

## Original Article

# MicroRNA-204 attenuates the migration and invasion of pancreatic cancer cells by targeting ZEB1/EMT axis

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Received March 8, 2018; Accepted April 13, 2018; Epub July 1, 2018; Published July 15, 2018

**Abstract:** Pancreatic cancer (PC) is one of the most aggressive malignancies worldwide. MicroRNAs play an important role in the development and progression of PC, but little is known about the role of miR-204 in PC. In this study, we revealed that miR-204 was downregulated in PC tissues and cell lines, and its expression was closely correlated with aggressive clinicopathological features of PC patients. Both gain- and loss-of-function studies showed that miR-204 overexpression inhibits the proliferation, migration and invasion of PC cells, whereas miR-204 knockdown had the opposite effects. Using mouse models, we found that miR-204 overexpression suppressed PC tumor growth in vivo. Moreover, miR-204 overexpression notably suppressed epithelial-mesenchymal transition (EMT) of PC cells, and through bioinformatics analysis and dual-luciferase reporter assay, ZEB1, a critical EMT promoter, was identified to be the functional target of miR-204 in PC cells. Rescue experiments further showed that ZEB1 overexpression abrogated the effects of miR-204 in PC cells. Collectively, these findings demonstrated the tumor suppressive role of miR-204 in PC through the ZEB1/EMT axis, therefore providing a novel therapeutic target for human PC.

**Keywords:** Pancreatic cancer, microRNA-204, epithelial-mesenchymal transition, ZEB1

## Introduction

Pancreatic cancer (PC) is one of the most aggressive malignancies worldwide [1], and the median survival time of PC patients is only 4 months after diagnosis [2]. Despite recent advances in diagnostic and treatment strategies, the prognosis of PC patients remains largely dismal [3]. In this regard, the molecular mechanisms implicated in the development and progression of PC are required to be further elucidated.

MicroRNAs (miRNAs, miRs) are an abundant group of endogenous, small non-coding, single-stranded RNAs with 21-25 nucleotides in length that inhibit the expression of their targeted genes [4]. Mounting evidence has demonstrated that miRNAs can serve as potential oncogenes or oncosuppressive genes, and their aberrant expression confers cancer development and progression [5]. Previous studies have demonstrated that miR-204 might serve as an oncosuppressor in various malignant tumors, including non-small-cell lung carcinoma [6], hepatocellular carcinoma [7] and cervi-

cal cancer [8]. miR-204 expression was also reported to be reduced in PC lines compared to normal cells [9]. Nonetheless, the potential involvement of miR-204 in PC remains to be further characterized.

Therefore, in the present study, we attempted to investigate the expression profile of miR-204 in PC tissues and cell lines. Gain- and loss-of-function assays were performed to determine the effects of miR-204 on PC cell behaviors. The molecular mechanisms underlying the function of miR-204 and its potential target in PC were also identified.

## Materials and methods

### Patient samples

PC and corresponding adjacent normal pancreas tissue samples were collected from 63 patients with PC who had undergone surgery at Lanzhou University Second Hospital (Lanzhou, China). The relevant characteristics of these PC patients are shown in **Table 1**. None of the patients had received chemotherapy or radio-

**Table 1.** The association between miR-204 expression and clinico-pathological characteristics of PC patients (n=63)

Characteristics	Total number	miR-204 expression		P value
		Low (n=34)	High (n=29)	
Age (years)				0.405
≥ 65	23	14	9	
< 65	40	20	20	
Gender				0.512
Male	43	22	21	
Female	20	12	8	
Tumor location in the pancreas				0.287
Head	39	19	20	
Non-head	24	15	9	
Tumor differentiation				0.336
Well + Moderate	44	22	22	
Poor	19	12	7	
TNM stage				0.022
I + II	43	19	24	
III + IV	20	15	5	
Vessel invasion				0.015
Present	30	21	9	
Absent	33	13	20	
Tumor size (cm)				0.042
> 4	26	18	8	
≤ 4	37	16	21	

therapy before surgery. All tumor and paired non-tumor tissues were confirmed by experienced pathologists in a double-blind manner. The collected tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until required. All participants provided written informed consent for sample collection. The study protocol was approved by the Ethics Committee of Lanzhou University Second Hospital.

#### *Cell culture, oligonucleotide transfection and lentiviral infection*

Human PC cell lines (Capan-2, ASPC-1, SW-1990, and Panc-1), normal pancreatic ductal epithelial cell line HPDE6c7 and HEK-293T cells, were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin in humidified air at 37°C with 5% CO<sub>2</sub>.

