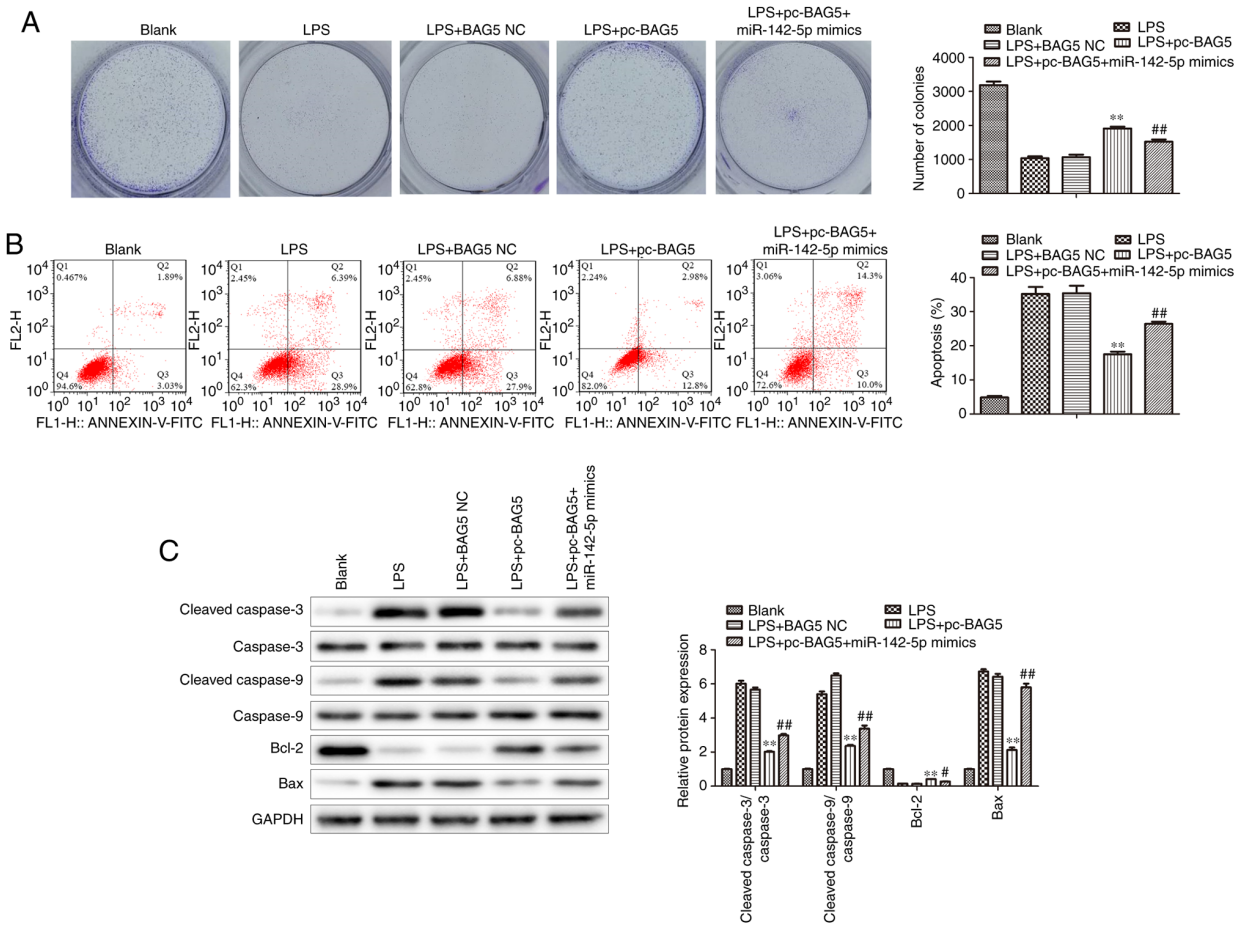

Figure 8. BAG5 regulates the proliferation and apoptosis of LPS-treated MAC-T cells. (A) Proliferation of MAC-T cells was examined by performing the clone formation assay. (B) Apoptosis of MAC-T cells. (C) Expression of cleaved-Caspase-3, cleaved-Caspase-9, Bcl-2 and Bax proteins. **P<0.01 vs. LPS. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS + pc-BAG5 group. LPS, lipopolysaccharide; NC, negative control; miR, microRNA; BAG5, Bcl-2 associated athanogene 5; ns, not significant.

and viability following the transfection of miR-142-5p mimics in LPS-treated cells indicated that miR-142-5p degraded bovine mammary epithelial cells. Additionally, the results indicated that miR-142-5p was required for the regulation of cell cycle-and apoptosis; Caspase-3, Caspase-9, Bcl-2 and Bax protein expression. These results further revealed that miR-142-5p negatively regulated the cell cycle progression of MAC-T cells or induced the degradation of MAC-T cells, which may further contribute to the progression of bovine mastitis. However, the underlying molecular mechanisms are still unknown.

BAG proteins are multifunctional (29). On one hand, the BAG domain suppresses interactions with the ATPase domain of Hsp70/Hsc70 molecular chaperones; on the other hand, BAG family proteins serve as regulators or co-chaperone proteins interacting with Hsp70 to regulate cell behavior, such as proliferation, apoptosis and inflammatory responses (30). For example, BAG3 functions as an antiapoptosis gene, suppressing colorectal cancer cell apoptosis, whereas BAG1 is involved in the development of chronic rhinosinusitis (31,32). BAG5 also participates in the progression of breast cancer and optic nerve head glial activation (33,34). Interestingly, BAG5 exerts a regulatory role in epithelial cells of epithelial ovarian cancer (35). The potential roles of BAG5 in the initiation and progression of inflammation and breast cancer indicated the potential of BAG5 to serve a crucial role in bovine mastitis (31,32). In the present study, BAG5 was shown to be a target of miR-142-5p. Bioinformatics analysis demonstrated that BAG5 and miR-142-5p shared a relative high Context++ score, indicating highly conserved. The present study investigated the potential roles of BAG5 in bovine mastitis. The results indicated that BAG5 overexpression mediated the upregulation of cytokines and the activation of NF-κB signaling pathways induced by LPS and/or miR-142-5p, which suggested that BAG5 served an anti-inflammatory role in bovine mastitis. Moreover, the present study suggested that BAG5 served an important role in regulating MAC-T cell viability, proliferation, cell cycle progression and apoptosis, which was further reiterated by the regulatory role of BAG5 in cell cycle- and apoptosis-related protein expression. Additionally, miR-142-5p mimics transfection abated the effects of BAG5 overexpression on MAC-T cell viability, proliferation, cell cycle and apoptosis, indicating that miR-142-5p may promote the degradation of MAC-T epithelial cells via targeting BAG5.

However, there were some limitations to the present study. To confirm the results shown here, *in vivo* experiments should be conducted It should also be noted that an miRNA may have several target genes and a gene may be targeted by several miRNAs. Overall, the present study suggested that miR-142-5p may serve a proinflammatory role in bovine mastitis via targeting BAG5, suggesting a novel therapeutic strategy for bovine mastitis.

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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

BG and JingL performed the experiments. BG, JinyL, WL and JiangL collected materials and interpreted the data. JingL designed and approved the current study. BG and JingL confirm the authenticity of all the raw data. BG and JiangL reviewed the results. JinyeL, WL and JingL reviewed the introduction, methodology and conclusion. All authors read and approved the final manuscript.

Ethics approval and consent for participation

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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