Effects of Egr1 on pancreatic acinar intracellular trypsinogen activation and the associated ceRNA network

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Abstract. Acute pancreatitis (AP) is a common digestive disorder with high morbidity and mortality. The present study aimed to investigate the expression of early growth response protein 1 (Egr1), and the effect of competing endogenous (ce)RNA network on trypsinogen activation. Pancreatic acinar intracellular trypsinogen activation (PAITA) is an important event in the early stage of AP; however, the underlying mechanisms remain unclear. The present study used taurolithocholic acid 3-sulfate (TLC-S)-treated AR42J cells (pancreatic cell line) to establish a PAITA model. A gene microarray and bioinformatics analysis was performed to identify the potential key targets in PAITA. The results demonstrated that Egr1, an important transcription factor, was significantly overexpressed in PAITA. In Egr1 small interfering (si)RNA-transfected cells, Egr1 expression was decreased and trypsinogen activation was significantly decreased compared with negative control siRNA-transfected cells, indicating that in TLC-S-induced PAITA, overexpression of Egr1 enhanced trypsinogen activation. A ceRNA network [mRNA-microRNA (miRNA/miR)-long non-coding (lnc)RNA] generated using the PAITA model revealed that the effects of Egr1 on PAITA may be regulated by multiple ceRNA pairs, and the lncRNAs (including NONRATT022624 and NONRATT031002) and miRNAs [including *Rattus norvegicus* (rno)-miR-214-3p and rno-miR-764-5p] included in the ceRNA pairs may serve roles in PAITA by regulating the expression of Egr1. The results of the present study may provide novel targets for researching the underlying mechanisms of, and developing treatments for AP.

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Introduction

Acute pancreatitis (AP) is a common digestive disorder with high morbidity and mortality. It has been reported that in the USA, AP is a leading cause of inpatient care among gastrointestinal conditions: >275,000 patients are hospitalized for AP annually (1). AP is the second leading cause of hospitalization, the largest contributor to total hospitalization cost and the fifth leading cause of inpatient mortality (2,3) In China, 20-30% of patients with AP are clinically dangerous, and the overall case fatality rate is 5-10% (4). At present, the pathogenic mechanisms of AP remain unclear. Pancreatic acinar intracellular trypsinogen activation (PAITA) is considered to be an important cause of AP and is an important event in the early stages of AP (5). Studies have demonstrated that normal activation of trypsinogen is a key factor for the pancreas to maintain normal function and that the abnormal activation of trypsinogen in pancreatic acinar cells is an initiating factor for the occurrence of AP (6,7). Thus, elucidation of the mechanisms underlying trypsinogen activation and identification of targets that serve key roles in this process are important for determining the pathogenesis of AP and providing clinical treatment.

As an important transcription factor, early growth response 1 (Egr1) is closely associated with the occurrence and development of various diseases, including AP (8-10). Ji *et al* (11) demonstrated that Egr1 was expressed in pancreatic acinar cells and served a role in the development of caerulein-induced AP in mice. Gong *et al* (12) reported that as a proinflammatory transcription factor, Egr1 may serve an important role in the development of early AP by regulating the expression of tissue factor. These studies suggest that PAITA is likely to be the most important early event of AP, and that Egr1 may serve a role in early AP. However, it remains unclear whether Egr1 serves a role in PAITA.

In recent years, non-coding RNAs have been demonstrated to serve important roles in the occurrence and development of a variety of diseases including cancer and leukemia (13,14). As an important non-coding RNA, long non-coding RNA (lncRNAs) have been used as a type of competing endogenous RNA (ceRNA) to affect multiple target genes and participate in

the regulation of various biological processes that are closely associated with the occurrence, development and prevention of human diseases (15,16). lncRNAs can affect mRNA expression by competing for a common microRNA (miRNA/miR) binding site; the mRNA-miRNA-lncRNA network is termed a ceRNA network (17). Studies have confirmed that the lncRNA has a close relationship with AP. For example, Zhao *et al* (18) demonstrated that the lncRNA Fendrr promoted the apoptosis of pancreatic acinar cells in caerulein-induced AP by interacting with annexin A2. Wang *et al* (19) reported that overexpression of lncRNA B3GALT5-AS1 may alleviate caerulein-induced cell injury in AR42J cells through the regulation of miR-203/NFIL3 axis and by inhibiting the activation of the NF-κB signals. These studies suggested that lncRNAs may be used as an important target for research and treatment of AP. However, it remains unclear whether lncRNAs serve a role in PAITA and whether there is an interaction between Egr1 and PAITA.

The present study used taurolithocholic acid 3-sulfate (TLC-S) to induce AR42J cells to establish a PAITA model. A gene microarray was used to detect the differential expression of lncRNAs, miRNAs and mRNAs in PAITA. Bioinformatics analyses were performed to identify a protein-protein interaction (PPI) network in PAITA in order to investigate the potential role of Egr1 in PAITA. Confocal laser microscopy and flow cytometry were then used to analyze the effects of Egr1 silencing on PAITA. Finally, a ceRNA regulatory network was established to predict the potential mechanisms underlying the influence of Egr1 on PAITA. The results of the present study may provide novel insight for studies into the pathogenesis and clinical treatment of AP.

Materials and methods

Cell culture and treatment. Cell culture and treatment were performed in accordance with a previous study (20). The rat pancreatic acinar AR42J cells were obtained from the China Center for Type Culture Collection (Wuhan, China) and cultured in F12K medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin (Beyotime Institute of Biotechnology) in a 5% CO_2 environment at 37˚C. A total of 200 μM TLC-S (Sigma-Aldrich; Merck KGaA) was used to treat AR42J cells for 40 min at 37˚C to establish the PAITA cell model as previously described (21,22).

Measurement of trypsinogen activation. Quantification of the activity of trypsin serine protease in intact living acinar cells was performed as previously described (22). Briefly, after an equilibration period of 30 min, 200 μM TLC-S was added for 40 min at 37˚C. Acinar cells were washed and resuspended in NaHEPES without TLC-S, and then supplemented with 10 μM of the cell-permeant synthetic trypsin substrate bis-(CBZ-Ile-Pro-Arg)-rhodamine 110 (BZiPAR; Molecular Probes; Thermo Fisher Scientific, Inc.) and allowed to react in the dark at 37˚C for 20 min. BZiPAR is a specific substrate for trypsin that emits fluorescence after cleavage of the two oligopeptide side chains. Activation may be observed by fluorescence of rhodamine 110 by using an excitation wavelength of 485 nm. Then 0.5 μg/ml DAPI (Beyotime Institute of Biotechnology) was used for 5 min at 37˚C to locate the nuclei, which fluoresced green under the laser confocal microscope). To ensure that the observed tryptic activity was solely from intracellular enzymes and not from trypsin released into the extracellular fluid, cells for these experiments were prepared in NaHEPES containing 5 mM soybean trypsin inhibitor (SBTI; Sigma-Aldrich; Merck KGaA); the solutions used all contained SBTI (SBTI and BZiPAR are added to the cell dish together). At this concentration, SBTI can inhibit 1,000 U/ml trypsin. Trypsin activity was investigated by flow cytometry [FACSCalibur II, BD Biosciences; CellQuest software (version 6.0; BD Biosciences) and Kaluza Analysis software program (version 2.1; Beckman Coulter, Inc.)] and confocal microscopy (A1R, Nikon Corporation). A total of 5 different fields in each group were randomly chosen to capture images at 200x magnification.