Synthetic miR-204 mimics, miR-204 inhibitor, mimics negative control, and inhibitor nega-

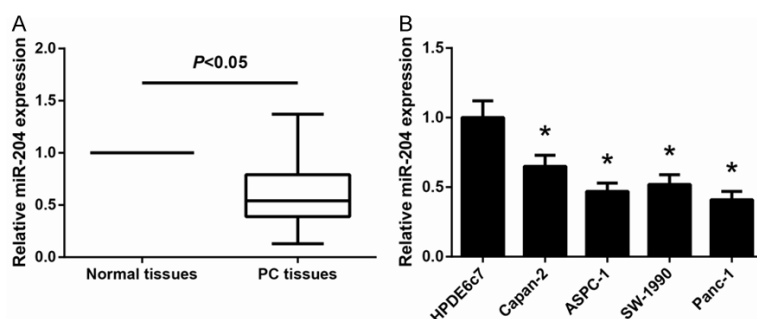
tive control were obtained from Guangzhou Ribobio Co., Ltd (Guangzhou, China). The pcDNA3.1-ZEB1 plasmid, which contains the full-length CDS sequence of ZEB1, was synthesized by GenePharma (Shanghai, China). The empty pcDNA3.1 vector served as a negative control. The cells were seeded onto 6-well plates and transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 48 h, transfection efficiency was determined through RT-qPCR analysis.

For in vivo study, the LV2-miR-204-mimics lentiviral vector was generated (Lv-miR-204; GenePharma) and confirmed by sequencing. The LV2 empty lentiviral vector (Lv-NC) served as a negative control. Lentivirus was packaged in the

HEK-293T cells and collected from the supernatants. Panc-1 cells were then infected with lentiviruses and screened by puromycin.

#### *RNA extraction and RT-qPCR analysis*

Total RNA was extracted from the tissues and cells using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesised using the Reverse Transcription System Kit (Takara; Dalian, China), and qPCR was performed using the fluorescent dye SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7500-HT Real-Time PCR System (Applied Biosystems). The relative quantification of target gene expression was calculated using the 2<sup>-ΔΔCt</sup> method [10], and normalized to the expression of U6 or GAPDH. All the primers were shown as follows: miR-204, RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACAGGC-AT-3', forward primer: 5'-TTCCCTTTGTCATCCT-3' and reverse primer: 5'-GTGCAGGGTCCGAGGT-3'; U6, RT: 5'-AACGCTTCACGAATTTGCGT-3', forward primer: 5'-CTCGCTTCGGCAGCACA-3' and reverse primer: 5'-AACGCTTCACGAATTTG-



**Figure 1.** MiR-204 expression is reduced in PC tissues and is associated with clinicopathological features. A. Relative miR-204 expression levels in human PC tissues were determined by RT-qPCR analysis. B. Relative miR-204 expression levels in PC cell lines were determined by RT-qPCR analysis. The data are presented as mean  $\pm$  SD. \* $P < 0.05$  versus HPDE6c7 cells.

CGT-3'; ZEB1, forward primer: 5'-GATGATGATGCGAGTCAGATGC-3' and reverse primer: 5'-CTGGTCTCTTCAGGTGCC-3'; GAPDH, forward primer: 5'-CGACTTATACATGGCCTTA-3' and reverse primer: 5'-TTCCGATCACTGTTGGAAT-3'.

#### Cell proliferation analysis

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, the transfected cells were seeded in 96-well plates about 5,000 cells/well. At different time points, 10  $\mu$ l of the CCK-8 solution was added to each well. After another 1 h of incubation, the optical density was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

#### Cell cycle analysis

In this assay, the transfected cells were fixed in ice-cold 70% ethanol for 12 h, and then stained with propidium iodide using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, USA) at room temperature for 30 min. Next, the cell cycle analysis was performed by a FACSCalibur flow cytometer (BD Biosciences). The ratios of cells in the G0/G1, S, G2/M phases were counted and compared.

#### Wound healing assay

For wound healing assay, the transfected cells were seeded in 6-well plates ( $1 \times 10^5$ /well). After monolayers formed, a sterilized 200  $\mu$ l plastic micropipette tip was used to make a straight scratch in the wells and then the cell were cultured in serum-free medium. After 48 h, the migrating distance was measured and photographed.

