European Review for Medical and Pharmacological Sciences 2018; 22: 8640-8648

MiR-210 knockdown promotes the development of pancreatic cancer via upregulating E2F3 expression

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-210 (miR-210) and E2F3 in the development of pancreatic cancer and to investigate the possible underlying mechanism.

PATIENTS AND METHODS: The expression level of miR-210 in pancreatic cancer tissues, para-cancerous tissues, and normal pancreatic tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between miR-210 expression and pathological indicators of pancreatic cancer was analyzed. Meanwhile, the expression of miR-210 in pancreatic cancer cells and normal pancreatic ductal epithelial cells was detected by qRT-PCR. After transfection with miR-210 mimics and inhibitor, the viability and cell cycle of pancreatic cancer cells were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. The binding condition of miR-210 and E2F3 was verified by Dual-Luciferase reporter gene assay.

RESULTS: MiR-210 was lowly expressed in pancreatic cancer tissues than that of para-cancerous tissues. The expression of miR-210 was negatively correlated with TNM stage and tumor size of pancreatic cancer. *In vitro* experiments showed that the miR-210 was downregulated in pancreatic cancer cells than that of normal pancreatic ductal epithelial cells. Meanwhile, overexpression of miR-210 arrested cell cycle decreased cell viability and downregulated E2F3 expression in pancreatic cancer cells. Dual-Luciferase reporter gene assay indicated that E2F3 bound to mi-210. Further experiments confirmed that E2F3 was negatively regulated by miR-210.

CONCLUSIONS: MiR-210 knockdown promotes cell proliferation by upregulating E2F3 expression, thereby promoting the progression of pancreatic cancer.

Key Words:
MicroRNA-210 (MiR-210), E2F3, Pancreatic cancer, Proliferation.

Introduction

Pancreatic cancer is a malignant tumor of the digestive tract, which is characterized by occult symptoms, rapid development and poor prognosis. Due to environmental pollution and accelerated lifestyle, the incidence of pancreatic cancer has increased rapidly. It is reported that the 5-year survival of pancreatic cancer is lower than 7%, ranking fourth and sixth among the causes of cancer death in the United States and China, respectively[1,2]. Nowadays, surgical resection is the first choice for the treatment of pancreatic cancer. However, because of occult clinical manifestations and lack of early diagnostic marker, 80% of pancreatic cancer patients with advanced stage or distant metastasis cannot be operated[3]. More seriously, the postoperative 5-year survival rate in pancreatic cancer patients is only 25%[4]. Recent studies have concerned about the role of miRNAs in pancreatic cancer[5].

MicroRNA (miRNA) is a type of small, single-stranded, non-coding RNA discovered in recent years, with 18-22 nucleotides in length. It's reported that miRNAs exert a crucial role in the post-transcriptional gene regulation. The function of miRNA is mediated by RNA-induced silencing complex (RISC), ultimately leading to the degradation or translational inhibition of target mR-

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NAs[6,7]. MiRNA participates in a series of biological processes, including embryonic development, organogenesis, hematopoiesis, cell proliferation, apoptosis and metastasis[8-10]. Meanwhile, miRNAs are also confirmed to be involved in tumor development[11]. As oncogenes or tumor-suppressor genes, differentially expressed miRNAs may regulate the proliferation, apoptosis and invasion of tumor cells[12,13]. Scholars have shown that miR-210 is involved in pathological regulation of different tumors[14,15]. For example, miR-210 is served as an independent prognostic indicator for patients with triple-negative breast cancer[16]. Meanwhile, miR-210 is highly expressed in liver cancer, which promotes the invasion and migration of liver cancer cells[17]. In addition, methylation of miR-210 is associated with H. pylori infection, thereby exerting an important role in the occurrence of gastric cancer[18].

