

miR-92a-3p regulates trypsinogen activation via Egr1 in AR42J cells

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Abstract. Acute pancreatitis (AP) exhibits high morbidity and mortality rates. The onset of AP is characterized by early trypsinogen activation. The present study aimed to investigate the expression of microRNA (miR)-92a-3p and early growth response protein 1 (Egr1), and the effect of miR-92a-3p on trypsinogen activation in the pancreatic exocrine cell line AR42J. mRNA and miRNA microarrays were used to identify differentially expressed mRNAs and miRNAs in AR42J cells. A miRNA-mRNA network was constructed using bioinformatics software, and Egr1 and its regulated miRNA subnetworks were identified by reviewing previous literature. The results suggested that miR-92a-3p could bind to Egr1 3'untranslated region sequence. Subsequently, miR-92a-3p mimic and inhibitor were used to transfect AR42J cells. Following transfection, reverse transcription-quantitative PCR and western blotting were performed to detect Egr1 expression. Furthermore, AR42J cells were cotransfected with miR-92a-3p inhibitor and small interfering (si)-Egr1. The trypsinogen activation rate of AR42J cells was measured by flow cytometry. Microarrays and bioinformatics results indicated that Egr1 may be a target gene of miR-92a-3p. In addition, the present study suggested that miR-92a-3p downregulated Egr1 *in vitro* and that miR-92a-3p and Egr1 expression was associated with trypsinogen activation. Furthermore, miR-92a-3p inhibitor reversed the effect of si-Egr1 on trypsinogen activation. In conclusion, miR-92a-3p may negatively regulate the activation of trypsinogen in AR42J cells via Egr1.

Introduction

Acute pancreatitis (AP) is a common acute abdominal disease with high morbidity and mortality rates. In the US, 220,000 patients with AP were hospitalized in 2013; of these cases, 1,661 were fatal (1). Additionally, there were also reports that the overall mortality rate in patients with acute pancreatitis was ~5%, with the mortality rate in patients with necrotizing pancreatitis ~17% (2). At present, the pathogenesis of AP remains unclear; however, trypsinogen activation is considered as the first step in the development of AP (3). Dawra *et al* (4) hypothesized that activation of trypsinogen in acinar cells results in the death of acinar cells, with this process ultimately leading to pancreatic damage. The rat pancreatic acinar cell line AR42J secretes digestive enzymes and has therefore been widely used as an *in vitro* model of AP (5). Ma *et al* (6) used tauroolithocholic acid 3-sulfate (TLC-S) to significantly induce trypsinogen activation in AR42J cells, and identified differentially expressed protein kinases by microarray analysis. Furthermore, Yang *et al* (7) reported that the JNK signaling pathway can promote trypsinogen activation. Although the development of AP is associated with gene regulation, it has been reported that changes in certain genes (*TMEM173*, *XIAP*, *BHLHA15*, *CTSB*) affect the severity of AP (8-12). Early growth factor 1 (Egr1) is an immediate early gene that contains three zinc finger domains (13). The expression level of Egr1 mRNA increased after 30 min of AP model establishment (14), whereas early pancreatitis may be caused by trypsinogen activation (15). In addition, reduced level of inflammation was measured in an Egr1-knockout mouse model of AP (16). These data confirmed the role of trypsinogen activation and Egr1 in AP and suggested novel targets that may also influence the treatment of the disease.

MicroRNAs (miRNAs) are small single-stranded RNAs (21-25 nucleotides in length) that are relatively conserved in biological evolution. Although these molecules do not encode proteins, they mediate numerous cellular processes (17). Previous studies reported that certain miRNAs are associated with AP. For example, miR-216a upregulation promotes the development of AP via the Akt and TGF- β pathway in mice (18), and miR-155 upregulation can inhibit zonula occludens-1 expression and aggravate AP (19). miRNAs can

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downregulate gene expression by binding to complementary sites of target mRNAs, and subsequently degrade or inhibit these mRNAs (20). Based on the negative regulation of mRNAs by miRNAs, the present study aimed to identify the mRNAs regulated by miRNAs that could affect trypsinogen activation. According to miRNA and mRNA microarrays, the results from the present study suggested that the expression of miR-92a-3p and Egr1 was decreased and increased, respectively, in AR42J cells treated with TLC-S. In addition, the TargetScan tool, which could predict the binding of miRNA seed regions to mRNAs, identified that miR-92a-3p may bind to the 3'untranslated region (UTR) sequence of Egr1 mRNA. Since our previous research (unpublished data) reported that small interfering (si)-Egr1 can reduce trypsinogen activation, the present study investigated whether miR-92a-3p could mediate trypsinogen activation in AR42J cells by modulating Egr1.