Detection of mRNAs, lncRNAs and miRNAs in PAITA. An Agilent-062716 Rat lncRNA Microarray (8x60K; Agilent Technologies, Inc.) was used to detect the expression of mRNA and lncRNA in the present study, and then data analyses of the TLC-S group (trypsinogen activation model; 3 samples) and control group (untreated AR42J cells; 3 samples) were performed. Total RNA was quantified using a NanoDrop™ ND-2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and the RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). Sample labeling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA was reverse transcribed to double-stranded cDNA, then synthesized into cRNA and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned by an Agilent G2505C Scanner (Agilent Technologies, Inc.). Feature Extraction software (version 10.7.1.1, Agilent Technologies, Inc.) was used to analyze array images.

The miRNA expression analysis was performed using Affymetrix® GeneChip® miRNA Arrays (Affymetrix; Thermo Fisher Scientific, Inc.). Poly (A) Tailing, FlashTag ligation, hybridization, washing, staining, and detection were performed in accordance with the protocols of the Affymetrix GeneChip miRNA Arrays using the Library file (http://www.affymetrix.com/support/downloads/manuals/agcc_command_console_user_guide.pdf). Data were normalized using the median normalization. After normalization, differentially expressed (DE) miRNAs were identified through fold change filtering.

Identification of the DE genes in PAITA. The limma package (http://bioconductor.org/packages/Limma/) (23) in R software (version 3.2.11, http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html) (24) was used to perform data preprocessing, including background correction, quantile normalization and probe summarization, to obtain the probe expression matrix (25). UCSC RefSeq database (http://genome.ucsc.edu/), miRBase database 21 (http://mirbase.org/) and NONCODE V4 database (http://www.noncode.org/) were used to perform the blast comparison (identity >95%, coverage >95) and obtain the gene ID (26). Combining the probe expression matrix, a gene expression matrix was obtained.

Limma was used to identify the DE mRNAs, miRNAs and lncRNAs. For each significant DE mRNA and lncRNA, the significance level of differential expression was set to log2 fold change ≥2 and P<0.05. For each significant DE miRNA, the significance level of differential expression was set to log2 fold change ≥2. In addition, MultiExperiment Viewer software 4.6.0 (http://mev.tm4.org/) was used to conduct the cluster analysis and draw the heatmaps.

PPI network of the DE mRNAs in PAITA. A PPI network of the DE mRNAs in trypsinogen activation was obtained from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (version 8.0; www.string-db.org) and analyzed using Limma (22,27). Cytoscape software (version 3.6.1; www.cytoscape.org) was used to visualize the PPI network. The node color of the genes was set to different gradients according to the fold change and the node size was set according to the P-value.

Analysis of gene function and signaling pathway enrichment. The function of DE genes and the enriched signaling pathway were investigated via Gene Ontology (GO) (28) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) (29) analysis through the Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov). Fisher's exact test was applied and P<0.05 was considered to indicate a statistically significant difference.

Small interfering (si)RNA transfection. siRNAs were transfected into cells using Lipofectamine® RNAiMAX transfection reagent (cat. no. 13778-150, Gibco; Thermo Fisher Scientific, Inc.). AR42J cells ($1x10^5$ cells) were seeded into six-well plates in complete DMEM and cultured overnight at 37˚C. The siRNA lipoRNAiMAX mixture was prepared and added to a culture wells containing cells suspended in 800 μl F12K medium, with a cell density of about 30-40% ($2x10^5$ cells). The siRNAs were used at a final concentrations of 50 nM. After 4-6 h of culture at 37˚C, the culture medium was replaced with fresh medium (containing fetal bovine serum and penicillin/streptomycin), followed by continuous culturing for 48 h at 37˚C before subsequent experimentation.

The siRNAs were purchased from Shanghai GenePharma Co., Ltd., and their sequences were as follows: Egr1-siRNA, 5'-CCAGGACUUAAAGGCUCUUTTAAGAGCCUUUAA GUCCUGGTT-3' and negative control (NC)-siRNA, 5'-UUC UCCGAACGUGUCACGUTT-3'.

AR42J cells were divided into four experimental groups: i) Control group, normally cultured AR42J cells; ii) NC-siRNA group, AR42J cells transfected with negative control siRNA; iii) TLC-S + NC-siRNA group, AR42J cells transfected with NC-siRNA and treated with 200 μM TLC-S for 40 min; and iv) TLC-S + Egr1-siRNA group, AR42J cells transfected with Egr1-siRNA and treated with 200 μM TLC-S for 40 min.

Reverse transcription-quantitative (RT-q)PCR. Total RNA from AR42J cell lines was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the isolated RNA was reverse transcribed (cat. no. FSQ-101; Toyobo Life Science): 37˚C for 15 min, 50˚C for 5 min and 98˚C enzyme inactivation reaction for 5 min. RT-qPCR was conducted using FastStart Universal SYBR-Green Master (ROX; Roche Diagnostics) and an ABI 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) 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, with a final extension at 72˚C for 10 min. The primers for Egr1 and GAPDH were synthesized by Guangzhou RiboBio Co., Ltd. The relative expression levels of Egr1 were normalized to GAPDH and quantified using the $2^{-\Delta\Delta Cq}$ method (30). The Egr1 and GAPDH primers were as follows: Egr1 forward, 5'-ACTGGAGGAGATGATGCTG CTGAG-3' and reverse, 5'-CCGCTGCTGCTGCTGCTG-3'; and GAPDH forward, 5'-ACAACTTTGGTATCGTGGAAG G-3' and reverse, 5'-GCCATCAGCCACAGTTC-3'.