Normal mitotic cycle is divided into G1 (pre-DNA synthesis), S (DNA synthesis), G2 (late DNA synthesis), and M phase (division). Among them, the G2 phase is closely related to the formation of the spindle. Meanwhile, the G2/M phase transition is also an important step in cell cycle regulation. Cell cycle is known to be strictly regulated by checkpoint mechanism, including G1/S checkpoint and G2/M checkpoint[19]. In G1/S checkpoint, cell status and growth materials are monitored to ensure that cells are suitable for mitosis. However, G2/M checkpoint mainly focuses on genetic material DNA. Only undamaged cells with complete DNA replication can enter in the M phase[20,21]. Relative studies have pointed out that miR-210 overexpression arrests cell cycle of kidney cancer and nasopharyngeal cancer cells in G1/S and G2/M phase, thereafter inhibiting cell proliferation[22,23]. E2F transcription factor 3 (E2F3) is a specific inhibitory target gene of miR-210. Researches have indicated that E2F3 knockdown can arrest cell cycle[24].

E2F is a kind of cytokines that can control the transcription of the E2 gene in adenovirus. At present, 8 genes have been found in the E2F family, namely E2F1-E2F8[25]. Among them, E2F3 is a transcriptional activator that regulates cell cycle, cell proliferation and apoptosis[26]. Moreover, E2F3 leads to excessive cell proliferation and apoptosis in Rb-mutant embryos, thus regulating tumor development[27]. For example, miR-125b can inhibit the development of bladder cancer by targeting E2F3[28].

However, the relationship between E2F3 expression and pancreatic cancer has not been clearly elucidated. This study aimed at exploring the specific role of miR-210 and E2F3 in pancreatic cancer development and to provide novel directions for the diagnosis and treatment of pancreatic cancer.

Patients and Methods

Sample Collection

From February 2014 to November 2017, totally 28 pancreatic cancer tissues surgically resected from patients undergoing duodenopancreatectomy in the Affiliated Yantai Yuhuangding Hospital of Qingdao University were enrolled in this study. All resected tissues were postoperatively diagnosed with pancreatic cancer. In addition, 28 para-cancerous tissues (5 cm away from the edge of pancreatic cancer tissues) and 8 healthy pancreatic tissues were collected as controls. None of the patients received preoperative treatment. Informed consent was obtained from each patient before the study. The Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University approved this study.

Cell Culture

4 pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPC-1, and PANC-1) and 1 normal pancreatic ductal epithelial cell line (HPC-Y5) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. Culture medium was replaced every 1-2 days.

Cell Transfection

Cells were transfected with miR-210 mimics or inhibitor according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the confluence reached 50%. Culture medium was replaced 4-6 h after transfection.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates at a density of 1×10^5/ml. A total of 10 μl CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well after culture for 24, 48 and 72 h, respectively. The absorbance at 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Cycle Detection

Cells were digested for the preparation of cell suspension. Then the cells were washed with

pre-cooled phosphate-buffered saline (PBS) twice. After adding pre-cooled 75% alcohol, the cells were placed in a 4°C refrigerator for overnight fixation. Subsequently, the ethanol was discarded, and the cells were washed with 1×PBS. After centrifugation at 1000 rpm/min for 5 min, the cells were incubated with 100 μL propidium iodide and 100 μL RNA enzyme. Cell cycle was detected by flow cytometry.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells and tissues was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed according to the instructions of PrimeScript RT Reagent Kit (TaKaRa, Otsu, Shiga, Japan). The concentration of extracted RNA was detected by a spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). Relative gene expression was calculated by the $2^{-\Delta Ct}$ method. Primers used in the study were as follows: GAPDH, forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; MiR-210, forward: 5'-ACACTCCAGCTGGGCTGTGCGTGTG-3', reverse: 5'-CAACTGGTGTCGTGGAGTCG-3'; E2F3, forward: 5'-GGGCCCATTGAGGTTTACTTATGTC-3', reverse: 5'-ATCGCTATGTCCTGAGTTGGTTGA-3'.

