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RESEARCH ARTICLE

MicroRNA-191 promotes pancreatic cancer progression by targeting USP10

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Abstract Recent studies have shown that microRNAs, a class of small and noncoding RNA molecules, play crucial roles in the initiation and progression of pancreatic cancer. In the present study, the expression and roles of miR-191 were investigated. Through both gain-of function and loss-of function experiments, a pro-oncogenic function of miR-191 was demonstrated. At the molecular level, bioinformatic prediction, luciferase, and protein expression analysis suggested that miR-191 could inhibit protein levels of UPS10, which suppressed the proliferation and growth of cancer cells through stabilizing P53 protein. Collectively, these data suggest that miR-191 could promote pancreatic cancer progression through targeting USP10, implicating a novel mechanism for the tumorigenesis.

Keywords Pancreatic cancer · MicroRNA · miR-191 · UPS10 · P53 · Gene regulation

Hua Liu and Xuan-Fu Xu contributed equally to this work.

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Introduction

Pancreatic cancer has become one of the most aggressive tumors with overall survival time of most patients less than 1 year after diagnosis [1]. Therefore, obtaining more pathogenetic mechanisms may help to improve its early detection rate and identify new therapeutic strategies.

MicroRNAs (miRNAs) belong to a class of short (18–25 nucleotides), phylogenetically conserved single-stranded RNA molecules, which suppress protein expression by either inhibition of translation initiation or elongation or by inducing mRNA decay [2, 3]. For a general overview on cancer-related miRNAs, their expression and roles in pancreatic cancer have been extensively explored [4]. A number of miRNAs were found to be deregulated in pancreatic cancer, and expression levels of certain miRNAs was furthermore associated with poor outcome, which provides potent novel targets for therapy [5, 6].

Previous studies have revealed an important role for miR-191 in several types of human cancers. For instance, high miR-191 expression was associated with clinical stage, lymph node metastasis, liver metastasis, and depth of colorectal carcinoma [7]. Besides, inhibition of miR-191 reduced cell proliferation in vitro and significantly reduced tumor masses in vivo in an orthotopic xenograft mouse model of hepatocellular carcinoma [8]. On the other hand, miR-191 is downregulated in thyroid follicular tumors [9]. Restoration of miR-191 expression in thyroid cancer cell reduced cell growth and migration rate by targeting cyclin-dependent kinase 6 (CDK6) [9]. Therefore, the precise roles of miR-191 may be tissue or cell-specific, which could act as either an oncomiRNA or tumor suppressor. In addition, recent studies have shown that miR-191 expression was upregulated in pancreatic adenocarcinomas [10, 11]. However, its biological functions and regulatory mechanisms remain unexplored. In the present

study, our data demonstrated that miR-191 potently promoted proliferation, metastasis, and invasion in pancreatic cancer cells through directly targeting ubiquitin-specific peptidase 10 (USP10).

Materials and methods

Tissue samples

Thirty-two pancreatic ductal adenocarcinoma and their matching adjacent nontumor tissues were obtained from our department. All tissue samples were histologically confirmed with hemotoxylin-eosin staining. Written consent forms were obtained from all patients before collection. This study was approved by the Research Ethics Committee of The Tenth Hospital Affiliated to Tongji University.

Cell cultures, RNA oligonucleotides, and transfection

Pancreatic cancer cell lines (PANC-1 and SW-1990) were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (CAS, Shanghai, China) and maintained in DMEM supplemented with 10 % fetal calf serum (Gibco, USA), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Gibco, USA) 37 °C with 5 % CO_2. miR-191 mimics and inhibitors were purchased from Invitrogen, Inc (Shanghai, China). For transient transfection of cells in 6-well plates, 100 μM mimics or inhibitor were used with Oligofectamine (Invitrogen) as the delivery agent.