Western blotting. For protein analysis, cells were lysed directly in RIPA buffer (cat. no. P0013K; Beyotime Institute of Biotechnology), and then centrifuged for 10 min at 12,000 x g at 4˚C, and the protein concentration was determined using the BCA method. The protein (40 μg) was separated by 8% SDS-PAGE and transferred onto 0.45 μm PVDF membranes. The membranes were blocked with 5% BSA (A8020; Beyotime Institute of Biotechnology) in PBST (0.1% Tween-20, cat. no. 9005-64-5; Beyotime Institute of Biotechnology) for 1 h at room temperature. The membranes were incubated with Egr1 (1:500; cat. no. 4153; Cell Signaling Technology, Inc.) and GAPDH (1:2,000; cat. no. TA-08; ZSGB-BIO) at 4˚C overnight. Membranes were then incubated with horseradish peroxidase secondary antibody (1:2,000; cat. no. ZB-2301; OriGene Technologies, Inc.) for 1 h at room temperature. A MiniChemi imager (Beijing Sage Creation Science Co., Ltd.) was used to detect the immunoreactivity. The intensity of the band was relatively quantified using ImageJ software (version 1.8.0; National Institutes of Health). All proteins were normalized to the internal control GAPDH.

Establishing the lncRNA-miRNA-mRNA ceRNA network. According to the results of the differential expression analysis, significant DE lncRNAs, miRNAs and mRNAs were filtered to construct an mRNA-miRNA-lncRNA regulatory network. DE lncRNA and DE miRNA pairs were generated based on the lnCeDB database (31). DE miRNA and DE mRNA pairs were generated based on the miRBase database. When screening the DE lncRNA-miRNA pairs and DE miRNA-mRNA pairs, a constraint that the regulatory directions of the miRNA and mRNA must be different was applied. Based on the results, Cytoscape(version 3.6.1; www.cytoscape.org) was used to construct the mRNA-miRNA-lncRNA ceRNA network.

Statistical analysis. All data are presented as the mean ± SD. RT-qPCR data were analyzed with unpaired Student's t-test. The group differences in flow cytometry analysis were determined with one-way ANOVA followed by Tukey's post hoc test. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

DE mRNAs, lncRNAs and miRNAs in PAITA. The gene microarray was used to identify the DE mRNAs, lncRNAs and

miRNAs in PAITA. Compared with the control group, 206 DE mRNAs, 19 DE lncRNAs and 23 DE miRNAs were observed in PAITA (Table I). The scatter plot and heatmap of DE genes are presented in Fig. 1A and B. In the scatter plot, the blue node represents the gene decrease, the yellow node represents the gene increase, and the node size is positively correlated with the P-value. In the heat map red sections represent an increase in expression and green sections represent a decrease in expression. The values -1 and +1 represent Z-score.

PPI network in PAITA. In order to further investigate the potential mechanisms involved in PAITA, the STRING database and Cytoscape software were used to establish a PPI network in PAITA. The results demonstrated 262 interaction pairs among the 206 DE mRNAs (Fig. 2). Important genes in the PAITA network are indicated by darker colors, larger sizes and higher degrees of interaction, such as nuclear receptor subfamily 4 group a member 1 (NR4A1), ARIIGAP5, FOS, COX11, BTG2, Egr1, dual specificity phosphatase 5 (Dusp5) and adrenomedullin (ADM; Fig. 2). A literature review of differentially expressed proteins was conducted to determine the association between proteins from regulatory networks and AP. The results suggested that although Egr1 did not display the most pronounced fold change, it is indeed most closely associated with AP.

Functional and pathway analysis of the PPI network in PAITA. The DAVID database was used to analyze the enriched functions and pathways of genes in the PPI network. GO analyses demonstrated that the network genes were mainly enriched in 'regulation of cell cycle', 'positive regulation of translation', 'cellular response to DNA damage stimulus', 'meiotic chromosome condensation' and 'mitotic nuclear division' ($P<0.05$; Fig. 3A).

KEGG analyses demonstrated that the network genes were mainly enriched in 'MAPK signaling pathway', 'RNA transportation', 'cell cycle', 'ubiquitin mediated proteolysis' and 'mRNA surveillance pathway' ($P<0.05$; Fig. 3B).

Egr1 knockdown. Among the important genes of the PPI network, Egr1 exhibited the closest relationship with AP according to a review of the literature (8-12). The microarray analysis demonstrated that Egr1 was upregulated in PAITA (Fig. 4A), but its effects remain unclear. Thus, siRNA was used to knockdown the expression of Egr1. RT-qPCR and western blotting were performed to detect the efficiency of knockdown. RT-qPCR and western blotting demonstrated that the expression levels of Egr1 were significantly decreased in the Egr1-siRNA group compared with in the of the NC-siRNA group; the knockdown rate was ~75% (Fig. 4B and C).

Egr1 regulates the activation of trypsinogen. To understand the effects of Egr1 on PAITA, laser scanning confocal microscopy was performed to detect the levels of trypsinogen activation in the TLC-S + Egr1-siRNA, TLC-S + NC-siRNA and NC-siRNA groups. The results demonstrated that the intracellular fluorescent area in the TLC-S + NC-siRNA group was notably larger compared with that of the NC-siRNA group. In addition, compared with the intracellular fluorescent area of the TLC-S + NC-siRNA group, the TLC-S + Egr1-siRNA group demonstrated a decreased intracellular fluorescent area (Fig. 5).

Table I. Number of DE mRNAs, miRNAs and lncRNAs in a cell model of PAITA.

Expression	DE mRNA	DE miRNA	DE lncRNA
Upregulated	172	9	16
Downregulated	34	14	3
Sum	206	23	19

DE, differentially expressed; lncRNA, long non-coding RNA; miRNA, microRNA; PAITA, pancreatic acinar intracellular trypsinogen activation.

To quantify the fluorescence levels, flow cytometry was performed to detect the levels of trypsinogen activation in the three groups. The result demonstrated that the percentage of PAITA-positive cells was 9.12±1.40, 8.95±2.43, 25.61±2.16 and 0.07±0.03% in the control, NC-siRNA, TLC-S + NC-siRNA and TLC-S + Egr1-siRNA groups, respectively (Fig. 6B). No significant difference was observed between the control group and the NC-siRNA group ($P>0.05$), but the percentage of positive cells in the TLC-S + NC-siRNA group was significantly higher compared with that of the NC-siRNA and TLC-S + Egr1-siRNA groups ($P<0.05$) and the percentage of positive cells in the NC-siRNA group was significantly higher compared with that of the TLC-S + Egr1-siRNA group ($P<0.01$; Fig. 6A and B).