#### Transwell invasion assay

Invasion of PC cells was detected through transwell invasion assay using transwell chambers with 8- $\mu$ m pores (Costar, Corning, NY, USA). In brief,  $1 \times 10^5$  transfected cells in serum-free medium were seeded into the upper chamber that is coated with Matrigel (BD Biosciences). Medium containing 10% FBS were added to the lower chamber as a chemoattractant. After 48 h of incubation, the cells were fixed in 70% ethanol

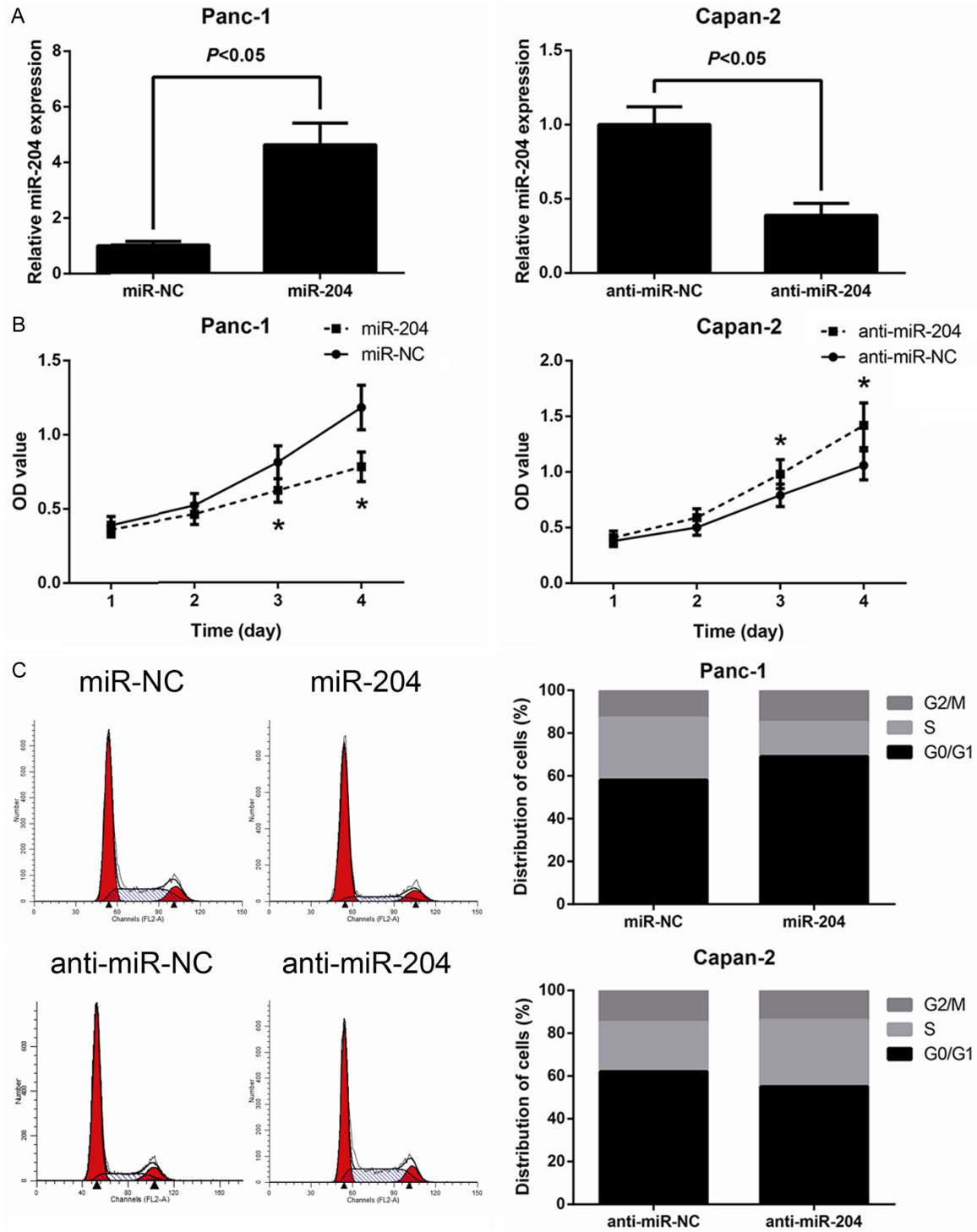
for 30 min and stained with 0.1% crystal violet for 10 min. The cells were then counted and photographed under an inverted microscope (Olympus Corporation, Tokyo, Japan).