Materials and methods

Cell culture and mRNA and miRNA microarrays. The AR42J cell line was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 μ g/ml streptomycin and 100 U/ml penicillin (Beyotime Institute of Biotechnology), and placed at 37°C in a humidified incubator containing 5% CO₂. The cells in the TLC-S group were treated with 200 μ M TLC-S (Sigma-Aldrich; Merck KGaA) for 40 min at 37°C (21,22), whereas cells in the control group were left untreated. Total RNA was extracted from cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Gene expression analyses (rat microarray v2.0; cat. no. Agilent-062716; Agilent Technologies, Inc.) and miRNA expression analyses (Affymetrix[®] GeneChip[®] miRNA 4.0 Array; cat. no. 902446, Affymetrix, Inc.; Thermo Fisher Scientific, Inc.) were performed by BioCloud, Inc. (Iyunbio).

Integrated analysis of miRNA-mRNA data and literature review. Gene names were integrated by referring to the abbreviations in the Rat Genome Database (<https://rgd.mcw.edu/>). Differences in mRNA/miRNA expression profiles between RNA samples from TLC-S-treated cells and RNA samples from control cells were analyzed using Student's t-tests and fold change (FC) assessment. Differentially expressed mRNAs were defined as those with a t-test false discovery rate <0.05 and $\log_2(\text{FC}) > 1$. Differentially expressed miRNAs had a $\text{FC} > 1.5$ or $\text{FC} < 2/3$. TargetScan version 7.2 (<http://www.targetscan.org>) was used to predict the differentially expressed miRNA target genes. Furthermore, to establish a miRNA-mRNA regulatory network, Cytoscape software (version 3.6.1; <https://cytoscape.org/>) was used to identify the downregulated miRNAs and the upregulated target gene mRNAs. To obtain potentially valuable mRNAs, a literature review was performed in order to determine the association between mRNAs from the regulatory network and AP. First, a literature review algorithm was designed using Perl 4 software (<http://www.perl.org/>). Then, data from the literature were retrieved from the PubMed database (<https://www.ncbi.nlm.nih.gov/pubmed/>) using ActivePerl 5.16.2 software (ActiveState Software, Inc.), and titles and

abstracts from the literature were searched using the keywords 'acute pancreatitis' and the mRNAs involved in AP were identified. Subsequently, the number of statistical documents was determined. Eventually, the target literature was manually screened to ensure the accuracy of the research content.

Cell transfection experiments. AR42J cells (1x10⁵ cells) were seeded into six-well plates in complete DMEM and cultured overnight at 37°C. Medium was removed and Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) was used for cell transfection according to manufacturer's instructions. Cells were transfected with miR-92a-3p mimic, miR-92a-3p mimic-NC, miR-92a-3p inhibitor, miR-92a-3p inhibitor-NC, si-Egr1 and si-Egr1-NC (Shanghai GenePharma Co., Ltd.) at final concentrations of 50 nM using 0.25% Lipofectamine 2000. After 4–6 h of transfection, the culture medium was replaced with fresh medium, and then the cells were collected by continuous culture for 48 h. Each transfected group was then treated with 200 μ M TLC-S at 37°C for 40 min before collecting the cells. The sequence of the oligonucleotides used were as follows: miR-92a-3p mimic, 5'-UUAUUGCACUUGUCCCCGGCCUGGGCCGGGACAAGUGCAAUAUU-3'; miR-92a-3p mimic-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-92a-3p inhibitor, 5'-CAGGCCGGGACAAGUGCAAUA-3'; miR-92a-3p inhibitor-NC, 5'-CAGUACUUUUGUGUAGUACAA-3'; si-Egr1, 5'-CCAGGACUAAAAGGCUCUUTTAAGAGCCUUUAAGUCCUGGTT-3'; si-Egr1-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'.

3'UTR sequence of Egr1 mRNA. According to the rat *EGR1* gene (gene ID: 24330), TargetScan was used to predict the miRNAs that may bind to Egr1 mRNA, and analyze their potential binding sites and the context score percentiles.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from AR42J cell lines (1x10⁶ cells) was isolated using Axygen reagent (Axygen; Corning Inc.), and first strand cDNA was synthesized using ReverTraAce qPCR RT kit (cat. no. FSQ-101; Toyobo Life Science) according to the manufacturer's protocol. qPCR was performed using SYBR[®] Green Real-time PCR Master Mix (Toyobo Life Science) under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 2 sec and 60°C for 30 sec, and a final extension at 72°C for 10 min. The primers for Egr1, GAPDH, miR-92a-3p and U6 were synthesized by Guangzhou RiboBio Co., Ltd. The relative expression levels of Egr1 and miR-92a-3p were normalized to endogenous control and were expressed as 2^{- $\Delta\Delta C_q$} (23). The RT-qPCR primers were as follows: miR-92a-3p, forward 5'-CGCGTATTGCACTGTCCC-3', reverse 5'-AGTGCAGGGTCCGAGGTATT-3'; U6, forward 5'-AGAGAAGATTAGCATGCCCCCTG-3', reverse 5'-AGTGCAGGGTCCGAGGTATT-3'; Egr1, forward 5'-ACTGGAGGAGATGATGCTGCTGAG-3', reverse 5'-CCGCTGCTGCTGCTG-3'; and GAPDH, forward 5'-ACAACCTTGGTATCGTGGAAGG-3' and reverse 5'-GCCATCAGCACAGTTTC-3'.