The results also demonstrated that the mean fluorescence intensity was 11.53±1.05, 11.61±1.71, 20.71±1.65 and 6.25±0.52 in the control, NC-siRNA, TLC-S + NC-siRNA and TLC-S + Egr1-siRNA groups, respectively. No significant difference was observed between the control group and the NC-siRNA group ($P>0.05$), but the mean fluorescence intensity in the TLC-S + NC-siRNA group was significantly higher than that of the NC-siRNA group and the TLC-S + Egr1-siRNA group ($P<0.01$), and the mean fluorescence intensity in the NC-siRNA group was significantly higher compared with the TLC-S + Egr1-siRNA group ($P<0.01$; Fig. 6C).

ceRNA network of PAITA. The microarray and bioinformatics analyses were used to establish an lncRNA-miRNA-mRNA network of PAITA, which contained 16 lncRNAs, 18 miRNAs and 25 mRNAs, indicating that PAITA has a complex ceRNA regulatory network (Fig. 7). In order to further clarify the mechanism of Egr1 in PAITA, it was demonstrated that the Egr1-associated ceRNA sub-network included four miRNAs [*Rattus norvegicus* (rno)-miR-214-3p, rno-miR-764-5p, rno-miR-338-5p and rno-miR-466b-5p] and 10 lncRNAs (NONRATT022624, NONRATT031002, NONRATT021194, NONRATT029766, NONRATT023334, NONRATT031103, NONRATT006386, NONRATT012585, NONRATT017440 and NONRATT015824) in total (Fig. 7).

The microarray and bioinformatics results also demonstrated that Egr1 displayed the top three degrees in the network, which suggested that Egr1 maybe serve an important role in PAITA. Additionally, two miRNAs and four lncRNAs

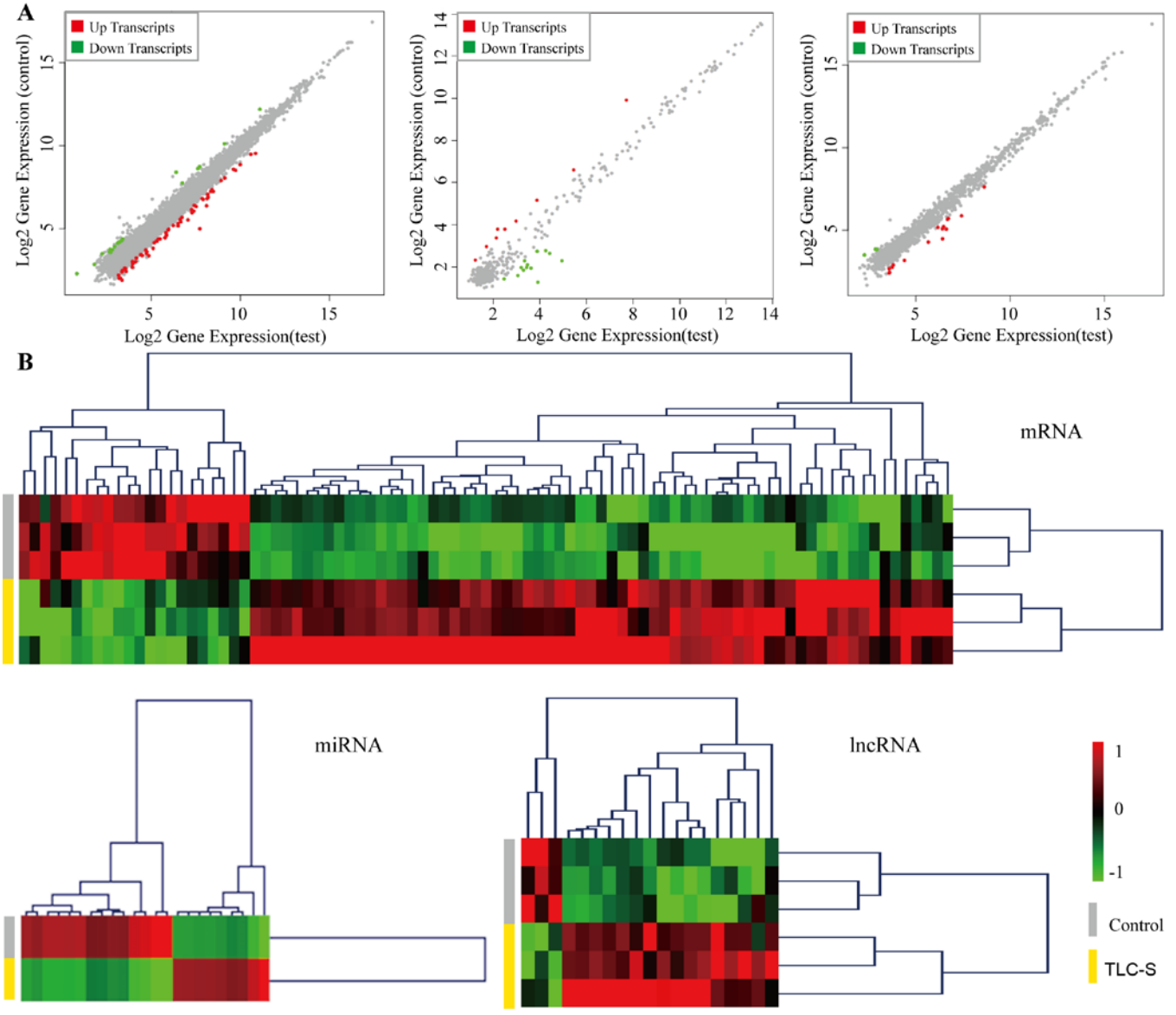

Figure 1. Identification of DE genes in PAITA. (A) Scatter plot of DE mRNAs, lncRNAs and miRNAs in a cell model of PAITA. (B) Heatmap of DE mRNAs, lncRNAs and miRNAs in a cell model of PAITA. There were 206 DE mRNAs, 19 DE lncRNAs and 23 DE miRNAs identified in a cell model of PAITA. PAITA, pancreatic acinar intracellular trypsinogen activation; DE, differentially expressed; lncRNA, long non-coding RNA; miRNA, microRNA; TLC-S, taurolithocholic acid 3-sulfate.

in the Egr1-associated ceRNA sub-network displayed the top five degrees in the network, including rno-miR-338-5p, rno-miR-466b-5p, NONRATT029766, NONRATT023334, NONRATT012585 and NONRATT015824. These results suggested that the effects of Egr1 on PAITA may be regulated by multiple ceRNA pairs, and the lncRNAs and miRNAs included in the ceRNA pairs may serve important roles in PAITA through increasing the expression of Egr1.