#### Protein extraction and western blotting

To extract the proteins, the cells were washed twice in cold PBS, and then lysed using Radio-Immunoprecipitation Assay (RIPA) protein extraction reagent (Beyotime, Beijing, China) with protease and phosphatase inhibitors. The protein concentration was determined using a BCA Protein Assay Kit (Solarbio, Beijing, China). Equivalent amounts of cell protein lysates were separated on SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked with 10% skim milk and then incubated with primary antibodies against E-cadherin (1:2000; Abcam, Cambridge, MA, USA), ZEB1 (1:2000; Abcam), N-cadherin (1:2000; Abcam), Vimentin (1:2000; Abcam) or GAPDH (1:2000; Abcam) at 4°C overnight, followed by incubation for 1 hour at room temperature with appropriate horseradish peroxidase-linked secondary antibodies (1:10000; Santa Cruz, Dallas, TX, USA). The bands were visualized using the electrochemiluminescence (ECL) kit (EMD Millipore, St. Charles, MO, USA), and the signals were analyzed by Image Lab software (Bio-Rad). GAPDH was used as a protein-loading control.

#### Dual-luciferase reporter assay

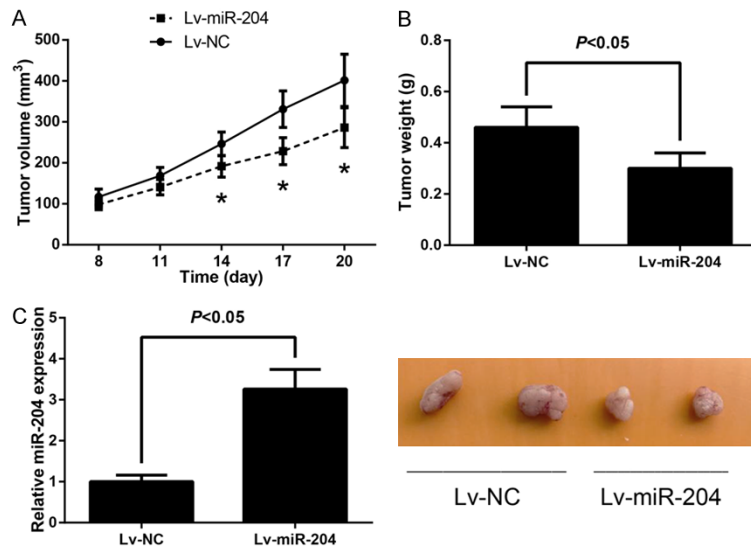
The 3'-UTR of ZEB1, containing putative miR-204-binding sites, was amplified by PCR and



**Figure 2.** miR-204 inhibits PC cell proliferation in vitro. A. The transfection efficiency was verified by RT-qPCR analysis. B. MTT assay was performed to determine the proliferation of Panc-1 and Capan-2 cells. C. The cell cycle profiles of Panc-1 and Capan-2 cells were determined by flow cytometric analysis. The data are presented as mean  $\pm$  SD. \* $P < 0.05$  versus NC cells.

subcloned into a pGL3 luciferase promoter vector (Promega, Madison, WI, USA). We also generated a mutant 3'-UTR reporter construct using Quickchange XL site-directed mutagenesis

kit (Agilent Technologies, Santa Clara, CA, USA). All constructs were verified by DNA sequencing. Cells were co-transfected with miR-204 mimics or mimics negative control, wild-type or mutant



**Figure 3.** MiR-204 suppresses PC tumor growth in vivo. A. Growth curves of tumor volumes. B. The average weight of the tumors derived from each group of mice. C. RT-qPCR analysis of miR-204 expression in the tumor tissues. The data are presented as mean  $\pm$  SD. \* $P < 0.05$  versus NC group.

reporter vector and Renilla plasmid. 48 h post-transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega). Renilla luciferase was used as an internal reference.

#### Tumor xenograft experiments

Male BALB/c nude mice (aged 4-6 weeks, weighing 18-22 g) were purchased from the Slac Laboratory Animal Center (Shanghai, China) and maintained under specific pathogen-free conditions. To establish a PC xenograft model,  $1 \times 10^6$  Panc-1 cells infected with Lv-miR-204 or Lv-NC were suspended in 100  $\mu$ l PBS and then injected subcutaneously into the upper right flank region of mice. Tumor volumes were routinely measured by using the following formula:  $V = 1/2 \times \text{Length} \times \text{Width}^2$  (V, volume; L, length; W, width of tumor). After 20 days, the mice were sacrificed, and the xenograft tumors were excised and weighted. All animal experiments were performed in accordance with protocols approved by the Ethics Committee of Lanzhou University Second Hospital.