Western blotting. Cells were lysed using RIPA buffer (cat. no. P0013K; Beyotime Institute of Biotechnology), and the protein concentration was measured using a Bicinchoninic

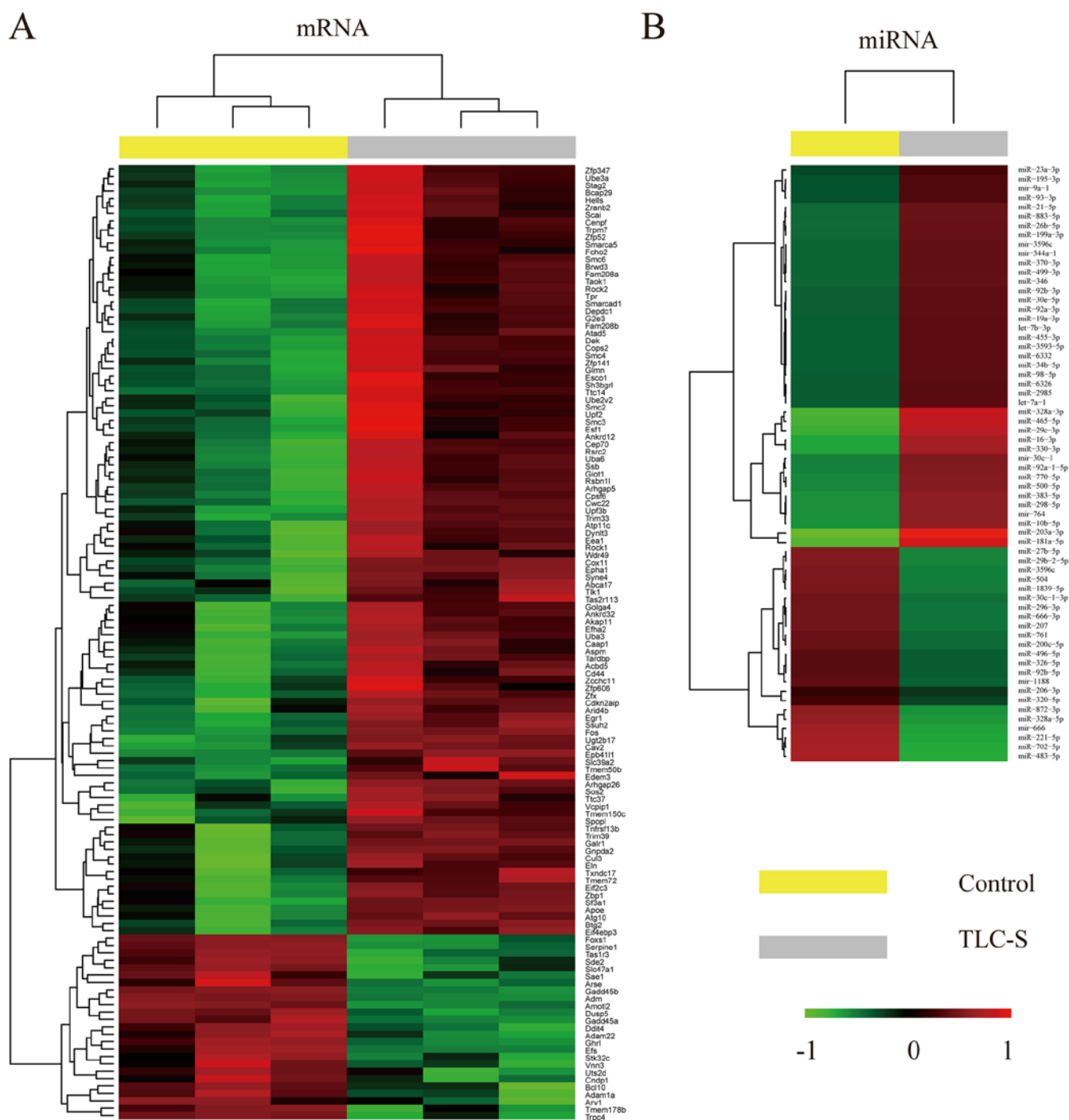


Figure 1. Hierarchically clustered heat map illustrating the changes in mRNA and miRNA expression profiles between TLC-S-treated AR42J cells and control group. Significantly expressed mRNA and miRNA clusters are presented. Red sections represent an increase in expression and green sections represent a decrease in expression. The values -1 and +1 represent Z-score. (A) mRNA hierarchically clustered heat map. (B) miRNA hierarchically clustered heat map. miRNA, microRNA; TLC-S, taurolicholic acid-3-sulfate.