Discussion

Studies have demonstrated that maintaining the normal activated form of trypsinogen is key to maintaining the normal function of the pancreas and that inappropriate early activation is the initiating factor for the occurrence of AP (32-34). However, the pathogenic mechanisms of AP and PAITA have not been studied thoroughly. Previous studies have observed that complex genetic network regulation, including coding genes and non-coding genes, is involved in the pathogenesis and development of diseases (35,36). Thus, in the present study, gene chips were used to discover the gene networks and corresponding important network nodes involved in a cell model of PAITA. The microarray results demonstrated that 206 mRNAs, 19 lncRNAs and 23 miRNAs were differentially expressed, and that these differential genes were mainly highly expressed. Based on this result, the present study established a genetic interaction network in PAITA. Through functional analysis, the present study demonstrated that these genes mainly served roles in regulating the cell cycle and positive translation, and that the network genes are mainly enriched in signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway, RNA transportation and the cell cycle. de Dios *et al* (37) observed that cell cycle changes are important features of AP and occur throughout the entire

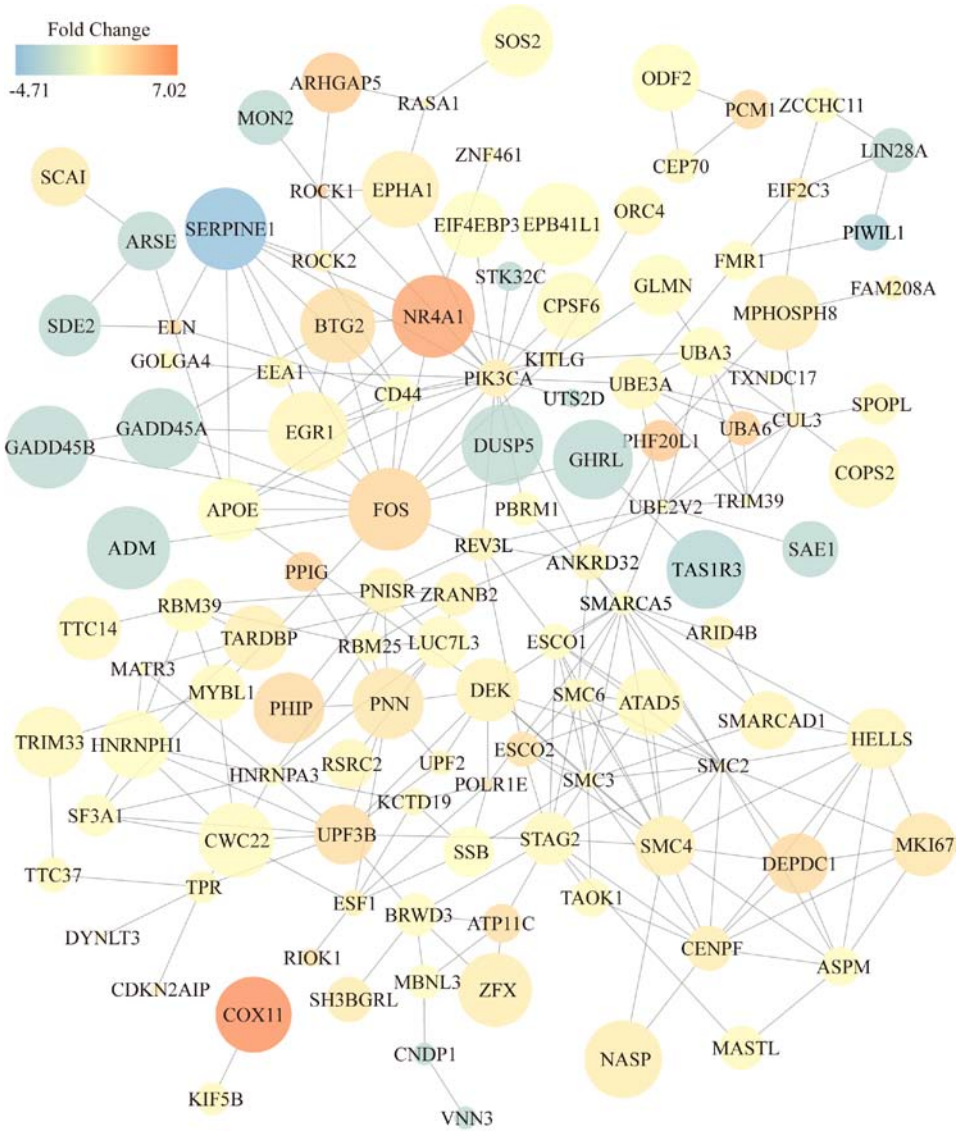

Figure 2. Protein-protein interaction network of DE mRNAs in PAITA. There were a total of 262 DE mRNA interaction pairs in a cell model of PAITA. The node color of the genes was set to different gradients according to the fold-change and the node size was set according to the P-value. DE, differentially expressed; PAITA, pancreatic acinar intracellular trypsinogen activation.

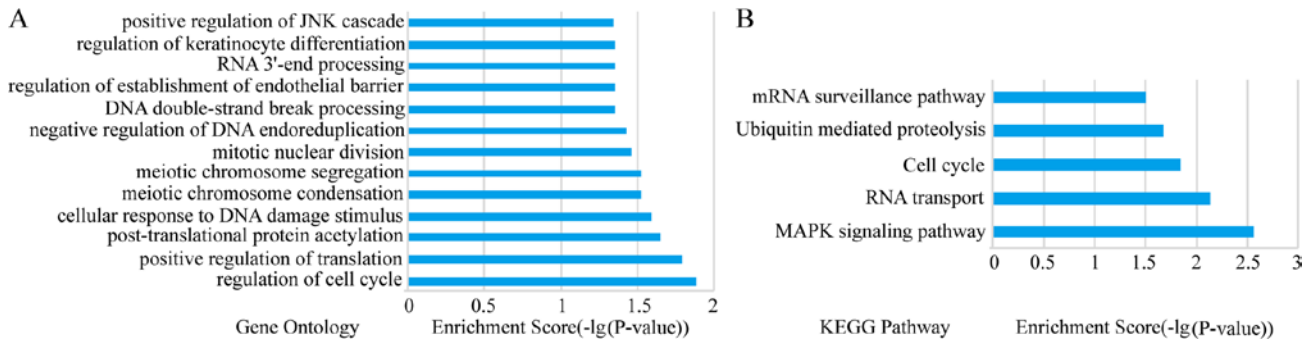

Figure 3. Function and pathway enrichment analysis of the protein-protein interaction network of DE mRNAs in PAITA. (A) GO analysis of DE mRNAs in a cell model of PAITA using the Database for Annotation, Visualization and Integrated Discovery database. The bar plot demonstrated the enrichment scores (-lg P-value) of the significant GO terms. mRNAs were mainly enriched in 13 GO terms. (B) Pathway analysis of DE mRNA in PAITA using the Kyoto Encyclopedia of Genes and Genomes database. mRNA were mainly enriched in five pathways. DE, differentially expressed; PAITA, pancreatic acinar intracellular trypsinogen activation; GO, Gene Ontology.