#### Statistical analysis

All data were analyzed by GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) and SPSS v. 16.0 software (SPSS, Inc., Chicago, IL, USA). The results of the experiments are displayed as the mean  $\pm$  standard

deviation (SD). To compare the significance of two groups, Student's t test or Chi-square test was performed. Pearson's correlation analysis was performed to determine the correlation between ZEB1 mRNA expression and miR-204 expression in PC tissues.  $P < 0.05$  was considered to indicate a statistically significant difference.

#### Results

*miR-204 expression is reduced in PC tissues and is associated with clinicopathological features*

We first determined the levels of miR-204 expression in 63 pairs of PC and correspond-

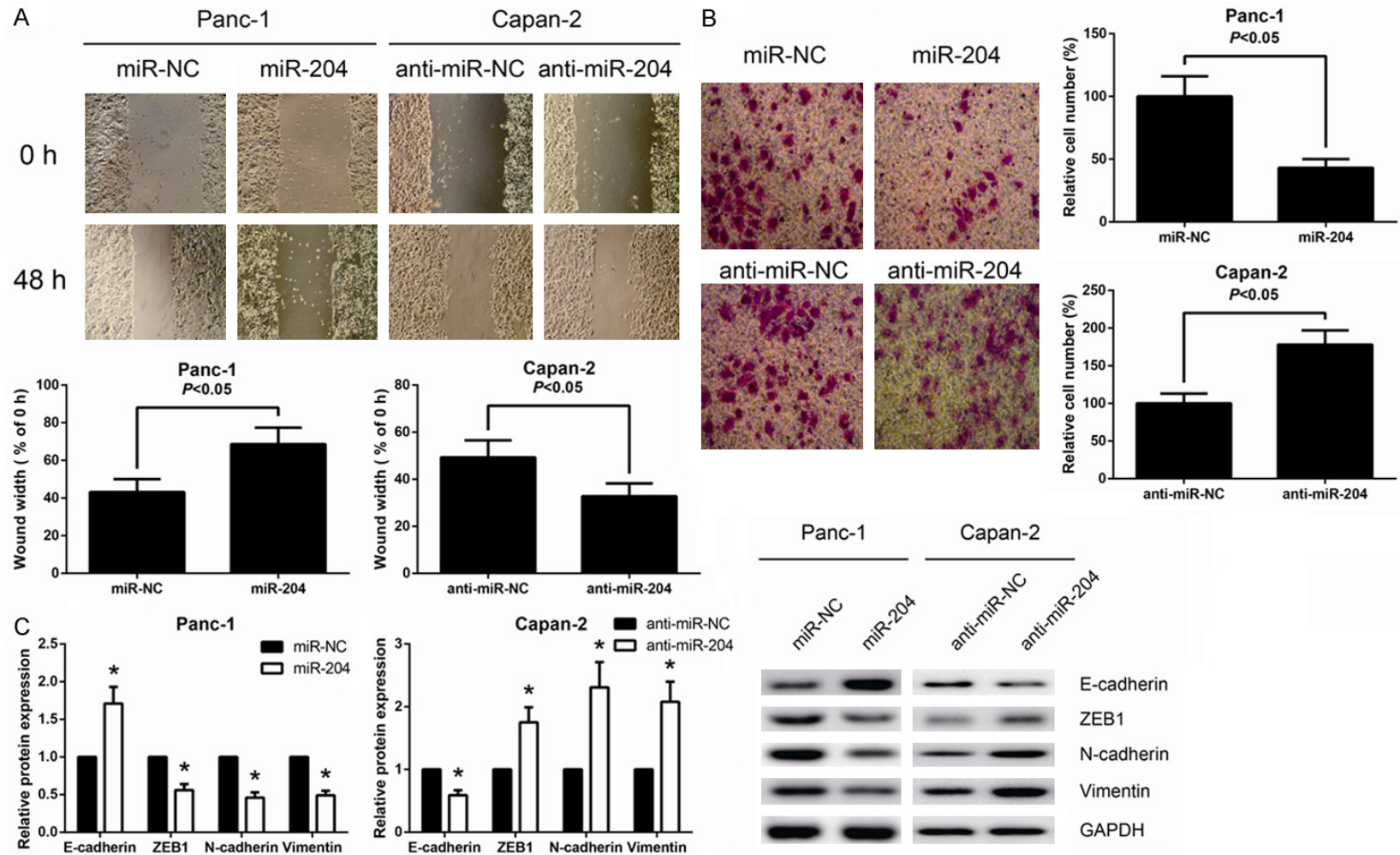
ing adjacent normal pancreas tissue samples by RT-qPCR analysis. As shown in **Figure 1A**, PC tissues showed significantly lower expression levels of miR-204 as compared with that of the normal tissues. In addition, miR-204 was also remarkably downregulated in the four PC cell lines (Capan-2, ASPC-1, SW-1990 and Panc-1) when compared with normal pancreatic ductal epithelial cell line HPDE6c7 (**Figure 1B**).