Acid Protein Assay kit (cat. no. P0011; Beyotime Institute of Biotechnology). Proteins (40 μ g) were separated via 8% SDS-PAGE and transferred onto 0.45- μ m PVDF membranes. Membranes were blocked with in 5% skimmed milk dissolved in PBST for 1 h at room temperature. Membranes were incubated with primary antibodies against Egr1 (1:500; cat. no. 4153; Cell Signaling Technology, Inc.) and β -actin (1:2,000; cat. no. PR-0255; ZSGB-BIO; OriGene Technologies, Inc.) at 4°C overnight. Membranes were then incubated with horseradish peroxidase secondary antibody (1:2,000; cat.

no. ZB-2301; ZSGB-BIO; OriGene Technologies, Inc.) for 1 h at room temperature. An ECL kit (cat. no. P0018; Beyotime Biotech, Inc.) was used to detect the signal on the membrane. Data were analyzed by densitometry using Image Lab software (version 2.0.1; Bio-Rad Laboratories, Inc.) and normalized to expression of the internal control β -actin.

Detection of trypsinogen activation by flow cytometry. Cells (1×10^6) were harvested and centrifuged at 500 x g for 5 min at room temperature and the supernatant was discarded. Cell

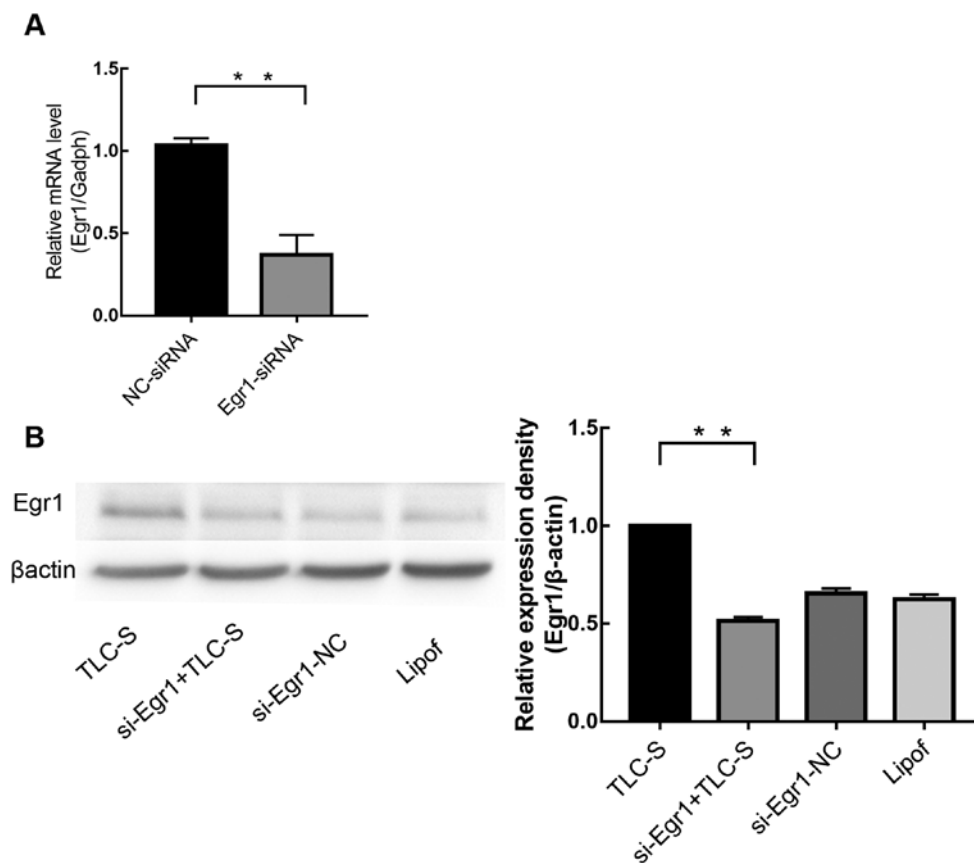


Figure 3. Egr1 expression following transfection. (A) Egr1 mRNA levels in AR42J cells following si-Egr1 transfection analyzed by reverse transcription-quantitative PCR. (B) Egr1 protein levels in AR42J cells following si-Egr1 transfection analyzed by western blotting. ** $P < 0.01$. Egr1, early growth response protein 1; lipof, lipofectamine; NC, negative control; si, small interfering; TLC-S, tauroolithocholic acid-3-sulfate.

resuspended with 1X HEPES and analyzed by flow cytometry (Calibur II, BD Biosciences). The cells were collected using CellQuest software (version 6.0; BD Biosciences), and the experimental data were analyzed using the Kaluza Analysis software program (version 2.1; Beckman Coulter, Inc.).