process of AP; in early AP, pancreatic cells exhibit S phase arrest, and with the aggravation of inflammation, G_2/M phase arrest occurs. The results of the present study suggested that PAITA, as an early AP event, is characterized by cell cycle

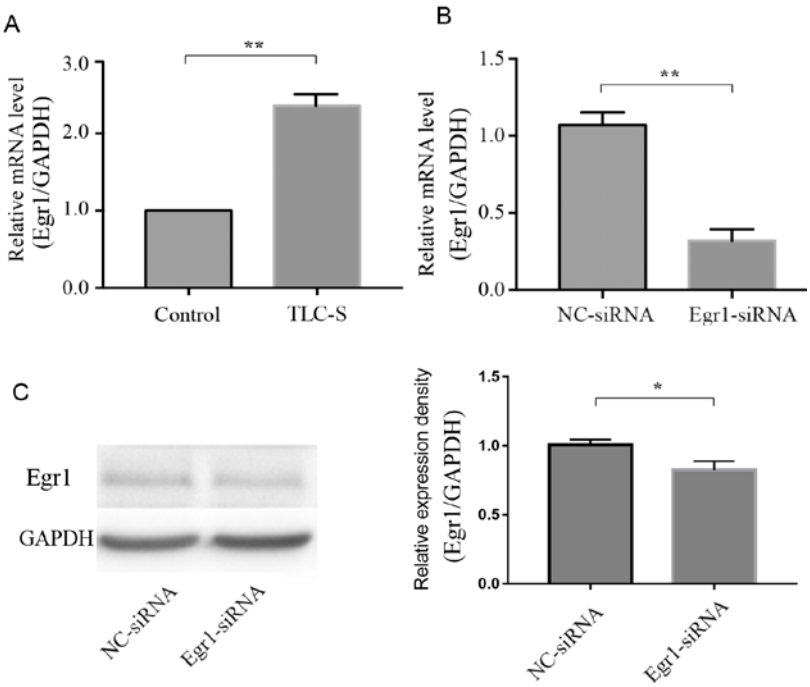

Figure 4. Expression and knockdown of Egr1 in PAITA. (A) Egr1 expression in AR42J cells (B) Egr1 expression after Egr1-siRNA transfection. (C) Egr1 protein expression in cells *P<0.05, **P<0.01. Egr1, early growth response protein 1; PAITA, pancreatic acinar intracellular trypsinogen activation; siRNA, small interfering RNA; NC, negative control.

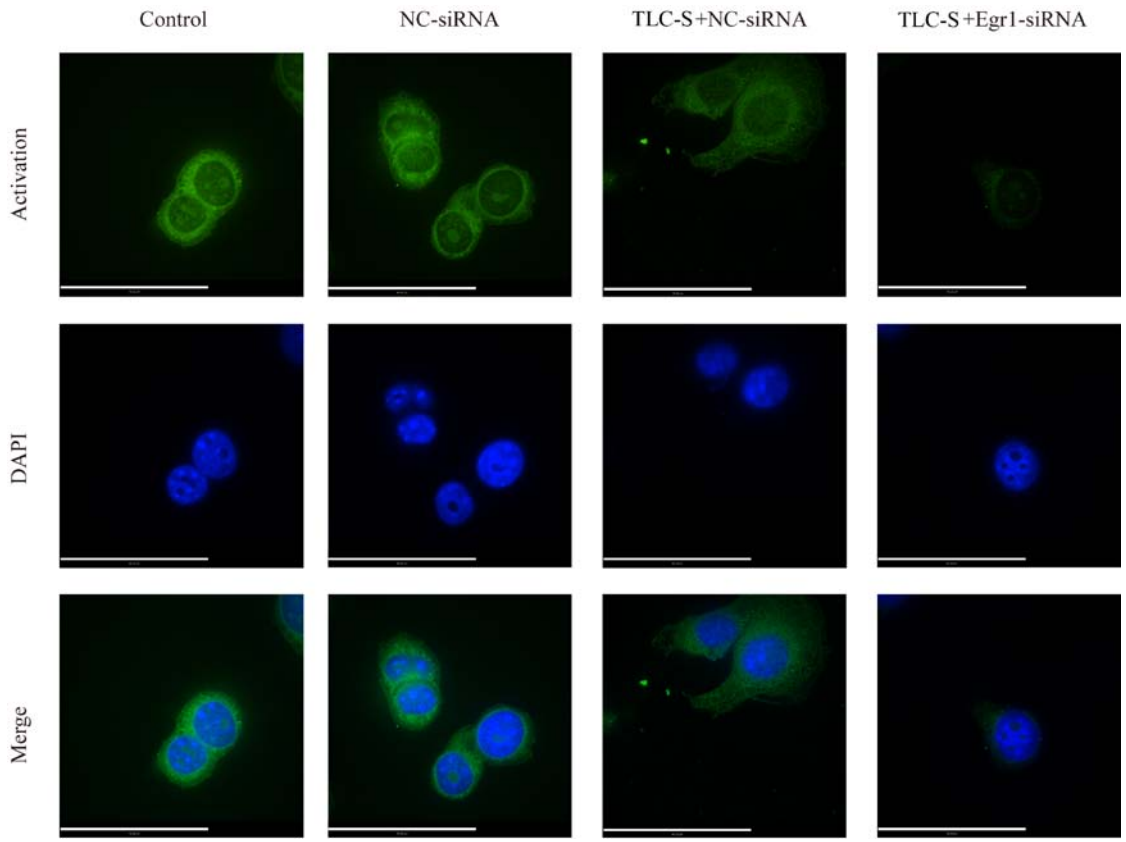

Figure 5. Trypsinogen activation detection using laser scanning confocal microscopy. Green fluorescence represents the degree of pancreatic enzyme activation of the cell; blue fluorescence represents the position of the nucleus. The lower left corner represents 40 μm. Magnification, x200. Egr1, early growth response protein 1; siRNA, small interfering RNA; NC, negative control; TLC-S, taurolithocholic acid 3-sulfate.

changes. The MAPK signaling pathway is one of the most classical signaling pathways in the occurrence and development of AP (38). For example, Cao *et al* (39) demonstrated that inhibition of MAPK signaling in mice effectively inhibited