Cell viability, BrdU incorporation, and cell-cycle analysis

To measure cell growth, 24 h after transfection with miRNA mimics or antisense, cells were trypsinized and reseeded in a 96-well plate. MTT assays were performed by incubating the cells with 200 μg/ml MTT (Sigma, USA) for 4 hr The formazan product was dissolved in dimethyl sulfoxide, and absorbance was read at 450 nm. A cell proliferation enzyme-linked immunosorbent assay kit (Beyotime, Shanghai, China) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. To assess cell-cycle properties, cells were transfected with miRNA mimics, antisense, or negative controls (NC) in 6-well plates. Thirty-six hours later, transfected cells were collected and incubated with 100 μg/ml propidium iodide (PI) and 0.5 μg/ml RNase A for 30 min at room temperature before subjecting to FACS analysis. PI-negative viable cells were gated out during the cell-cycle analysis.

Migration and invasion assays

Cell migration and invasion were analyzed using Transwell migration and extracellular matrix-coated invasion chambers (Millipore, CA, USA), and quantitated with a colorimetric microplate reader at 570 nm, according to the manufacturer's instructions.

RNA isolation and real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, USA), and reverse transcription was performed using the PrimeScript RT reagent kit (Takara, Dalian, China). In order to determine the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara) on an ABI 7900 machine. TaqMan quantitative real-time PCR detection of miRNAs was performed using the miRNA assay kit (Applied Biosystems, USA), and the U6 snRNA was used for normalization.

Western blot analysis

Tissues or cells were lysed with radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris–HCl (pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 2 Mm EDTA, 1 mM NaF, 1 % NP40, and 0.1 % SDS after treatments as indicated). Equal amounts of protein were subjected to 8 % SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes. The membranes were blocked in 10 % skim milk for 2 h at room temperature. The immunoblots were incubated overnight at 4 °C with antibodies. The next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 2 h at room temperature. The immunoreactive bands were detected with a chemiluminescence substrate kit (ProteinSimple, Santa Clara, CA, USA) under the Fluor Chem FC2 system. Antibodies were purchased from Abcam (anti-GAPDH, anti-USP10, and anti-P53).

Luciferase reporter assays

3′-Untranslated region (3′-UTR) of human USP10 gene that were predicted to interact with miR-191 were synthesized and inserted into pMir-Report (Ambion, USA), yielding pMir-Report-USP10. Mutations within potential miR-191 binding sites were generated by nucleotide replacement of wild-type sequence to inhibit miR-191 binding. The pRL-SV40 vector (Promega) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega).

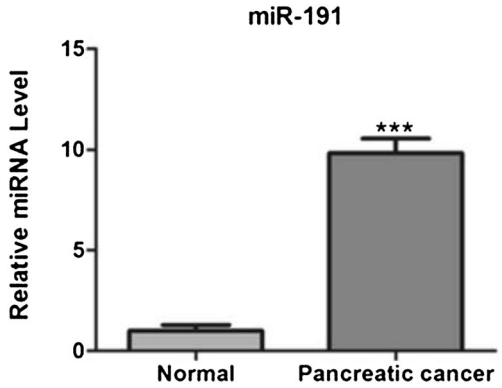

Fig. 1 Expression levels of miR-191 in PDAC tissues. miR-191 expression was determined by quantitative real-time PCR in human PDAC tissues and adjacent noncancerous tissues. ***$P<0.001$ between two groups

Tumor growth assay

Male BALB/c nude mice aged 5 weeks were purchased from Shanghai Laboratory Animal Company (SLAC, Shanghai); 5×10^6 PANC-1 cells stably expressing miR-191 or NC were injected subcutaneously to the two bilateral sites on the lower back of 5 weeks old BALB/c nude mice. The mice were observed over 4 weeks for tumor formation. After the mice were killed, the tumors were recovered and the wet weights of each tumor were determined.

Statistical analysis

Statistical analysis was performed in the GraphPad Prism 5.0 environment. All pairs of columns were compared; bars denote mean±SEM. Statistical significance is shown as *$P<0.05$, **$P<0.01$, or ***$P<0.001$.

