

# Circ-LDLRAD3 Regulates Cell Proliferation, Migration and Invasion of Pancreatic Cancer by MiR-876-3p/STAT3

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## Abstract

**Background:** Pancreatic cancer (PC) is one of the most lethal types of cancer in the world. The complex network of non-coding RNAs has been demonstrated to involve in the PC progression, however, the potential mechanism was still unclear.

**Methods:** The clinical tumor tissues and the adjacent-tumor tissues of PC were obtained from the surgery. Real-time PCR and western blot were performed to detect gene expression in an appropriate manner. The interaction between circ-LDLRAD3 and miR-876-3p was determined using a luciferase reporter assay and RNA pull-down. The interaction between miR-876-3p and STAT3 was determined using luciferase reporter assay.

**Results:** Overexpressed circ-LDLRAD3 and STAT3, while down-regulated miR-876-3p was observed in both PC tissues and cell lines. Knockdown of circ-LDLRAD3 suppressed PC cell proliferation, migration and invasion. circ-LDLRAD3 directly regulated the expression of miR-876-3p. STAT3 is the target molecule of miR-876-3p. circ-LDLRAD3 regulated the expression of STAT3 by miR-876-3p. circ-LDLRAD3 regulated cell proliferation, migration and invasion by miR-876-3p.

**Conclusion:** Down-regulated circ-LDLRAD3 suppressed cell proliferation, invasion and migration by directly regulating miR-876-3p/STAT3.

**Keywords:** pancreatic cancer; circ-LDLRAD3; miR-876-3p; STAT3; cell proliferation; migration and invasion

## INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive cancer with high lethality. It was estimated the 5-year survival rate of PC is 6%, and the mortality rate is almost equal to the incident rate [1]. The risk factors of PC are included in the smoking, obesity, family history, as well as the environment. Even though great progress has been achieved on the PC mechanism, the clinical therapy still needs to further be improved. Thus, to detect the biomarkers of PC in the early diagnosis seemed important in the PC therapy [2-4].

Signal transducer and activator of transcription 3 (STAT3) is reported to mediate angiogenesis, apoptosis, cell cycle, cell migration and drug resistance [5, 6]. Additionally, STAT3 is involved in the phosphorylation of specific tyrosine residue [7, 8] and transcriptional expression

of autophagy-associated genes [8]. What's more, STAT3 promotes mitochondrial transcription and oxidative respiration [9]. STAT3 has been identified to involve in various cancers, such as colon cancer [10], breast cancer [11], bladder cancer [12], as well as PC [13]. Study reported that STAT3 promoted cell proliferation, migration and invasion, knockdown of STAT3 is important for alleviating cell proliferation and colony formation of PC cell lines [14]. However, the potential regulatory mechanism of STAT3 is still need further identification.

Circular RNAs (circRNAs) are a class of noncoding RNAs that are formed by a close loop in the 5' end and 3' end [15], which different with long non-coding RNA (lncRNA) and microRNA (miRNA). Recently, due to the important role of circRNAs on disease progression, it has attracted much attention. Functional study revealed that circRNA is involved in the gene expression in transcriptional and post-translational level. Moreover, much studies shown that circRNAs are dysfunctional in nervous system disease [16], heart disease [17] and cancers [18]. Mounting evidences showed that circRNAs are involved in tumor cell proliferation, apoptosis, migration, invasion and metastasis [19]. In PC studies, much circ-RNA has been reported, such as circ-IARS [20], circ-0006215 [21]. Circ-LDLRAD3,