Western Blot

Radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) was used to extract total protein. The concentration of extracted protein was measured by the bicinchoninic acid (BCA) reagent kit (Pierce Biotechnology, Rockford, IL, USA). Extracted proteins were separated on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. Then the membranes were washed with phosphate buffered saline-tween (PBST) (Beyotime, Shanghai, China) 3 times, followed by incubation with secondary antibodies at room temperature for 2 h. Finally, immunoreactive bands were exposed by the Enhanced-chemiluminescence (ECL) method (Thermo Fisher, Waltham, MA, USA).

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant E2F3 were first constructed. Subsequently, the cells were co-transfected with miR-210 mimic/negative control and wild-type/mutant E2F3, respectively. Luciferase activity was detected according to the instructions of the Dual-Luciferase reporter gene assay kit (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS 18.0 Software) (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were expressed as mean ± standard deviation ($\overline{x}\pm s$). The *t*-test and chi-square test were performed to compare the differences between measurement data and classification data, respectively. $p<0.05$ was considered statistically significant.

Results

MiR-210 Was Lowly Expressed in Pancreatic Cancer

To investigate the role of miR-210 in the development of pancreatic cancer, we first used qRT-PCR to detect its expression in pancreatic cancer tissues, para-cancerous tissues and normal pancreatic tissues, respectively. Results showed that the expression of miR-210 in pancreatic cancer tissues was significantly lower than that of para-cancerous tissues (Figure 1A). Moreover, miR-210 expression was obviously lower in pancreatic cancer tissues than in normal pancreatic tissues (Figure 1B). The basic characteristics of 28 pancreatic cancer patients were shown in Table I. Correlation analysis demonstrated that miR-210 expression was negatively correlated with TNM stage and tumor size of pancreatic cancer (Figure 1C and 1D).

Knockdown of MiR-210 Promoted the Proliferation of Pancreatic Cancer Cells

We, then, detected the expression of miR-210 in pancreatic cancer cells and normal pancreatic ductal epithelial cells by qRT-PCR. Findings found that miR-210 was highly expressed in pancreatic cancer cells than that of HPC-Y5 cells (Figure 2A). In particular, AsPC-1 and PANC-1 cells showed the highest and lowest expression of miR-210, which were selected for the following experiments. MiR-210 mimics and inhibitor were first constructed, and the transfection efficiency was verified by qRT-PCR (Figure 2B and 2C).

Table I. The basic characteristics of pancreatic cancer patients (n=28).

		miR-210 expression		
Clinicopathologic features	**No. (n=28)**	**Low (n=14)**	**High (n=14)**	***p*-value**
Age (years)				
≤60	11	6	5	0.6988
>60	17	8	9	
Gender				
Male	9	4	5	0.6857
Female	19	10	9	
Tumor location				
Head	17	9	8	0.6988
Body/tail	11	5	6	
Clinical stage				
I-II	16	4	12	0.0023*
III-IV	12	10	2	
Tumor size				
<4 cm	18	5	13	0.0016*
≥4 cm	10	9	1	
Lymph node metastasis				
Absent	15	10	5	0.0581
Present	13	4	9	