Based on the median expression of miR-204, we classified the PC patients into two groups: high miR-204 expression group ( $n=29$ ) and low miR-204 expression group ( $n=34$ ). **Table 1** summarizes the correlation between miR-204 expression and clinicopathological features of PC patients, and we found that low miR-204 expression levels were closely correlated with TNM stage ( $P=0.022$ ), vessel invasion ( $P=0.015$ ) and tumor size ( $P=0.042$ ).

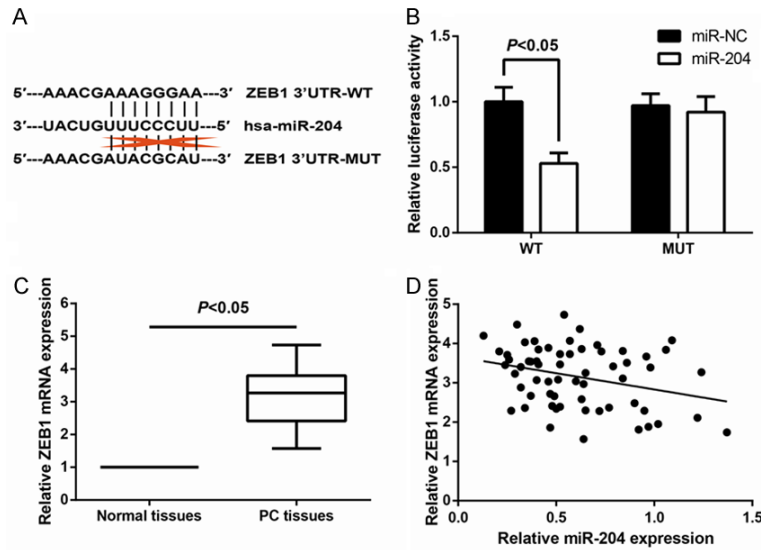
#### MiR-204 inhibits PC cell proliferation in vitro

Furthermore, we modulated miR-204 expression to examine whether miR-204 regulated the proliferation of PC cells in vitro. Panc-1 and Capan-2 cells were respectively transfected using miR-204 mimics or miR-204 inhibitor, and the results of transfection efficacy indicated that miR-204 expression was increased significantly in Panc-1 cells and was significantly reduced in Capan-2 cells by RT-qPCR analysis





**Figure 4.** MiR-204 attenuates PC cell migration, invasion, and EMT in vitro. A. Wound healing assay was performed to analyze the migration ability of Panc-1 and Capan-2 cells. B. Cell invasion capacity was detected by transwell assay in Panc-1 and Capan-2 cells. C. The expression levels of EMT-associated protein markers in Panc-1 and Capan-2 cells were measured by western blot analysis. The data are presented as mean  $\pm$  SD. \* $P < 0.05$  versus NC cells.



**Figure 5.** ZEB1 is a direct target of miR-204 in PC cells. A. A schematic diagram of ZEB1 3'-UTR as a putative target for miR-204. B. Relative luciferase activity was analyzed in Panc-1 cells cotransfected with pGL3-ZEB1-WT or pGL3-ZEB1-MUT and miR-204 mimics or mimics control. The data are presented as mean  $\pm$  SD. C. Relative ZEB1 mRNA expression levels in human PC tissues were determined by RT-qPCR analysis. D. Pearson's correlation analysis showed a negative correlation between ZEB1 mRNA and miR-204 expression in PC tissues.

(Figure 2A). CCK-8 assay displayed a significant decrease in the proliferation of Panc-1 cells transfected with miR-204 mimics, and miR-204 knockdown in Capan-2 cells led to a significant increase in cell proliferation (Figure 2B). We further analyzed the effect of miR-204 on cell cycle distribution of PC cells. A larger proportion of Panc-1 cells transfected with miR-204 mimics accumulated in the G0/G1 phase, whereas the S phase population reduced. However, an opposite result was found in Capan-2 cells with miR-204 knockdown (Figure 2C).