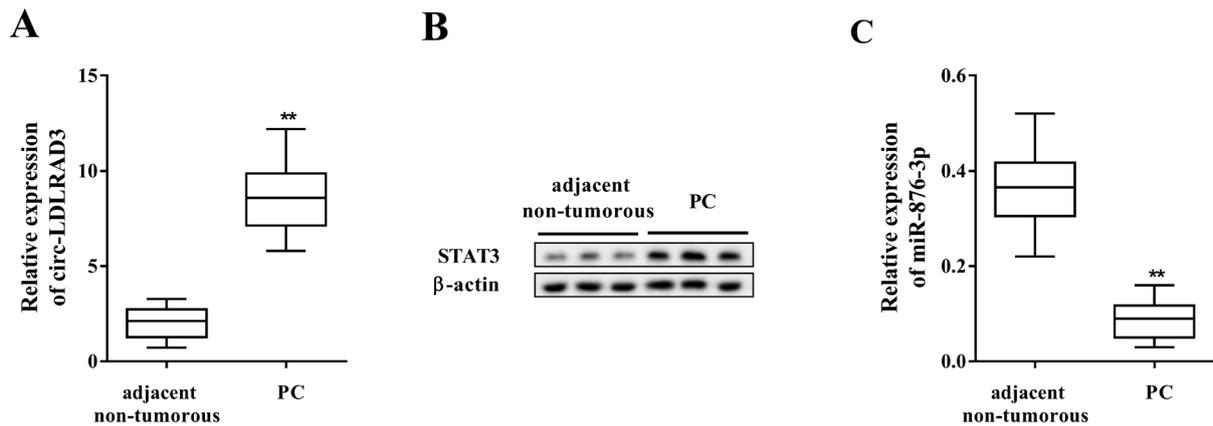
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**Figure 1.** The expression pattern of circ-LDLRAD3, miR-876-3p and STAT3 in clinical PC tissues. **A:** the expression of circ-LDLRAD3 was determined using real-time PCR; **B:** the protein expression of STAT3 was detected using western blot in 3 cases of PC tissue and 3 paired adjacent non-tumorous. **C:** the expression of miR-876-3p was detected using real-time PCR. \*\* $P < 0.01$  vs adjacent non-tumorous.

also known as hsa-circ-0006988, is increased in cell lines and clinical tissues of PC [22], while the potential role on PC was still no documented.

microRNA (miRNA) has shown to play a key regulatory role in the cellular physiological process and the cancer biological process [18]. Numerous miRNAs have been described as having altered expression in pancreatic cancer, including miR-21 [23, 24], miR-155 [25, 26], miR-146a [27]. MiR-876-3p showed significantly decreased in PC tissues, and overexpressed miR-876-3p significantly promoted cell apoptosis of BXP-3 and PANC-1 cell lines [28], while the potential mechanism of miR-876-3p in PC was still need further exploration.

In the present study, we performed the clinical experiments and in vitro study to explore the insight of miRNA and circ-RNA on PC. This study highlights the diagnostic potential for noninvasive evaluation of PC.

## MATERIALS AND METHODS

### Clinical tissues

Twenty samples of pancreatic cancer, from whom were first confirmed to contain tumor cells after evaluation by two experienced pathologists, and all patients treated at Affiliated Hospital of Inner Mongolia University for Nationalities from September 2016 to June 2017. Paired normal tissue samples were obtained 5 cm away from the pancreatic cancer tissue. All samples were immediately stored in liquid nitrogen for the following experiments. All participants provided written informed consent before the experiments. The Affiliated Hospital of Inner Mongolia University for Nationalities approved this study based on the Helsinki Declaration.

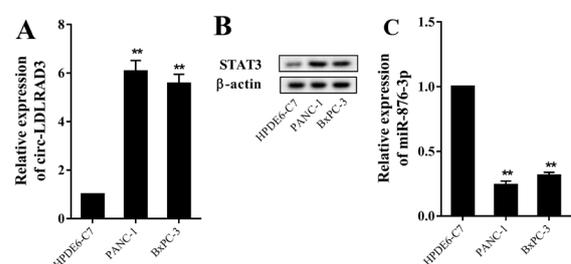
### Real-time PCR

Total RNA from tissues or cells were isolated using TRIzol

reagent assay (Invitrogen, Carlsbad, CA). Purified RNA was reversely transcribed into cDNA using the Reverse Transcription Kit (Ambion, Carlsbad, CA) according to the manufacturer's instruction. Real-time PCR was carried out using a Roche 480II system (Roche, Basel, Switzerland) and SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Dalian, China), following the manufacturer's instructions. The relative mRNA expression was normalized to GAPDH and calculated using the  $2^{-\Delta\Delta Ct}$  method. All experiments were repeated for three independent times.

### Western blot

Total protein collected after the cells or tissues were lysed by lysis buffer. The protein was measured using bicinchoninic acid protein assay (Beyotime, Shanghai, China). SDS-PAGE was performed to separate the protein and equal amount of the separated protein was transferred onto the PVDF and incubated with



**Figure 2.** The expression pattern of circ-LDLRAD3, miR-876-3p and STAT3 in PC cell lines. **A:** the expression of circ-LDLRAD3 was determined using real-time PCR; **B:** the protein expression of STAT3 was detected using western blot. **C:** the expression of miR-876-3p was detected using real-time PCR. \*\* $P < 0.01$  vs HPDE6-C7.

the primary antibodies (Abcam) at 4°C for 24 h. Then the PVDF was washed using PBS and incubated with the secondary antibody (Beyotime, Shanghai, China) at room temperature for 1 h. Protein bands were normalized to  $\beta$ -actin and visualized using the enhanced chemiluminescence ECL method.