*$p<0.05$

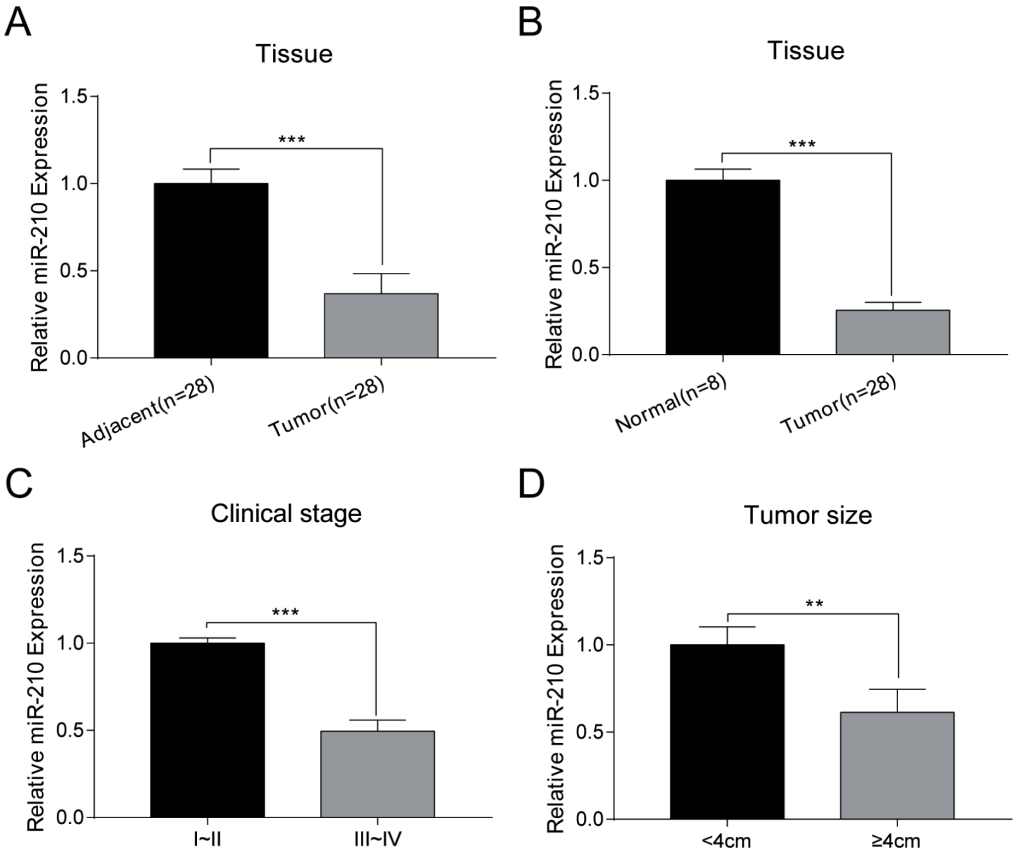

Figure 1. MiR-210 was lowly expressed in pancreatic cancer. ***A,*** MiR-210 was lowly expressed in pancreatic cancer tissues than that of para-cancerous tissues. ***B,*** Expression of miR-210 in pancreatic cancer tissues was lower than that of normal pancreatic tissues. ***C, D,*** Correlation analysis showed that miR-210 expression was negatively correlated with TNM stage and tumor size of pancreatic cancer.