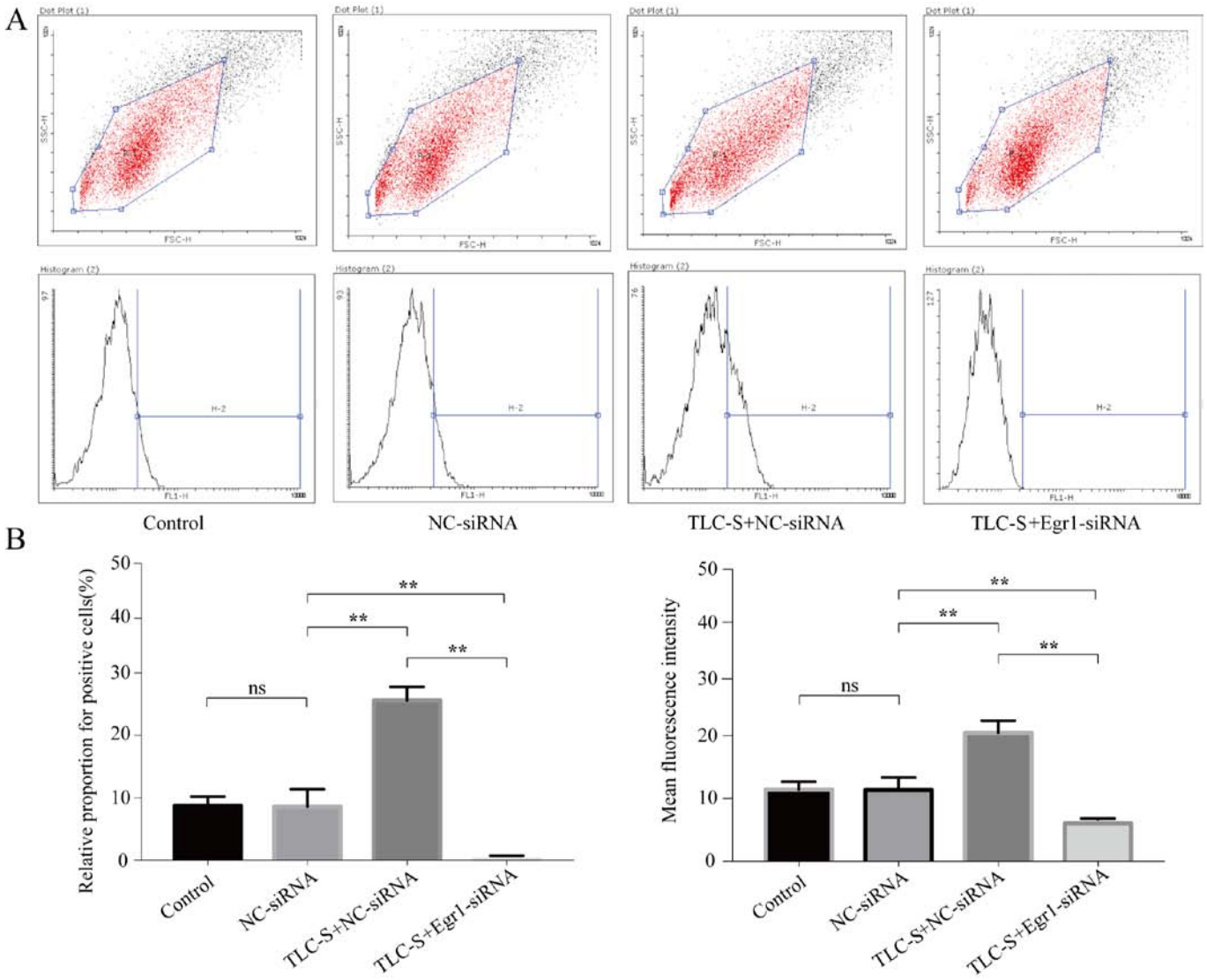

Figure 6. Trypsinogen activation detection using flow cytometry. (A) Percentage of pancreatic acinar intracellular trypsinogen activation-positive cells and (B) mean fluorescence intensity in the control, NC-siRNA, TLC-S + NC-siRNA and TLC-S + Egr1-siRNA groups. **P<0.01. siRNA, small interfering RNA; NC, negative control; Egr1, early growth response protein 1; ns, not significant; TLC-S, taurolithocholic acid 3-sulfate.

the development of pancreatitis. Additionally, another study demonstrated that inhibition of MAPK signaling accelerated the apoptosis of pancreatic acinar cells and decreased the AP-associated inflammatory response (40). In the present study, the DE network genes in PAITA were mainly enriched in the MAPK signaling pathway, suggesting that activation of the MAPK signaling pathway affects the process of PAITA and may generate a subsequent cascade reaction, ultimately promoting further development of AP.

In addition, the gene network in PAITA revealed some potentially important genes, including Fos, Egr1, Dusp5, ADM and NR4A1. These genes exhibited higher degree scores in the network, larger fold changes and smaller P-values. Among these genes, the transcription factor Egr1 has a very close relationship with AP. For example, Sandoval *et al* (41) observed that Egr1 was expressed at high levels in the early inflammatory reaction of AP, and Ji *et al* (11) demonstrated that after initiating caerulein-induced AP in mice, the severity of AP in Egr1 gene-deficient mice was significantly lower compared with that in normal mice. Their study suggested that Egr1 may be a key regulator of early development of AP. However, it has not been reported whether Egr1 is involved in PAITA (the initial stage of AP). The microarray and gene network results of the present study suggested that Egr1 may serve a role in PAITA. To further verify this effect, siRNA was used to silence Egr1, and then confocal laser microscopy and flow cytometry were used to detect the effect of Egr1 on PAITA. The results demonstrated that silencing of Egr1 significantly inhibited PAITA.

Previous studies have observed that Egr1 is located on the q31.1 'cytokine aggregation' region of human chromosome 5, and as a transcription factor, it is closely associated with cell proliferation, differentiation, apoptosis and inflammatory responses (42-45). A number of transcription factors, including activator protein 1, NF-κB and Egr1, can regulate the expression of tumor necrosis factor-α (TNF-α), transforming growth factor β1 and other cytokines by various stimulating factors in different types of cells, such as macrophages and epidermal keratinocytes (46,47). In addition, a number of studies have observed that the transcription factor Egr1 serves an important role in regulating the expression of these stimulating factors