Results

Upregulation of miR-191 in pancreatic cancer tissues

Firstly, the expression levels of miR-191 were determined in pancreatic ductal adenocarcinoma (PDAC) tissues by quantitative real-time PCR from 32 patients who underwent

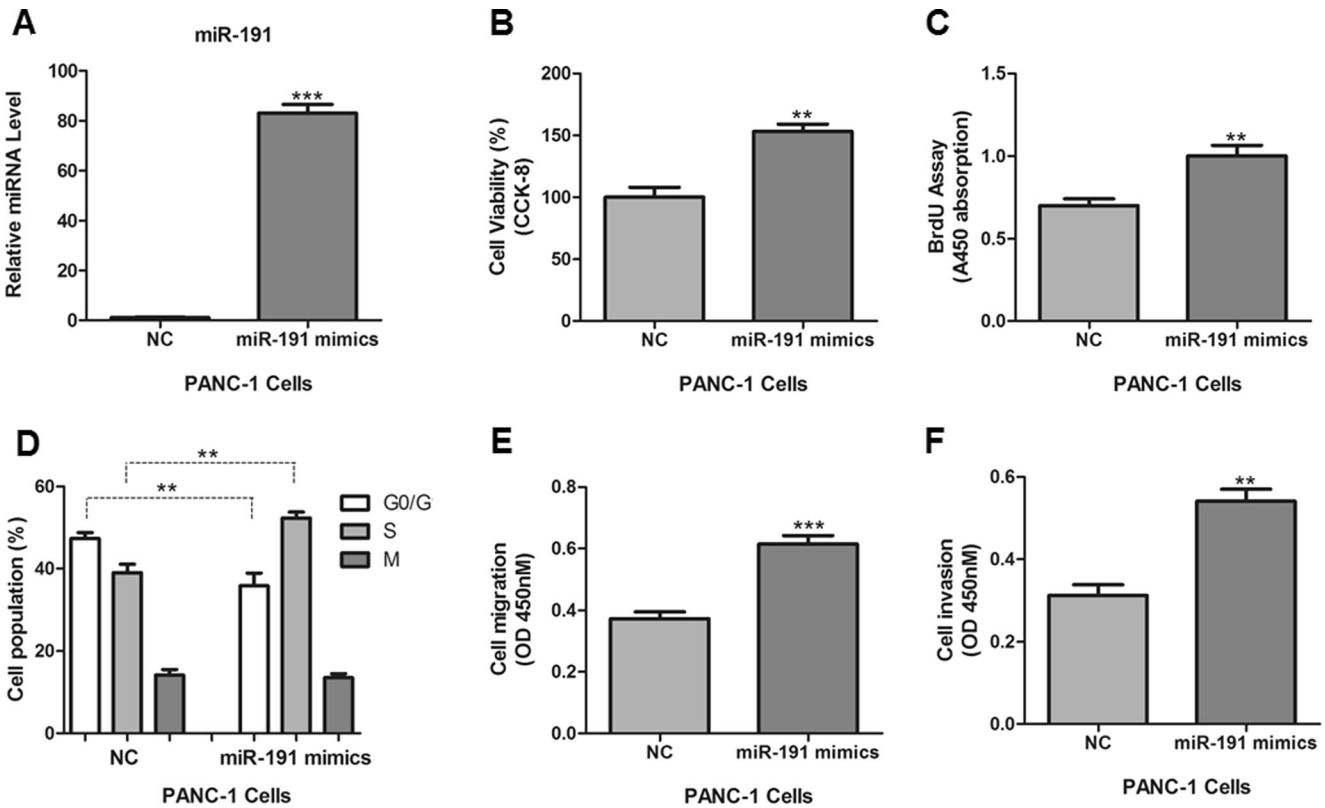

Fig. 2 miR-191 mimics promoted PDAC cell growth. **a** Relative expression levels of miR-191 were determined by real-time PCR in PANC-1 cells after transfection of miR-191 mimics or negative controls (*NC*) for 36 h. **b**, **c** The cell viability (CCK-8 assays, **b**) and proliferative potential (BrdU assays, **c**) were determined in PANC-1 cells transfected with miR-191 mimics or NC. **d** The cell-cycle phase of PANC-1 cells transfected with miR-191 mimics or NC was analyzed by flow cytometry. **e**, **f** Cell migration and invasion abilities were determined in PANC-1 cells. After transfected with miR-191 mimics or NC for 24 h, cells were seeded in Transwell filters (**e**) or ECM gel-coated Transwell culture chambers (**f**) and incubated for 24 h, then the Transwell migration assay (**e**) or invasion assay (**f**) were conducted. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with NC