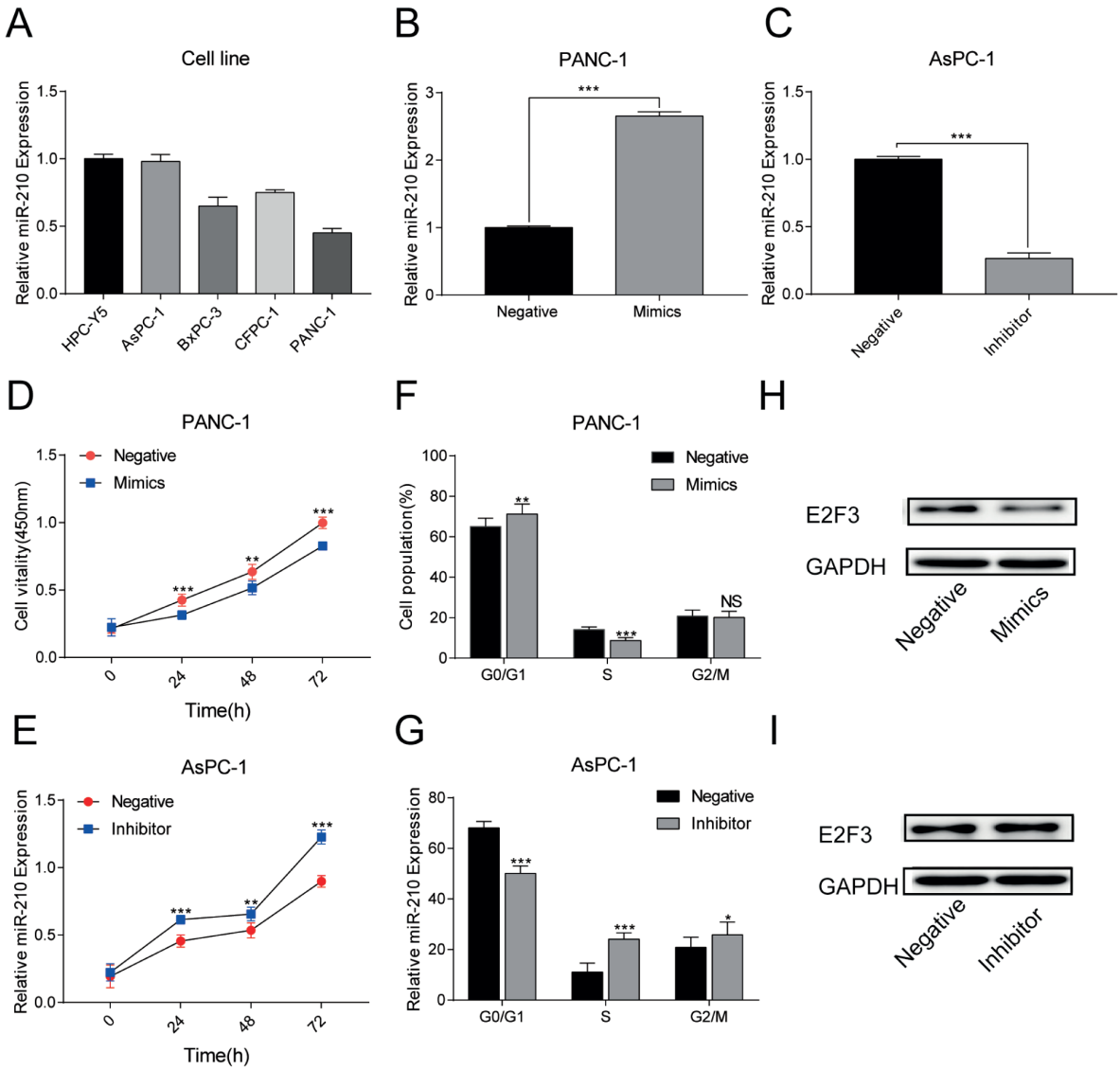

Figure 2. Knockdown of miR-210 promoted the proliferation of pancreatic cancer cells. ***A,*** MiR-210 was highly expressed in pancreatic cancer cells than that of HPC-Y5 cells. ***B, C,*** Transfection efficiency of miR-210 mimics and miR-210 inhibitor. ***D,*** CCK-8 assay showed that compared with the control group, the viability of PANC-1 cells was significantly inhibited after overexpression of miR-210. ***E,*** The viability of AsPC-1 cells was remarkably increased after miR-210 knockdown. ***F,*** Cell cycle detection indicated that cell amount in G0/G1 phase was larger in PANC-1 cells transfected with miR-210 mimics, and fewer cells were found in S phase. ***G,*** Knockdown of miR-210 in AsPC-1 cells reduced cell amount in G0/G1 phase, whereas increased the amount in S phase and G2/M phase. ***H, I,*** Protein expression of E2F3 was negatively regulated by miR-210.

CCK-8 assay showed that compared with the control group, the viability of PANC-1 cells was significantly inhibited after overexpression of miR-210 (Figure 2D). On the contrary, the viability of AsPC-1 cells was remarkably increased after miR-210 knockdown (Figure 2E). Cell cycle detection showed that after transfection with miR-210 mimics, more cells were found in the G0/G1 phase and fewer cells were found in S phase, suggesting cell cycle arrest (Figure 2F). Knockdown of miR-210 in AsPC-1 cells significantly reduced cell amount in G0/G1 phase, whereas significantly increased cell amount in S phase and G2/M phase (Figure 2G). Subsequently, bioinformatics predicted that E2F3 was the target gene of miR-210. Western blot confirmed that the protein expression of E2F3 was negatively regulated by miR-210 (Figure 2H and 2I).