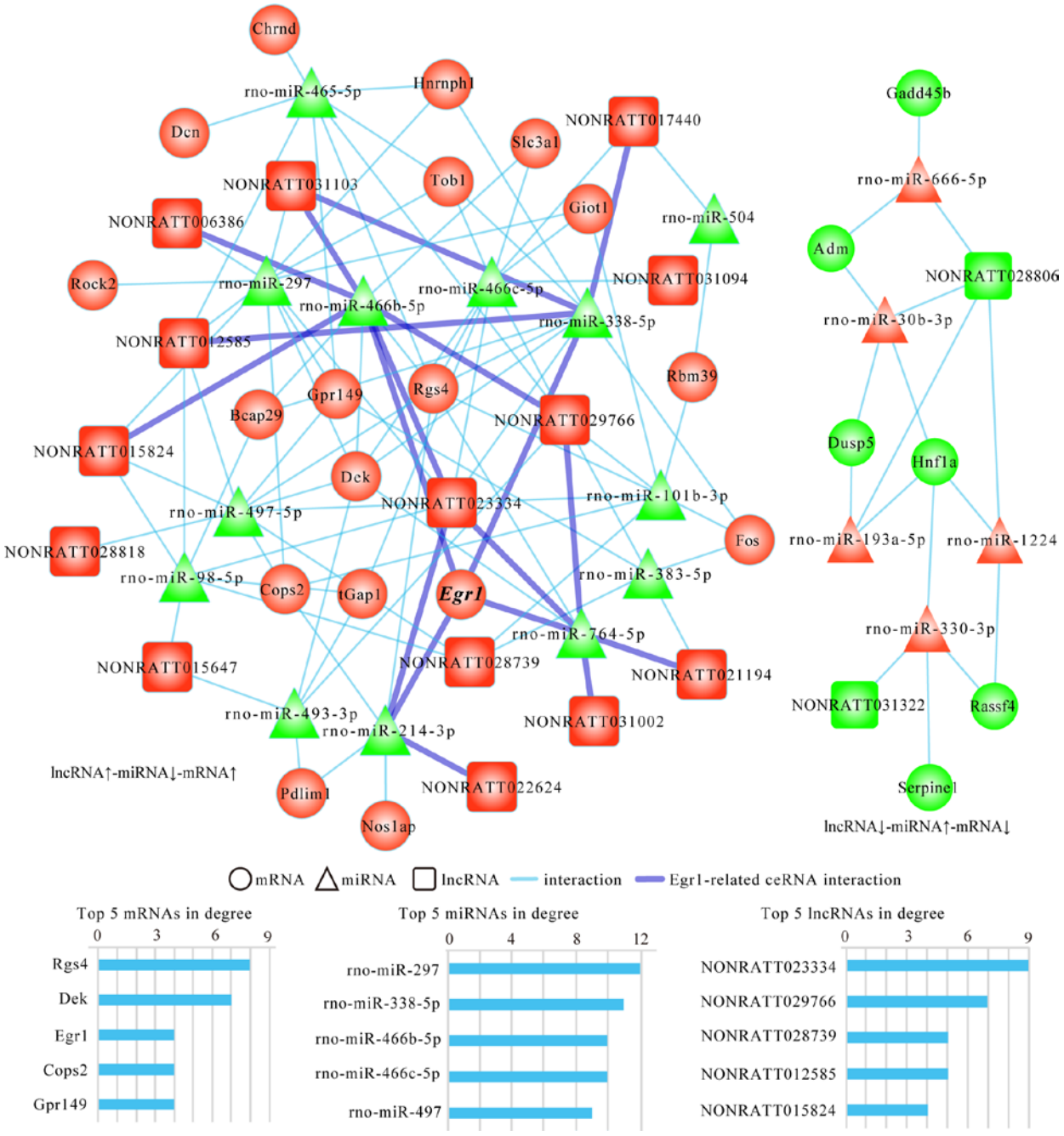

Figure 7. ceRNA network in a cell model of pancreatic acinar intracellular trypsinogen activation. Circular nodes represent mRNAs, triangular nodes represent miRNAs, square nodes represent lncRNAs, thin lines represent interactions, and thick lines represent Egr1-related ceRNA interactions. Egr1, early growth response protein 1; ceRNA, competing endogenous RNA; miRNA/miR, microRNA; lncRNA, long non-coding RNA; rno, *Rattus norvegicus*.

and affects the occurrence and development of diseases (48-52). These results suggested that Egr1 may cause PAITA via the regulation of PAITA-associated pathogenic factors. However, the effect of Egr1 on the regulation of PAITA through the regulation of downstream genes require validation in further molecular experiments.

With the generation of large quantities of basic experimental data, bioinformatics has been widely used as an effective means for big data analysis in various diseases. To further investigate the potential mechanisms underlying the effects of Egr1 on PAITA, gene mapping and bioinformatics methods were used to establish an mRNA-miRNA-lncRNA network in the cell model of PAITA. The expression of lncRNAs was increased, and these can reduce miRNA inhibition of mRNA by competing to bind miRNA binding sites, thereby causing increased mRNA expression. The results of the present study demonstrated that there was an Egr1-associated ceRNA sub-network in PAITA, including four miRNAs and 10 lncRNAs. miRNA was the first type of non-coding RNA to be discovered, and its function has been extensively studied (53). Of the four miRNAs in the Egr1-related ceRNA network, two have been clearly associated with inflammation. Liu *et al* (54) reported that miR-338 inhibits TNF-α and thereby inhibits the occurrence of sebaceous inflammation. Zhao *et al* (55) demonstrated that miR-214 is closely associated with inflammatory responses, and could promote the release of

the inflammatory cytokines TNF-α and interleukin-6. These four miRNAs (miR-214-3p, rno-miR-764-5p, rno-miR-338-5p and rno-miR-466b-5p) displayed low expression in the cell model of PAITA, suggesting that they may serve a role in processes involved in PAITA. Notably, these miRNAs all have complementary binding base sites to Egr1, suggesting that Egr1 may be regulated by these upstream miRNAs. A previous study suggested that the regulation of target RNAs by miRNAs is negative and unidirectional (54), but later studies have reported that lncRNAs can compete with their encoded genes for miRNAs via conserved miRNA response elements, resulting in decreased miRNA content and thereby reduced inhibition by miRNAs of target RNAs (56,57). The Egr1-related ceRNA sub-network contains 10 lncRNAs; currently, the role of these lncRNAs in PAITA remains unclear. Combined with the results of microarray and bioinformatics analysis, these 10 lncRNAs were determined to be highly expressed in PAITA and exhibited a competitive relationship with Egr1, suggesting that these lncRNAs may become therapeutic targets for PAITA.

In summary, the results of the present study suggested that Egr1 was highly expressed in PAITA and may affect the development of PAITA. Further analysis revealed that a lncRNA-miRNA-Egr1 regulatory network may be the basis for the effects of Egr1 on PAITA. The results of the present study may provide novel insight for studies into the pathogenesis of AP and PAITA, as well as the development of strategies for the clinical treatment of AP.

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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 XZ provided study material, performed the experiments and assembled the data. BG performed experiments and wrote the manuscript. All authors 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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