### Cell culture

Both the PC cell lines PANC-1 and BxPC-3 were purchased from the ATCC, and the normal pancreatic cell line HPDE6-C7 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM complemented with 10% of bovine serum (FBS) in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% air at 37 °C.

### Cell transfection

miR-876-3p mimic, inhibitor siRNAs targeting circRNA-LDLRAD3, STAT3 and their negative control were purchased from RiboBio (Guangzhou, China). Cell transfection was performed using INTERFERin (Polyplus transfection, Illkirch, France) according to the man-

ufacturer's instructions. The transfection efficiency was determined using real-time PCR.

### Cell migration

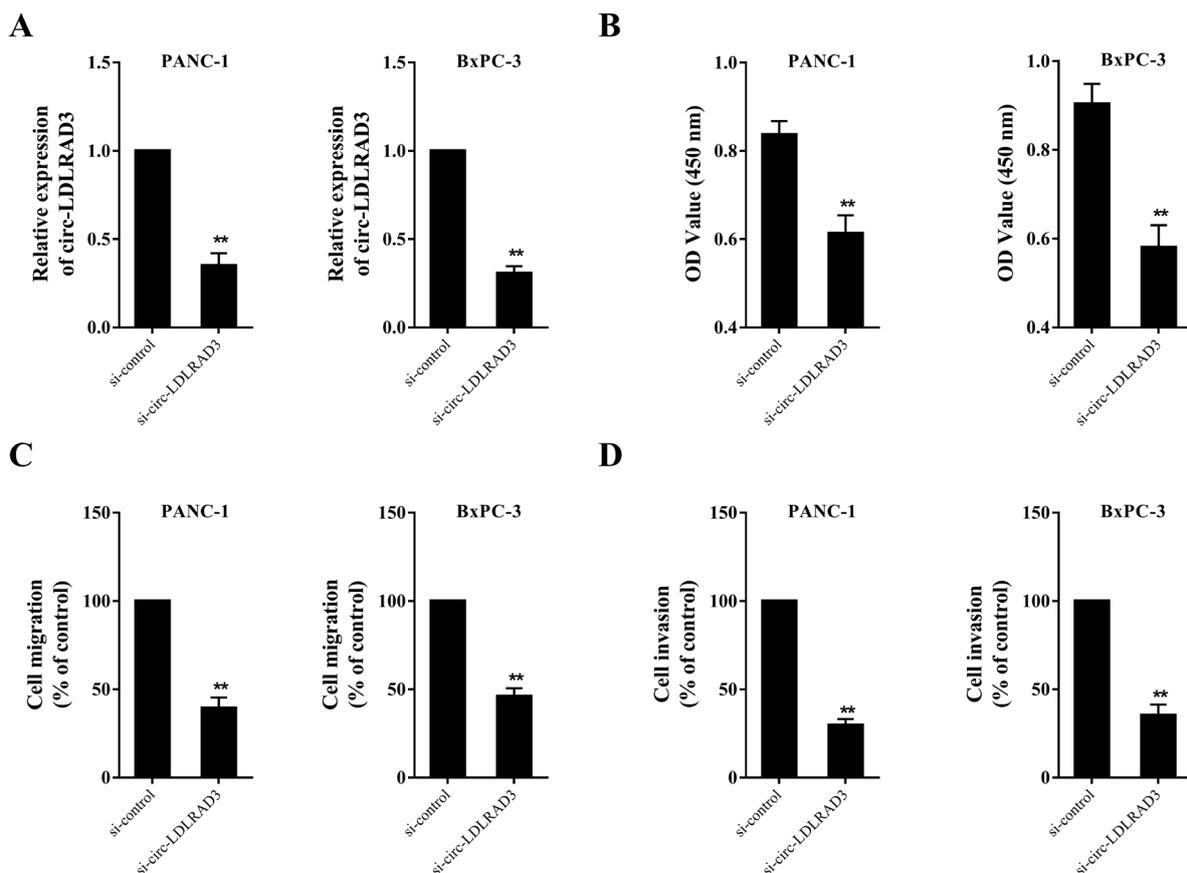
Cells were seeded in a 24-well plate at a concentration of 1×10<sup>4</sup> cells per well. After 24 h, the cell monolayers were scratched using a plastic tip across the plate. The wells were washed three times with PBS and incubated in low-serum (2%) medium with treatments. After 24 h, 48 h, and 72 h, wound healing pictures were taken.