pancreaticoduodenectomy at our department. In agreement with previous reports [10, 11], our results confirmed that miR-191 was upregulated in PDAC tissues compared with adjacent normal tissues (Fig. 1a).

miR-191 increased pancreatic cell growth, migration, and invasion in vitro

Next, to investigate the roles of miR-191 in pancreatic cancer progression, its mimics or NC were transfected into PANC-1 cells (Fig. 2a). The viability of the miR-191 mimics group was higher compared with the NC group, as measured by CCK-8 assays (Fig. 2b). The enhanced cell proliferative ability was further confirmed by BrdU incorporation assays (Fig. 2c). Besides, cell-cycle analysis revealed that miR-191 overexpressing cells had a significantly decreased percentage of cells in the G1/G0 phase and increased percentage of cells in the S phase, compared with theNC-transfected cells (Fig. 2d). Cell migration and invasion abilities were also enhanced by miR-191 mimics (Fig. 2e, f). Similar results were also observed in the SW-1990 cells with miR-191 overexpression (Supplementary Fig. 1a–f).

Given that miR-191 mimics could promote pancreatic cancer growth, it would be anticipated that block of miR-191 might inhibit cell proliferation and invasion. Indeed, transfection of miR-191 antisense oligos into PANC-1 and SW-1990 cells resulted in a dramatic suppression in the cell viability, proliferation, migration, and invasion (Fig. 3a–d; Supplementary Fig. 2a–d). Collectively, our data support the notion that miR-191 could promote pancreatic cancer progression in vitro.

miR-191 promotes xenograft tumor growth in vivo

To further demonstrate its function, we tested if forced expression of miR-191 promotes the ability of PANC-1 cells to form xenograft tumors in nude mice. We injected approximately 5×10^6 stable PANC-1 cells subcutaneously into two bilateral sites on the lower back of 5 weeks old BALB/c nude mice. The tumors were measured weekly for four consecutive weeks, and each tumor was individually weighed after the mice were euthanized. As a result, the tumor volume and weight were markedly increased in miR-191-overexpressed tumors compared with the control tumors (Fig. 4a, b), suggesting that miR-191 could also promote tumor growth in vivo.