#### MiR-204 suppresses PC tumor growth in vivo

We then determined the role of miR-204 in PC tumor growth in vivo. Based on the periodic observation, we found that overexpressing miR-204 significantly attenuated the growth rate of PC tumors (Figure 3A). After sacrificed, the tumors were resected and the average weight of the tumors from the miR-204-overexpressing group was dramatically decreased (Figure 3B). The overexpression of miR-204 in the tumor tissues derived from Lv-miR-204-transfected cells was further confirmed by RT-qPCR analysis (Figure 3C).

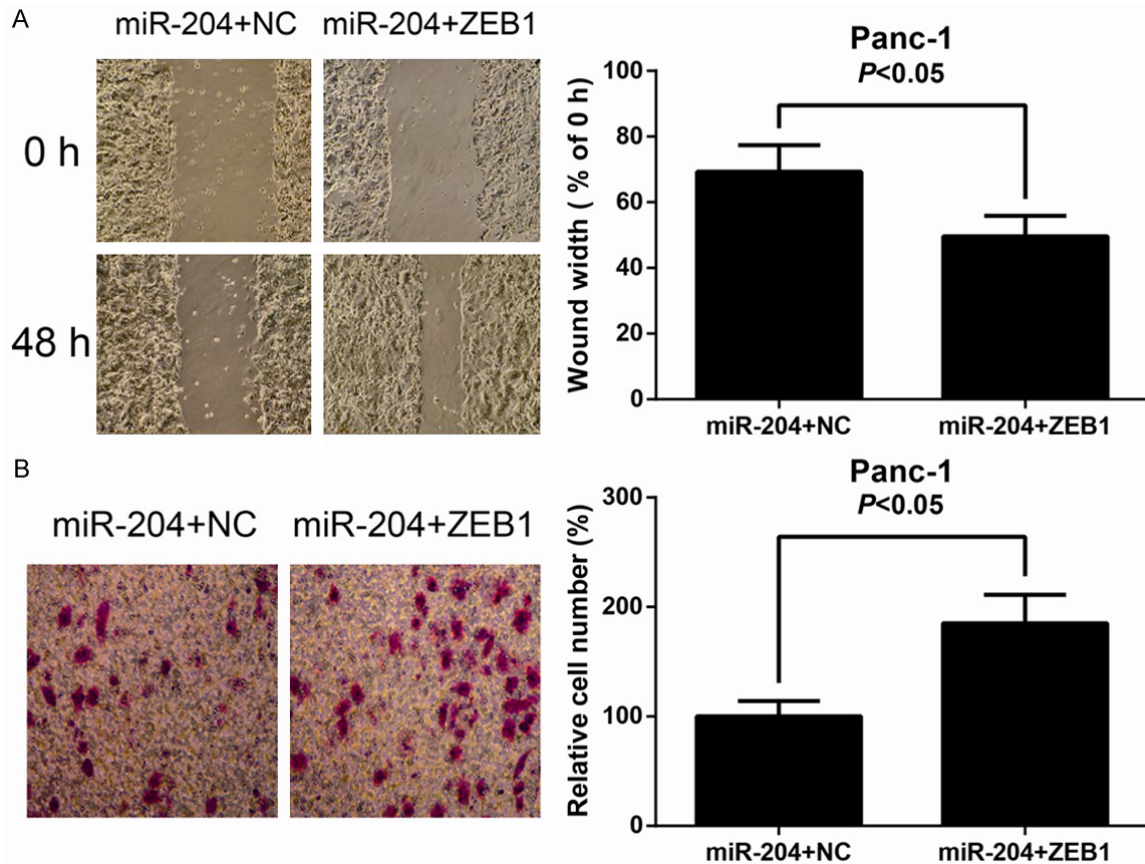
#### miR-204 attenuates PC cell migration, invasion and EMT in vitro

We then assessed the role of miR-204 in regulating PC cell migration using wound healing assay. Panc-1 cells transfected with miR-204 mimics resulted in a slower closing of scratch wounds, and knockdown of miR-204 had the opposite effect in Capan-2 cells (Figure 4A). Moreover, transwell invasion assay showed that miR-204 overexpression reduced the number of invaded Panc-1 cells, whereas the number of invaded Capan-2 cells was increased when miR-204 was knocked down (Figure 4B). In addition, western blot analysis was performed to investigate the expression levels of EMT-associated markers in PC cells. Our results demon-

strated that overexpression of miR-204 promoted E-cadherin expression, while reduced ZEB1, N-cadherin and vimentin expression in Panc-1 cells, and knockdown of miR-204 had the opposite effects in Capan-2 cells (Figure 4C).