Statistical analysis. Experiments were repeated at least three times. All data are presented as the mean \pm SD. Comparisons between groups were made using Student's *t*-test or one-way ANOVA followed by Bonferroni test. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Screening of differentially expressed mRNAs and miRNAs. Analysis of mRNA and miRNA microarrays revealed significant differences in mRNA and miRNA expression levels between TLC-S-treated AR42J cells and control group. In total, 129 mRNAs and 64 miRNAs were found to be differentially expressed (Fig. 1).

Construction of the miRNA-mRNA regulatory network. Based on TargetScan bioinformatics predictions and differentially expressed mRNAs and miRNAs, Cytoscape software was used to construct a regulatory network containing upregulated mRNAs and downregulated miRNAs (Fig. 2A). Through

a literature review, the results demonstrated that Egr1 was associated with AP. Subsequently, five miRNAs (miR-199a-3p, miR-203a-3p, miR-23a-3p, miR-181a-5p and miR-92a-3p) that exhibited regulatory association with Egr1 were identified (Fig. 2B).

si-Egr1 transfection efficiency. Following 4-6 h transfection of AR42J cells with si-Egr1, Egr1 expression was analyzed by RT-qPCR (Fig. 3A) and western blotting (Fig. 3B). The results demonstrated that si-Egr1-transfected cells presented significantly reduced Egr1 expression level, whereas there was no significant difference between the siRNA-NC group and the lipofectamine-treated group ($P > 0.05$).

Potential miR-92a-3p binding site was identified in the Egr1 3'UTR sequence. The structure of miR-92a-3p is presented in Fig. 4A. Bioinformatics analysis using TargetScan revealed that the 3'UTR sequence of Egr1 contained a potential binding site for miR-92a-3p (Fig. 4B). Furthermore, potential binding sites for miR-181a-5p, miR-203a-3p, miR-199a-3p and miR-23a-3p were also found in the Egr1 3'UTR sequence (Fig. 4C). In addition, the context score percentiles of miR-181a-5p, miR-203a-3p, miR-199a-3p and miR-23a-3p were reduced compared with that of miR-92a-3p, which indicated that miR-92a-3p was more likely to bind Egr1 compared with the other miRNAs. The present bioinformatics analysis suggested that miR-92a-3p may regulate Egr1 expression.