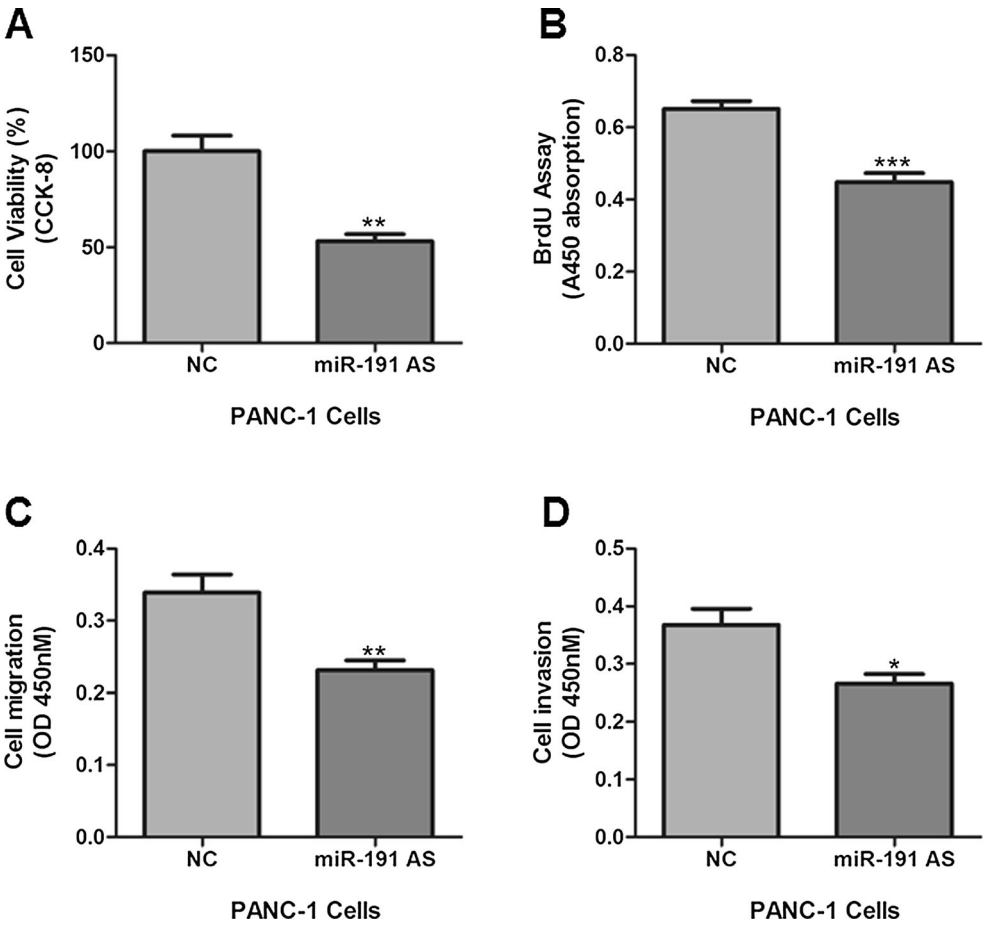

Fig. 3 miR-191 antisense inhibited PDAC cell growth. **a**, **b** The cell viability (CCK-8 assays, **a**) and proliferative potential (BrdU assays, **b**) were determined in PANC-1 cells transfected with miR-191 antisense (*AS*) or negative control (*NC*). **c**, **d** Cell migration (**c**) or invasion assays (**d**) were conducted in PANC-1cells transfected with miR-191 AS or NC. **P*<0.05; ***P*<0.01; ****P*<0.001 compared with NC

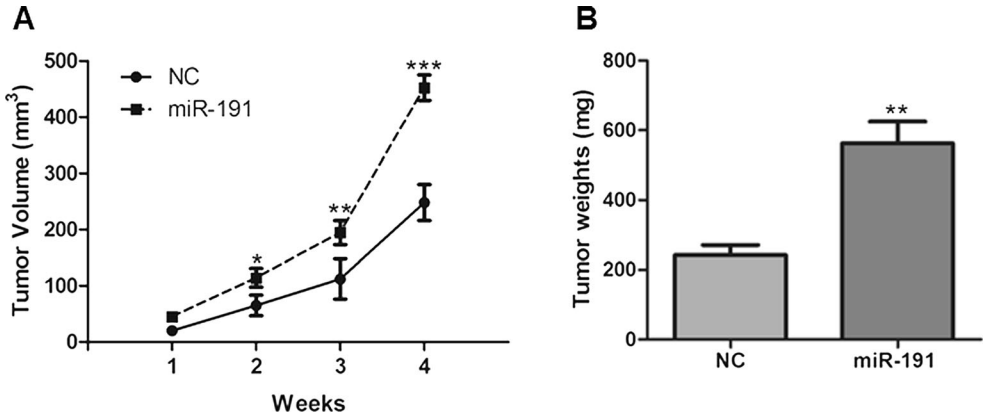

Fig. 4 miR-191 overexpression promotes tumor growth in vivo; **a**, **b** 5×10^6 PANC-1 cells stably expressing miR-191 or negative control (*NC*) were injected subcutaneously to the two bilateral sites on the lower back of 5 weeks old BALB/c nude mice ($n=8$ for each group) and followed up for tumorigenesis. Growth curve of tumor volumes (**a**) and tumor weights (**b**) were taken 4 weeks after injection. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with NC