#### ZEB1 is a direct target of miR-204 in PC cells

To identify the potential target of miR-204 in PC cells, we performed in silico analysis using the TargetScan database (<http://www.targetscan.org>) [11]. A conserved miR-204-binding site in the 3'-UTR of ZEB1 was thus identified (Figure 5A). Besides, as demonstrated in Figure 5B, miR-204 mimics significantly inhibited the luciferase activity of the reporter with WT 3'-UTR sequence and failed to obviously suppress that of MUT 3'-UTR in Panc-1 cells. We further analyzed the ZEB1 mRNA expression in human PC tissues, and the results showed that the expression levels of ZEB1 mRNA were remarkably higher in PC tissues than in corresponding adjacent normal pancreas tissues (Figure 5C). Additionally, correlation analysis demonstrated that ZEB1 mRNA expression levels were negatively correlated with miR-204 expression levels in 63 cases of PC tissues ( $r=-0.303$ ,  $P=0.016$ ; Figure 5D).



**Figure 6.** ZEB1 restores the effects of miR-204 in PC cells. A. Wound healing assay was performed to analyze the migration ability of Panc-1 cells. B. Cell invasion capacity was detected by transwell assay in Panc-1 cells. The data are presented as mean  $\pm$  SD.

#### ZEB1 restores the effects of miR-204 in PC cells

Next, we determined whether miR-204 inhibits the migration and invasion of PC cells partly through regulating ZEB1. When pcDNA3.1-ZEB1 was transfected into Panc-1 cells, the ZEB1 protein expression was remarkably increased (Data not shown). Both wound healing assay and transwell invasion assay showed that ZEB1 overexpression significantly blocked the inhibitory role of miR-204 on the migration and invasion of Panc-1 cells (**Figure 6A, 6B**).

#### Discussion

PC remains a major clinical challenge. Great efforts have been made to identify PC-related miRNAs and elucidate their biologic functions [12]. For example, from a clinical perspective, gemcitabine-treated PC patients with high miR-204 expression had more favorable prognosis than those with low miR-204 expression [13]. In

the present study, we found that miR-204 expression was significantly decreased in human PC tissues, and low expression of miR-204 was closely associated with the aggressive clinicopathological features of PC patients.

miRNAs are implicated in the regulation of various biological processes in PC, including proliferation, invasion, and development [14]. Here we investigate the functional role of miR-204 in PC cells through gain- and loss-of-function approaches, and the results demonstrated that overexpression of miR-204 markedly inhibited PC cell proliferation, migration and invasion, whereas knockdown of miR-204 exerted the opposite effects. In line with the *in vitro* findings, we found that miR-204 overexpression inhibited the growth of PC xenografts *in vivo*. Taken together, our results suggested that miR-204 plays a tumor-suppressive role in PC.

Metastasis is the major cause of death in PC patients, and epithelial-mesenchymal transi-



tion (EMT) is an essential prerequisite process for cancer metastasis [15]. EMT is also associated with poor survival in surgically resected PC [16]. MiRNAs have been frequently shown to exert critical regulatory functions in EMT [17]. In our present study, we also observed the inhibition of EMT in PC cells following miR-204 overexpression. To further explore the underlying molecular mechanisms, we investigated the potential targets of miR-204. Through bioinformatics analysis and dual-luciferase reporter assay, ZEB1 was confirmed as a direct downstream target of miR-204 in PC cells. ZEB1 is an important EMT promoter in cancer cells [18], and several lines of evidence show that ZEB1 is overexpressed in PC [19, 20]. Consistently, our results also showed the upregulation of ZEB1 in PC tissues, and more importantly, ZEB1 overexpression largely abrogated the effects of miR-204 in PC cells.

In summary, we confirmed that miR-204 acts as a tumor suppressive miRNA in PC tumorigenesis. We further identified ZEB1 as functional target of miR-204, and proved the involvement of EMT in PC progression. These findings provided new insight into the pathogenesis of PC and a novel potential therapeutic target for the treatment of PC in the future.

## Acknowledgements

The work of our research was supported by grant from Gansu Natural Science Foundation (Grant number: 145RJZA177).

## Disclosure of conflict of interest

None.

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