### Cell invasion

Cell invasion was tested using a transwell assay. Cells were seeded with serum-free DMEM medium into the upper layer polycarbonate membrane filter and 20% FBS was added to the bottom chambers. After 48 h, the cells in the upper layer were removed, while the cells on the bottom were fixed with 4% PFA, stained with 0.05% crystal violet and counted.

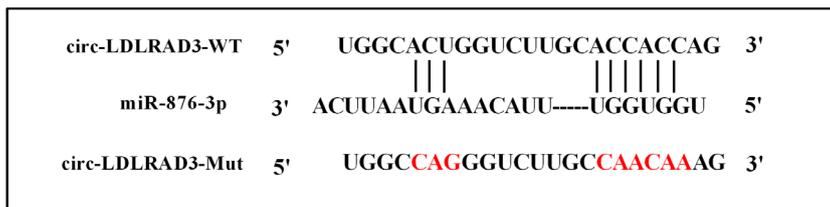
### Cell proliferation

Cell proliferation was determined using Cell Counting

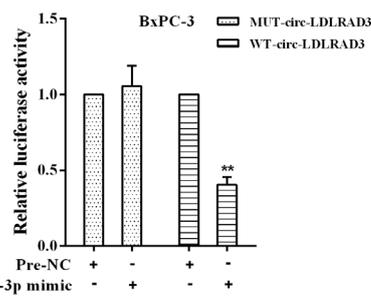
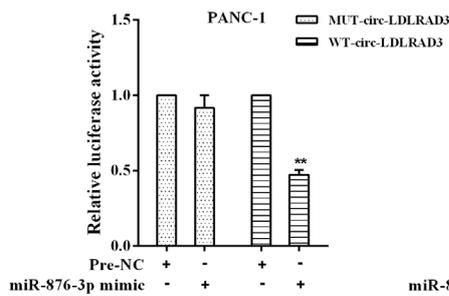


**Figure 3.** Role of circ-LDLRAD3 on PC cell proliferation, migration and invasion. Both PC cell lines PANC-1 and BxPC-3 were transfected with si-circ-LDLRAD3 or si-control, cells were divided into two groups, including si-control and si-circ-LDLRAD3; A: transfection efficiency was detected using real-time PCR. B: CCK-8 assay was performed to detect cell proliferation; C: cell migration was determined in different treatments; D: cell invasion was detected using transwell assay. \*\*P<0.01 vs si-control.

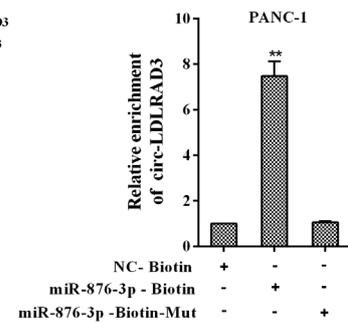
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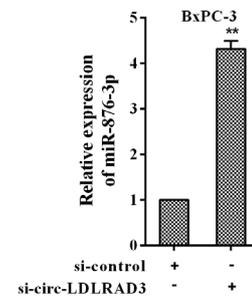
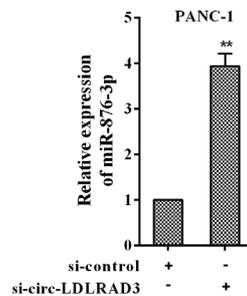
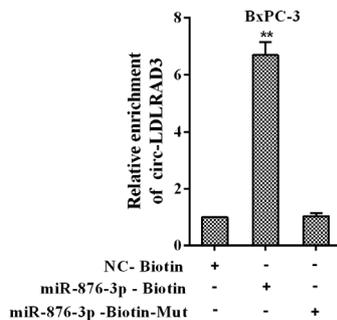
B



C



D



**Figure 4.** The interaction between circ-LDLRAD3 and miR-876-3p. **A:** online prediction revealed that miR-876-3p could bind with circ-LDLRAD3-WT; **B:** luciferase reporter assay was performed to determine the luciferase activity; **C:** RNA pull-down was performed to determine the relative enrichment of circ-LDLRAD3 under the biotin-miR-876-3p; **D:** real-time PCR was performed to determine the expression of miR-876-3p under the treatment of si-circ-LDLRAD3. \*\* $P < 0.01$  vs Pre-NC or NC-Biotin or si-control.

Kit-8 (CCK-8, Dojindo Chemical Laboratory, Kumamoto, Japan). Approximately  $5 \times 10^3$  cells per well were seeded onto 96-well plates subjected to the CCK-8 assay according to the manufacturer's instruction. The absorbance at 450 nm was measured following the addition of 10  $\mu$ L of the CCK-8 solution. All experiments were conducted in 5 replicates.