miR-191 directly targets the USP10 in pancreatic cancer cells

It has been demonstrated that several cell-cycle regulators, such as CDK6 and Cyclin D2, were regulated by miR-191 in human cancers [9, 12]. However, our Western blot experiments revealed that overexpression of miR-191 mimics did not affect protein levels of these genes (Fig. 5a), suggesting that additional mechanisms might be involved in the antitumorgenic roles of miR-191 in pancreatic cancer. Therefore, bioinformatics software (TargetScan) was employed to

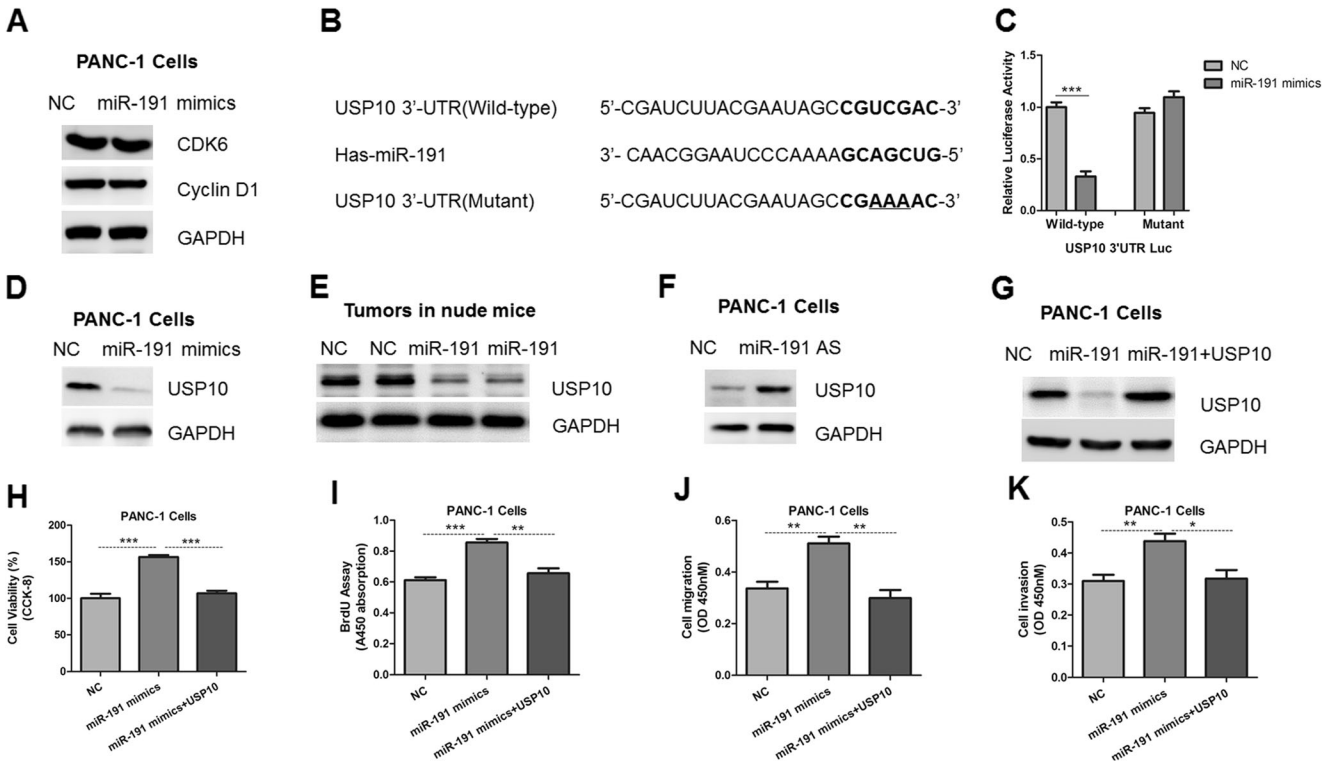

Fig. 5 miR-191 negatively regulates USP10 expression in PDAC cells. **a** Protein levels of CDK6 and Cyclin D2 were determined by western blot analysis in PANC-1 cells transfected with miR-191 mimics or negative control (*NC*). **b** Computer prediction of miR-191 binding sites in the 3′-UTRs of human USP10 genes. Potential binding sites and point mutations were set in *bold* and *underlined*, respectively. **c** Luciferase reporter assays in PANC-1 cells. Cells were transfected with 100 ng of wild-type 3′-UTR-reporter or mutant constructs together with miR-191 mimics or NC. **d** Protein levels of USP10 were determined by western blot in PANC-1 cells transfected with miR-191 mimics or NC. **e** Protein levels of USP10 were determined by western blot in tumors from nude mice. **f** Protein levels of USP10 were determined by western blot in PANC-1 cells transfected with miR-191 AS or NC. **g** USP10 protein expression in PANC-1 cells. Cells were pretransfected with miR-191 mimics or NC for 24 h, and then transfected with expression plasmids for USP10 or empty vector (*EV*) for another 24 h. **h**–**k** The cell viability (**h**), proliferative (**i**), migration (**j**), and invasion (**k**) abilities were determined in PANC-1 cells. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with NC