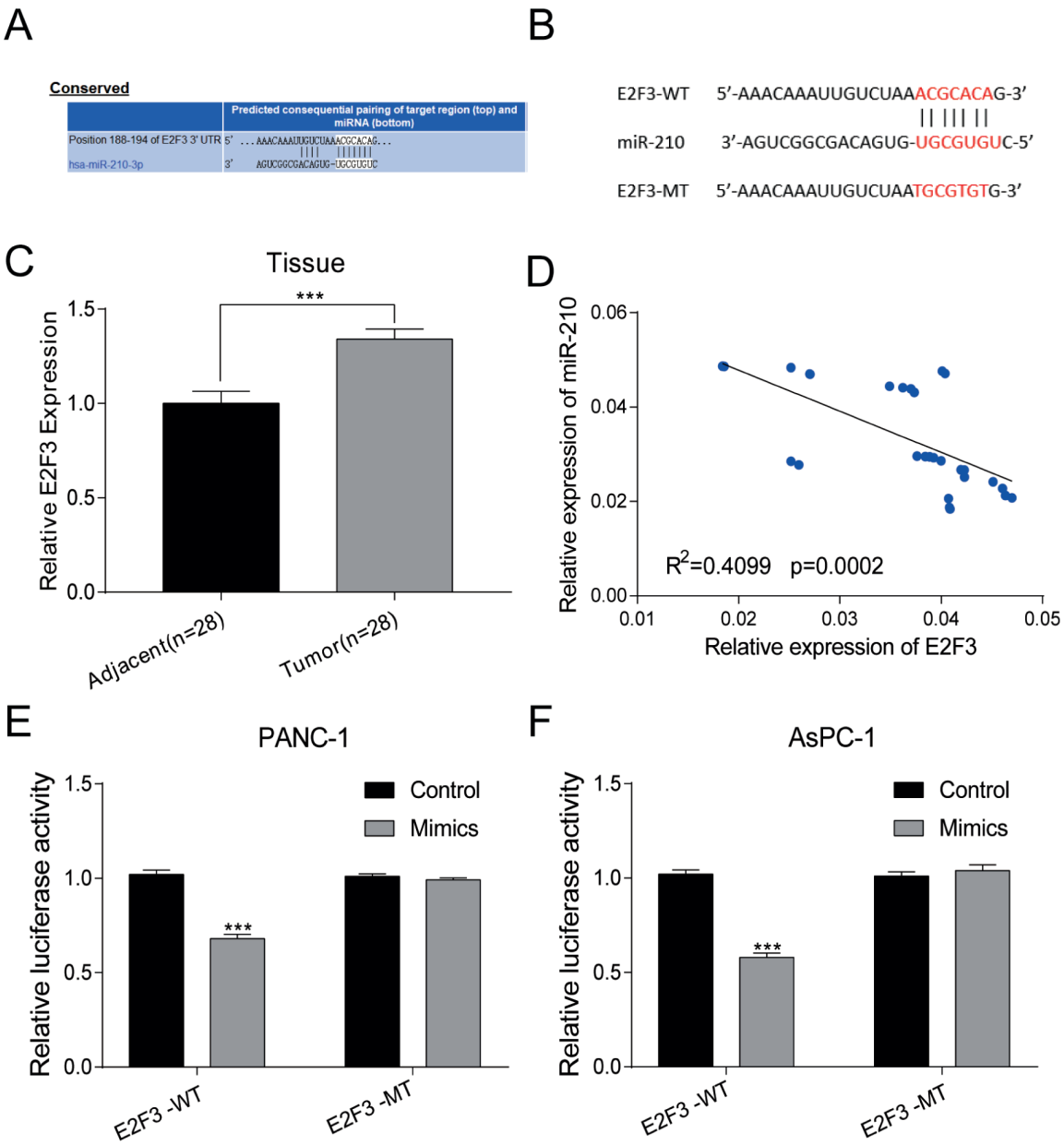

Figure 3. MiR-210 inhibited pancreatic cancer development *via* suppressing E2F3. ***A,*** The binding sites of E2F3 and miR-210. ***B,*** Construction of wild-type and mutant E2F3. Luciferase activity in cells co-transfected with miR-210 mimics and wild-type E2F3 was significantly lower than those co-transfected with miR-210 mimics and mutant E2F3. ***C,*** QRT-PCR results showed that E2F3 expression in pancreatic cancer tissues was remarkably higher than that of para-cancerous tissues. ***D,*** MiR-210 expression was negatively correlated with E2F3.