#### Luciferase reporter assay

Cells were co-transfected with pmirGLO-circRNA-LDLRAD3 (WT/Mut) or pmir-GLO-vector with miR-876-3p mimic using Lipofectamine 3000 (Thermo Scientific). Additionally, HEK293T cells were co-transfected with pmirGLO-STAT3 3'UTR (WT/Mut) and miR-876-3p mimic/miR-876-3p inhibitor. After 48 h, cells were collected and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

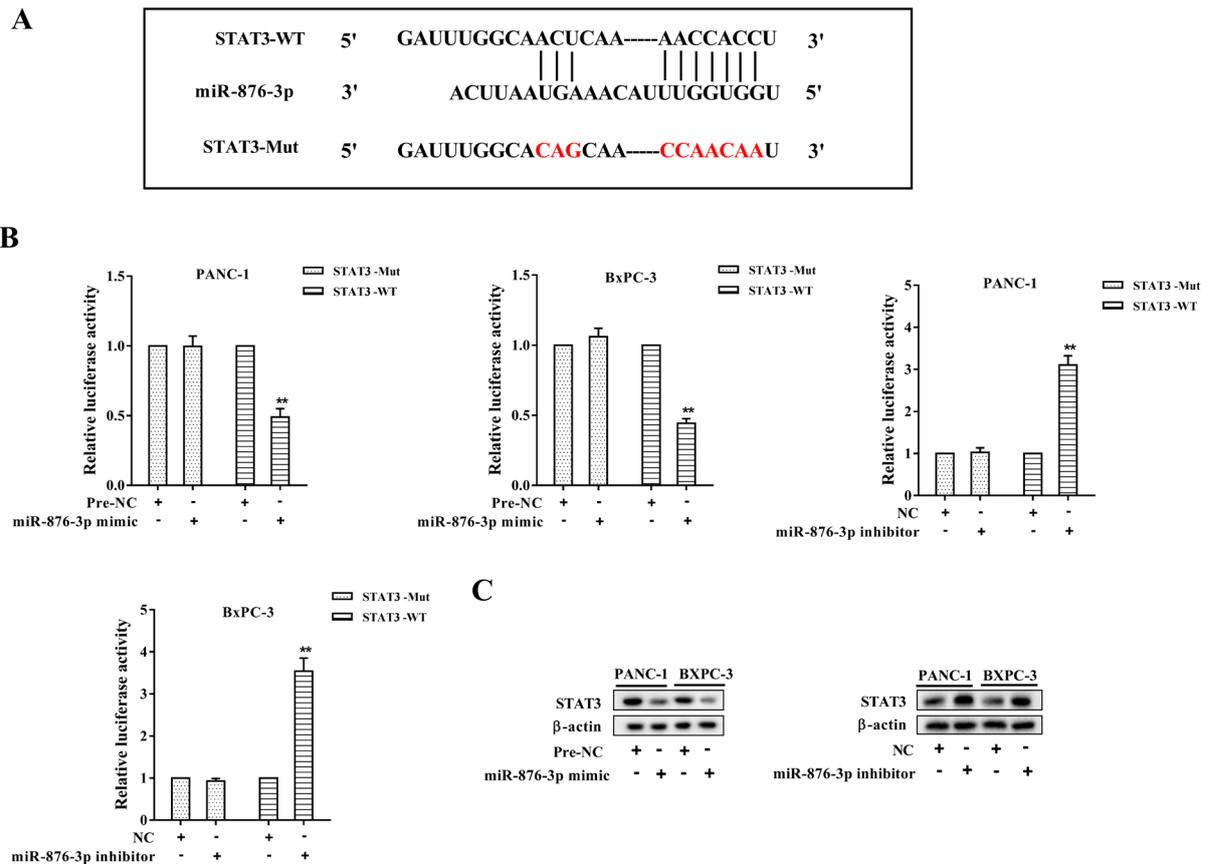
#### RNA pull-down

The Biotin-coupled RNA pull down assay was performed as described previously [29]. Briefly, the biotinylated miR-876-3p mimic/inhibitor or control (RiboBio) were transfected into cells for 24 h. The biotin-coupled RNA complex was pull-downed by incubating the cell lysates with streptavidin-coated magnetic beads (Life Technologies). The abundance of circRNA-LDLRAD3 in bound fractions were evaluated by western blot.

#### Data analysis

Data were presented as means  $\pm$  SD. Statistical analyses were performed using GraphPad Prism. Statistically significant differences were calculated using one-way ANOVA with Geisser-Greenhouse correction.  $P < 0.05$  were considered to be statistically significant difference.