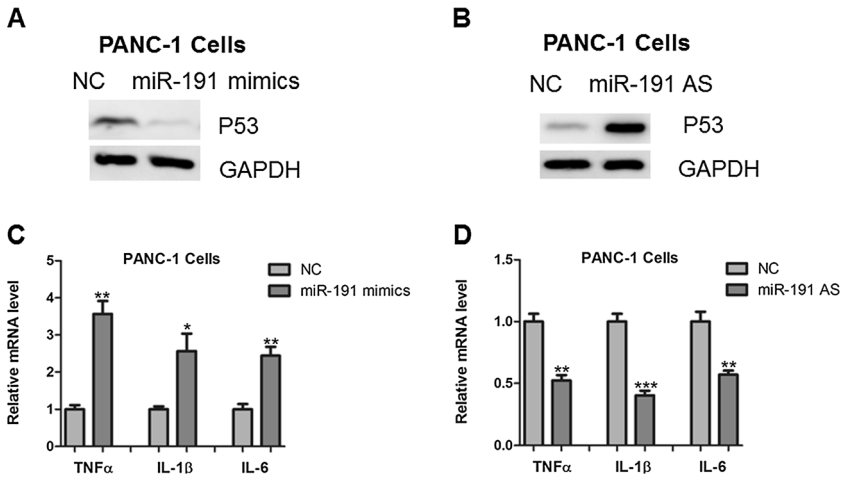

Fig. 6 miR-191 regulates downstream targets of USP10 in PDAC cells. **a**, **b** Protein levels of P53 were determined by western blot in PANC-1 cells transfected with miR-191 mimics (**a**), antisense (**b**), or negative control (*NC*). **c**, **d** Relative mRNA levels of TNF-α, IL-1β, and IL-6 were determined by real-time PCR in PANC-1 cells transfected with miR-191 mimics (**c**), antisense (**d**), or NC. **P*<0.05; ***P*<0.01; ****P*<0.001 compared with NC

identify potential target genes for miR-191. Among which, we found that USP10, a deubiquitinating enzyme [13], harbored a potential miR-191 binding site in its 3′-untranslated region (3′-UTR) (Fig. 5b). Therefore, the 3′-UTR of USP10 gene was cloned and inserted into a luciferase reporter construct. As expected, overexpression of miR-191 mimics led to a reduction of luciferase activity containing the wild-type USP10 3′-UTR (Fig. 5c). However, mutation of the potential binding site abolished the inhibitory roles of miR-191 (Fig. 5c). Moreover, transfection of miR-191 mimics resulted in a reduced USP10 protein expression (Fig. 5d; Supplementary Fig. 3a). Besides, protein levels of USP10 in tumors from nude mice were also reduced by miR-191 overexpression (Fig. 5e). In agreement, a dramatic upregulation of USP10 was observed in cells with miR-191 inhibition (Fig. 5f; Supplementary Fig. 3b).