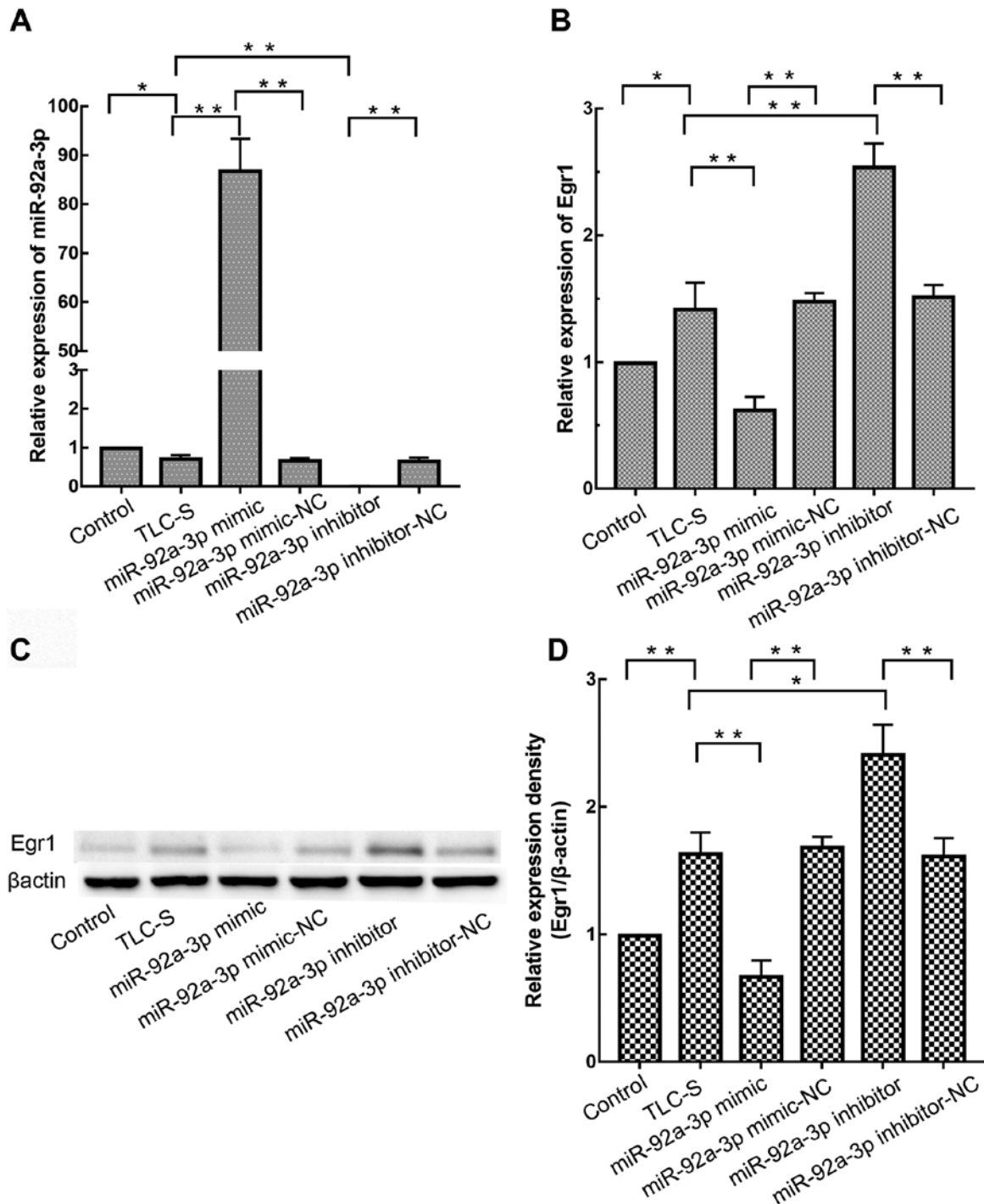


Figure 5. Effects of miR-92a-3p on Egr1 expression. miR-92a-3p mimics and inhibitors were transfected into AR42J cells. (A) Relative miR-92a-3p expression level in each group. * $P < 0.05$, ** $P < 0.01$. (B) Effect of miR-92a-3p mimic and inhibitor on Egr1 mRNA expression levels. * $P < 0.05$, ** $P < 0.01$. (C) Effect of miR-92a-3p mimic and inhibitor on Egr1 protein levels. (D) Egr1 protein levels represent the means \pm SD from three replicates. * $P < 0.05$, ** $P < 0.01$. Egr1, early growth response protein 1; miRNA, microRNA; NC, negative control; TLC-S, taurolichocholic acid-3-sulfate.

of AP development and to determine the genes or pathways of interest (14,16,30,31). Studies using AP animal models will be performed.

In the present study, AR42J cells treated with TLC-S were used to establish an *in vitro* trypsinogen activation model of AP. Microarray and bioinformatics assays were also used to construct mRNA and miRNA interaction networks based on Egr1 as an entry point, and to study the role of miR-92a-3p binding with Egr1 in AP.

Egr1 is located on the q23-31 'cytokine aggregation' region of human chromosome 5 and is an early gene that serves crucial roles in regulating cell cycle, proliferation and differentiation, and damage repair (32). Abnormal expression of Egr1 was reported to be associated with various types of disease, including ischaemic injury, atherosclerosis, inflammation, cardiovascular pathogenesis and cancer (33). Egr1 serves a key role in cell damage (34). In unstimulated cells, Egr1 is rarely expressed; however, when cells are