## RESULTS



**Figure 5.** The interaction between miR-876-3p and STAT3. **A:** online prediction revealed that miR-876-3p could bind with STAT3 3'UTR -WT; **B:** luciferase reporter assay was performed to determine the luciferase activity in cells; **C:** western blot was conducted to determine the expression of STAT3 under the treatment of mR-876-3p mimic or inhibitor. \*\*P<0.01 vs pre-NC or NC.

### Overexpressed circ-LDLRAD3 in PC patients

To study the expression pattern of circ-LDLRAD3, miR-876-3p and STAT3, the clinical PC tissues and the matched normal tissues were collected. Real-time PCR revealed that circ-LDLRAD3 was significantly increased (Figure 1A), while miR-876-3p was decreased in PC tissues (Figure 1C). Western blot showed that STAT3 was increased in PC in comparing with the adjacent normal tissues (Figure 1B).

### Circ-LDLRAD3 was increased in PC cell lines

To verify the expression of circ-LDLRAD3, miR-876-3p and STAT3, the human normal pancreatic duct epithelial cell line HPDE6-C7, PC cell lines PANC-1 and BxPC-3 were employed. The results revealed that circ-LDLRAD3 (Figure 2A) and STAT3 (Figure 2B) were increased, while miR-876-3p (Figure 2C) was decreased in PC cell lines in comparing with HPDE6-C7.

### Knockdown of circ-LDLRAD3 inhibited cell proliferation, migration and invasion

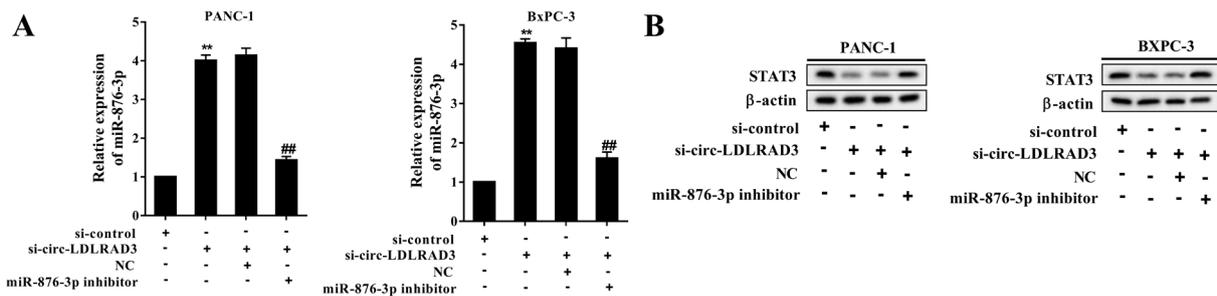
To determine the role of circ-LDLRAD3 on PC cells,

both PANC-1 and BxPC-3 were divided into two groups, including si-control and si-circ-LDLRAD3. The transfection efficiency was detected (Figure 3A). Knockdown of circ-LDLRAD3 significantly suppressed cell proliferation (Figure 3B). Cell migration (Figure 3C) and invasion (Figure 3D) was also significantly decreased in cells transfected with si-circ-LDLRAD3.

### Circ-LDLRAD3 regulated the expression of miR-876-3p

Online LncBase Predicted V.2 revealed that circ-LDLRAD3 could combined with miR-876-3p (Figure 4A). The luciferase reporter assay revealed that the luciferase activity in cells co-transfected with WT-circ-LDLRAD3 and miR-876-3p mimic is significantly decreased (Figure 4B). Additionally, RN pull-down assay revealed that circ-LDLRAD3 was enriched in WT biotin- miR-876-3p (Figure 4C). The expression of miR-876-3p under the treatment of si-circ-LDLRAD3 was also determined, the results showed that down-regulated circ-LDLRAD3 significantly promoted the expression of miR-876-3p in PC cell lines (Figure 4D).

### MiR-876-3p targets STAT3 to regulate its expression



**Figure 6.** The interaction role of circ-LDLRAD3 and miR-876-3p on STAT3. Both PANC-1 and BxPC-3 cells were divided into 4 groups, including si-control, si-circ-LDLRAD3, si-circ-LDLRAD3+NC and si-circ-LDLRAD3+miR-876-3p inhibitor. **A:** the expression of miR-876-3p was determined using real-time PCR; **B:** the expression of STAT3 was determined using western blot. \*\* $P < 0.01$  vs si-control, ## $P < 0.01$  vs si-circ-LDLRAD3+NC.