To further verify the functional connection between miR-191 and USP10, PANC-1 cells were transfected with USP10 expression plasmids after transfection of miR-191 mimics (Fig. 5g). As shown in Fig. 5h–k, USP10 re-introduction reversed the pro-oncogenic roles of miR-191, underlining the specific importance of the USP10 for miR-191 action in the cell proliferation and invasion.

miR-191 regulates P53 expression and NF-κB signaling

Previous studies have demonstrated that USP10 increased P53 protein abundance by deubiquitinating P53 and antagonized nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcriptional activation by deubiquitination of NEMO [14, 15]. Consistent with the downregulation of USP10 by miR-191, protein levels of P53 were regulated by miR-191 mimics or antisense (Fig. 6a, b). Besides, mRNA levels of proinflammatory cytokines, such as TNF-α, interleukin (IL)-1β, and IL-6, down-stream target of NF-κB signaling, were also affected by miR-191 (Fig. 6c, d).

USP10 was downregulated in pancreatic cancer tissues

Finally, we detected USP10 protein expression in PDAC tissues and corresponding nontumor tissues. As a result, protein contents of USP10 were decreased in PDAC tissues (Fig. 7), which was inversely correlated with miR-191 level (Fig. 1).

Discussion

Understanding the roles of miRNAs may provide clues for the diagnosis and treatment of patients with pancreatic cancer. In the present study, we found that miR-191 was upregulated in PDAC tissues, which is consistent with previous reports. It has been indicated that hypomethylation of the hsa-miR-191 locus caused high expression of miR-191 in hepatocellular carcinoma [16]. Therefore, whether epigenetic mechanisms participated in the upregulation of miR-191 in PDAC remains to be defined in the future.

Besides, our in vitro and in vivo studies demonstrated a pro-proliferative, promigratory, and proinvasive effect of miR-191, while its antisense inhibited this effect. At the molecular level, we showed that USP10 was a direct target of miR-191 in PDAC cells. USP10 was shown to suppress tumor formation by enhancing P53 stability [14, 17]. Besides, USP10 antagonized c-myc transcriptional activation through

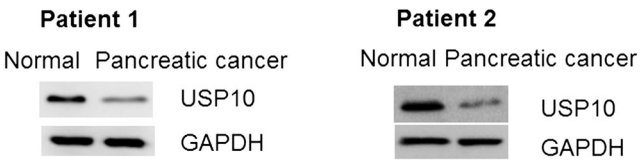

Fig. 7 Expression levels of USP10 in PDAC tissues. Representative protein levels of USP10 in human PDAC tissues and adjacent noncancerous tissues from two patients

SIRT6 stabilization to suppress tumor formation [18]. Recent studies also demonstrated that expression level of USP10 in gastric cancer tissues was lower than that in noncancerous mucosa tissues [19]. Given that P53 and c-myc play central roles in the regulation of tumorigenesis [20, 21], our study provides the potential for manipulations that target miR-191 could hold tremendous promise for the identification of new, more selective cancer treatments. On the other hand, USP10 inhibited genotoxic NF-κB activation upon DNA damage [15]. In agreement with this point, we found that miR-191 positively regulate expression levels of proinflammatory cytokines. It has been well-established that inflammatory signaling, including NF-κB, was constitutively activated in human cancers [22–24], although the molecular basis remains poorly understood. Therefore, our data add a novel mechanism for the aberrant activation of NF-κB signaling in pancreatic cancer.

Taken together, our findings strongly support the importance of miR-191 in the initiation and (or) progression of pancreatic cancer. Future studies, for example generating miR-191 knockout or transgenic mice, are needed to confirm the physiological role of miR-191 in tumorigenesis.

Conflicts of interest None

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