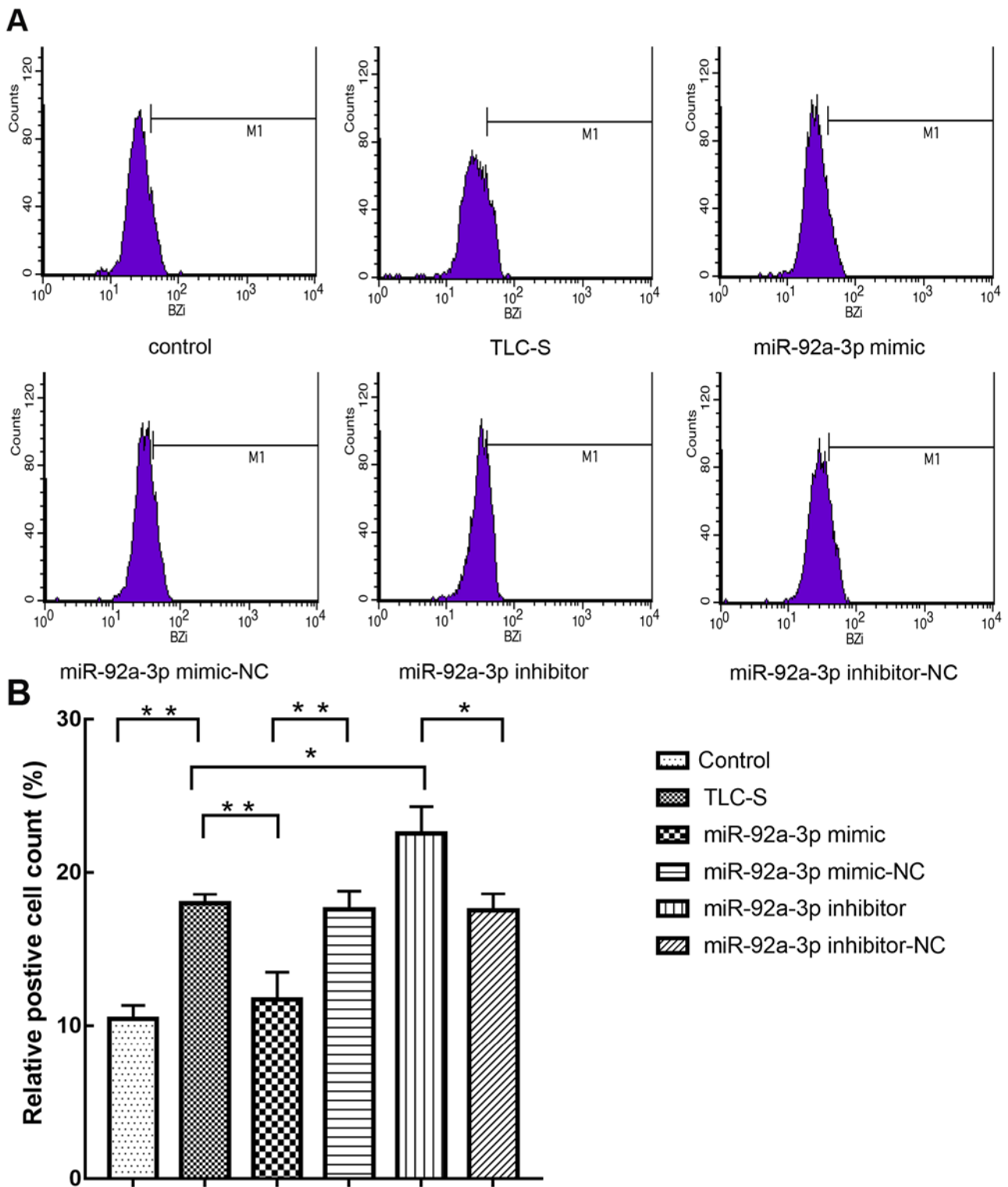


Figure 6. Trypsinogen activation detected by flow cytometry in each group. (A) Flow cytometry results. (B) Quantification of flow cytometry results. *P<0.05, **P<0.01. miRNA, microRNA; NC, negative control; TLC-S, taurolicholic acid-3-sulfate.

stimulated by extracellular signaling molecules (growth factors, hormones and neurotransmitters), Egr1 is immediately synthesized (35,36).

It has been demonstrated that Egr1 is early synthesized in pancreatic acinar cells following AP induction by cerulein injection (14). Sandoval *et al* (16) used TLC-S to induce AP in rats and AR42J cells and reported that Egr1 is an early proinflammatory gene. Furthermore, the expression of

inflammation-associated genes, including monocyte chemoattractant protein 1, macrophage inflammatory protein 1 and intercellular adhesion molecule 1, and lung inflammation were reduced in Egr1-deficient mice, and it was demonstrated that Egr1 activity can affect the severity of AP (37-39). Stimulation of Egr-1 synthesis is transient, which indicates that cells are adapting to prevent constitutive synthesis of Egr-1. The inhibitory domains NGFI-A binding protein 1 and 2 (NAB1 and