Online TargetScan predicted that miR-876-3p could bind with STAT3 3'UTR (Figure 5A). cells co-transfected with WT-STAT3 3'UTR and miR-876-3p mimic significantly decreased the luciferase activity, while co-transfected with WT-STAT3 3'UTR and miR-876-3p inhibitor significantly promoted luciferase activity (Figure 5B). Western blot revealed that cells transfected with miR-876-3p mimic decreased the expression of STAT3, while transfected with miR-876-3p inhibitor significantly promoted the expression of STAT3 (Figure 5C).

#### Circ-LDLRAD3 regulated the expression of STAT3 by miR-876-3p

To determine the role of circ\_LDLRAD3 on STAT3, both PC cell lines PANC-1 and BxPC-3 were employed. Cells were divided into 4 groups, including si-control, si-circ-LDLRAD3, si-circ-LDLRAD3+NC and si-circ-LDLRAD3+miR-876-3p inhibitor. Results revealed that si-circ-LDLRAD3 transfection promoted the expression of miR-876-3p, then cells co-transfected with miR-876-3p inhibitor decreased the expression of miR-876-3p (Figure 6A). What's more, the expression of STAT3 was decreased along with the treatment of si-circ-LDLRAD3, then cells co-transfected with si-circ-LDLRAD3 and miR-876-3p inhibitor significantly promoted the expression of STAT3 (Figure 6B).

#### Circ-LDLRAD3 regulated cell proliferation, migration and invasion by miR-876-3p

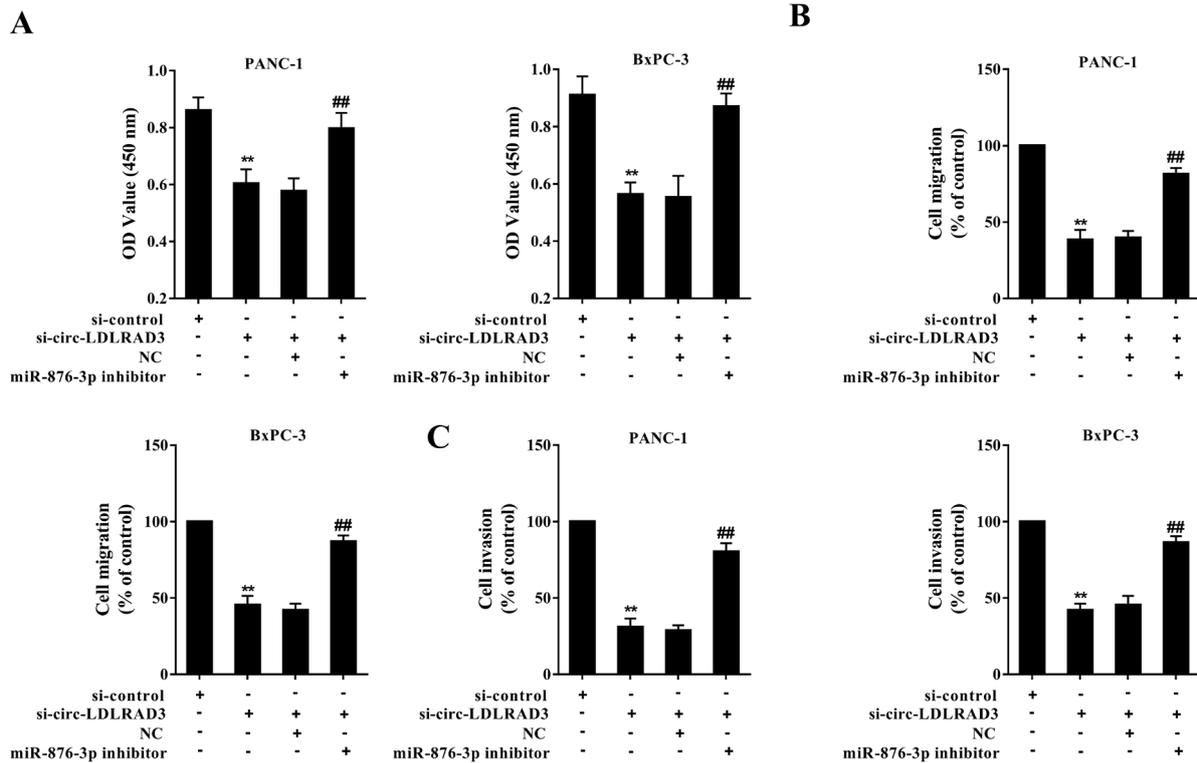
To explore the mechanism of circ-LDLRAD3 on PC, PANC-1 and BxPC-3 were divided into 4 groups, including si-control, si-circ-LDLRAD3, si-circ-LDLRAD3+NC and si-circ-LDLRAD3+miR-876-3p inhibitor. Results revealed that si-circ-LDLRAD3 transfection decreased cell proliferation (Figure 7A), migration (Figure 7B) and invasion (Figure 7C), while then cells co-transfected with miR-876-3p inhibitor significantly reversed the effect of si-circ-LDLRAD3.