MiR-210 Inhibited Pancreatic Cancer Development via Suppressing E2F3

Based on the condition of complementary pairing of miR-210 and conservation of target sequences, it was determined that the target miRNA of E2F3 was has-miR-210-3p. The binding sites were shown in Figure 3A. To further validate the binding condition of E2F3 and miR-210, wild-type and mutant-E2F3 were constructed and transfected into cells (Figure 3B). Dual-Luciferase reporter gene assay confirmed that the Luciferase activity was remarkably lower in cells co-transfected with miR-210 mimics and wild-type E2F3 than those co-transfected with miR-210 mimics and mutant E2F3 (Figure 3E and 3F). Meanwhile, qRT-PCR re-

sults showed that E2F3 expression in pancreatic cancer tissues was remarkably higher than that of para-cancerous tissues (Figure 3C). All these results indicated that miR-210 expression was negatively correlated with E2F3 (Figure 3D).

Discussion

Uncontrolled cell cycle is reported as the major reason for the occurrence and development of malignant tumors. E2F was first discovered in the 1980s, which could regulate cell proliferation, apoptosis, differentiation and cell cycle[29,30]. In the E2F family, E2F1, E2F2 and E2F3 positively regulate the cell cycle, whereas E2F4 and E2F5 negatively regulate cell cycle. Recent studies have indicated that E2F3 is greatly involved in the development and progression of tumors[31]. Scholars[32,33] have found that E2F3 is highly expressed in multiple tumors. For example, E2F3 overexpression is closely related to the invasion and proliferation of prostate cancer[34]. Meanwhile, upregulation of E2F3 and down-regulation of pRb promote the development of lung cancer[35]. In addition, E2F3 is served as a crucial prognostic factor in gastric cancer[36].

MiRNAs are involved in multiple pathological processes, especially in tumor development, which can be used as diagnostic biomarkers. Surgical resection can remove lesioned organs and tissues, but cannot completely kill tumor cells. Postoperative chemotherapy and radiotherapy not only kill cancer cells, but also destroy healthy tissues and organs. Hence, it is of great significance to find out safe and effective treatment for tumor. Some studies[37,38] have found that multiple miRNAs, such as miR-449a and miR-125b, can inhibit the proliferation and induce the apoptosis of tumor cells *via* inhibiting E2F3. Overexpression of miR-449a in lung cancer cells inhibits cell proliferation *via* downregulating E2F3. Moreover, E2F3 is highly expressed in melanoma, eventually leading to E2F3 downregulation, proliferative capacity decline and senescence[38]. Furthermore, miR-34a inhibits the proliferation of human papillomavirus-positive cervical cancer *via* targeting E2F3[39].

Pancreatic cancer is the most common malignancy in the digestive tract, whose 5-year survival rate is lower than 7%. Moreover, pancreatic cancer may bring great burden to affected patients and their families. In the present study, we found that miR-210 was lowly expressed in pancreatic cancer tissues than that of para-cancerous tissues. The expression of miR-210 was negatively correlated with TNM stage and tumor size of pancreatic cancer. *In vitro* experiments showed that miR-210 knockdown promoted the proliferation of pancreatic cancer cells. Dual-Luciferase reporter gene assay indicated that E2F3 bound to miR-210. Further experiments confirmed that E2F3 was negatively regulated by miR-210.

Conclusions

We showed that miR-210 knockdown promotes cell proliferation by upregulating E2F3 expression, thereby promoting the progression of pancreatic cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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