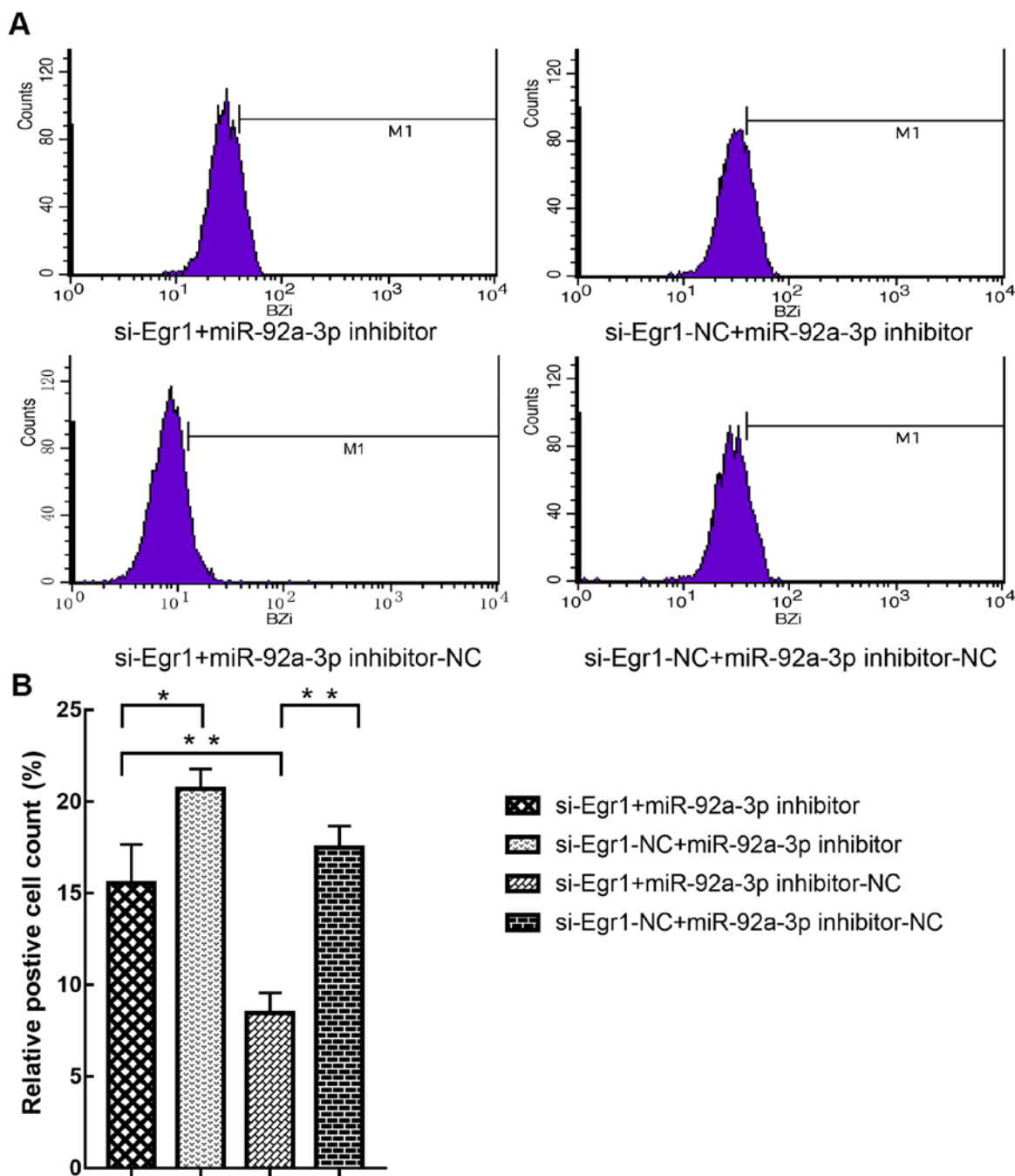


Figure 7. Trypsinogen activation was detected by flow cytometry following cotransfection of si-Egr1 and miR-92 mimic or inhibitor. (A) Flow cytometry results. (B) Quantification of flow cytometry results. * $P < 0.05$, ** $P < 0.01$. Egr1, early growth response protein 1; miRNA, microRNA; NC, negative control; si, small interfering; miR, microRNA.

NAB2) are comprised between the Egr1 activation domain and the DNA binding domain. Both NAB1 and NAB2 block the biological activity of Egr-1 (40,41). Achieving Egr-1 over-expression has therefore been a challenge, and experimental methods attempting to do so were not always successful (42). In addition, the results of the present study indicated that miR-92a-3p may regulate trypsinogen activation via Egr1, suggesting the importance of Egr1 in AP.

Previous work from our laboratory suggested that high Egr1 expression is associated with the development of AP and that AR42J cell transfection with si-Egr1 reduces trypsinogen activation (unpublished data). By using microarray

analysis and literature review, the present study demonstrated that Egr1 was associated with AP. Furthermore, microarrays and bioinformatics analyses demonstrated that miR-92a-3p regulated Egr1 expression. It has been demonstrated that miR-92a-3p is associated with cancer invasion (43) and insulin secretion (44); however, its role in pancreatitis has not yet been reported. In the present study, the results from miRNA microarray and RT-qPCR demonstrated that miR-92a-3p levels were decreased in the trypsinogen activation *in vitro* model of AP. In addition, miR-92a-3p mimic and inhibitor were used to study the function of miR-92a-3p. The present results demonstrated that trypsinogen activation was significantly increased in the

miR-92a-3p mimic group but was significantly decreased in the miR-92a-3p inhibitor group.

Present bioinformatics analyses suggested that Egr1 may be a target gene of miR-92a-3p. The results of the present study suggested that AR42J cells transfected with miR-92a-3p mimics expressed low Egr1 mRNA and protein levels, whereas miR-92a-3p inhibitors induced overexpression of Egr1 mRNA and protein levels. These data suggested that miR-92a-3p overexpression may downregulate Egr1 expression. Furthermore, the effect of AR42J cell transfection with si-Egr1 and miR-92a-3p inhibitor on trypsinogen activation were investigated. The present results suggested that transfection with miR-92a-3p inhibitor could reverse the si-Egr1-induced decrease in trypsinogen activation. The present findings suggested that miR-92a-3p may regulate Egr1 expression, reducing the transcription and translation levels of trypsinogen-associated genes in AR42J cells. Further studies are required to investigate the role and underlying mechanisms of genes that could potentially interact with Egr1. In conclusion, the results of the present study suggested that miR-92a-3p was significantly reduced in AR42J cells treated with TLC-S compared with control group. The present data indicated that a decrease in miR-92a-3p may not completely inhibit Egr1 gene and may lead to trypsinogen activation.

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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

DX and WZ designed the study. BG and YH provided study material. YZ, ZL, DZ and BM performed the experiments and assembled the data. XZ, BG, YH and YZ analyzed and interpreted the data. XZ performed experiments and wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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