## DISCUSSION

Recently, to explore the sensitive biomarkers for identifying cancers at a early stage is prevalence, and various biomarkers has been used in the clinical diagnosis. The specific biomarkers of PC have been discovered for a few years before, such as arbohydrate antigen 19-9 (CA19-9) and Glypican-1 (GPC1)<sup>[30-33]</sup>. In the present study, we employed circ-LDLRAD3, miR-876-3p and STAT3 to explore the potential mechanism on PC.

Recently, miRNAs played an important role in PC in its development and progression. By conducting miRNA expression profiling, those aberrant expressed miRNAs that revealed in the serum and in cancer tissues from patients with PC attracted our attention. Studies have demonstrated that these aberrantly expressed miRNAs are critically correlated with the disease stage, and drug resistance<sup>[34,35]</sup>. Hence, targeting these specific miRNAs, could provide an efficient and optimal approach in the therapy of pancreatic cancer. Indeed, the previous experiments also showed that nanoparticle delivery of synthetic oligonucleotides or treatment with natural agents could be useful to modulate the expression of miRNAs and thereby inhibit PC growth and progression, suggesting that targeting miRNAs could be a novel therapeutic strategy for increasing drug sensitivity and achieving better therapeutic outcomes of patients diagnosed with PC. In the present study, miR-876-3p was significantly decreased in the clinical PC tissues and PC cell lines, indicating the potential role of miR-876-3p in PC.

circRNAs have been identified to exhibit species conservation and tissue specificity<sup>[36]</sup>. With the emergence of RNA sequencing technology, circRNAs have been found to be extensively expressed in diseases. In addition, circRNAs are characterized by stable ring structure formed by a covalently closed continuous loop. Without free 3' and 5' ends, these molecules are not easily degraded by nucleases, which makes them ideal biomarkers for



**Figure 7.** The interaction role of circ-LDLRAD3 and miR-876-3p on cell proliferation, migration and invasion. Both PANC-1 and BxPC-3 cell lines were divided into 4 groups, including si-control, si-circ-LDLRAD3, si-circ-LDLRAD3+NC, si-circ-LDLRAD3+miR-876-3p inhibitor. **A:** cell proliferation was determined. **B:** cell migration was determined; **C:** cell invasion was determined. \*\*P<0.01 vs si-control, ##P<0.01 vs si-circ-LDLRAD3+NC.

detection of disease [37]. Investigators have identified disease-specific patterns of circRNA expression, which can serve as biomarkers for diseases [38], especially cancer [39]. However, there has been little investigation into the association of circRNAs with PC. In the present study, circ-LDLRAD3 was studied, with the results that overexpressed circ-LDLRAD3 was observed in both PC cancer tissues and cell lines, down-regulated circ-LDLRAD3 inhibited PC cell proliferation, invasion and migration, indicating that circ-LDLRAD3 played an important role in regulating the progression of PC.

As our knowledge of the transcriptome space has expanded, it has become increasingly clear that numerous miRNA-binding sites exist on a wide variety of RNA transcripts, leading to the hypothesis that all RNA transcripts that contain miRNA-binding sites can communicate with and regulate each other by competing specifically for shared miRNAs, thus acting as competing endogenous RNAs [40, 41]. The discovery of functional ceRNA regulation in diverse species — including viruses, plants, mice and humans — by multiple independent groups suggests that it may represent a widespread layer of gene regulation [42, 43]. We discuss literature describing the effect of miRNA competition on the regulation of both non-coding and coding RNAs, additional factors

that may affect ceRNA activity and potential directions for future studies, as well as the implications of miRNA competition for development and disease. Recent studies have demonstrated that circRNAs could function as miRNA sponges or potent competitive endogenous RNA (ceRNA) molecules [44-46]. In the present study, circ-LDLRAD3 was identified to regulate the expression of miR-876-3p by ceRNA mechanism, and then regulated the expression of STAT3.

Taken together, the present study revealed that the overexpressed circ-LDLRAD3 was observed in clinical PC tissues, circ-LDLRAD3 regulated the expression of miR-876-3p, while miR-876-3p is the upstream target gene of STAT3. Down-regulated circ-LDLRAD3 suppressed PC cell proliferation, invasion and migration by regulating miR-876-3p/STAT3.

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