Inhibitory effect of RNA-mediated knockdown of zinc finger protein 91 pseudogene on pancreatic cancer cell growth and invasion

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Abstract. Worldwide, human pancreatic cancer is a rare malignancy with a poor prognosis. Long non-coding RNAs (lncRNAs) are known to have a crucial role in cancer occurrence and progression; however, the role of pseudogene-expressed lncRNAs, a major type of lncRNA, have not been thoroughly analyzed in cancer. Therefore, the present study focused on zinc finger protein 91 pseudogene (*ZFP91*-P). *ZFP91*-P expression was initially detected in two pancreatic cancer cell lines by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and the highest expression of *ZFP91*-P was found in the BXPC-3-H cell line. Subsequently, BXPC-3-H cells were transfected with *ZFP91*-P short hairpin RNA (shRNA) using a plasmid vector and termed sh*ZFP91*-P. Cells transfected with negative control plasmid vector were termed shCon. MTT and Transwell assays were performed to analyze the proliferation and migration of BXPC-3-H cells, respectively, and western blotting was used to detect epithelial-mesenchymal transition markers, including vimentin and β-catenin. The present study showed that depletion of *ZFP91*-P markedly decreased pancreatic cancer cell proliferation and inhibited cell migration capacity. In addition, the expression of β-catenin increased while vimentin expression decreased. The current findings suggest that high expression of *ZFP91*-P promotes the migration of BXPC-3-H cells and may be a novel marker for early diagnosis for pancreatic cancer.

Introduction

Pancreatic cancer is one of the most lethal neoplastic diseases with an overall 5-year survival rate of ~7% in USA (1). It is the fourth leading cause of cancer-related mortality in the USA, and its mortality rate of ~7% has not decreased in Western countries over the past few decades (1,2). Advanced stage pancreatic cancer with a <5-year survival rate is more common in elderly individuals than in younger patients (3,4). To better solve this health problem, numerous studies are focusing on the molecular mechanism of pancreatic cancer occurrence in order to develop novel treatment strategies (5-8).

Non-coding RNAs (ncRNAs) were previously considered to be transcriptional noise, however, they have more recently been proven to have a pivotal role in cellular development and various pathologies. Long ncRNAs (lncRNAs), which have a length of >200 nt, are a major group of ncRNAs that can be classified into five categories: Sense, antisense, bidirectional, intronic and intergenic (9). lncRNAs are involved in almost every step of the life cycle of genes and regulate diverse functions (10). Pseudogenes have been emerging as a novel class of lncRNAs and have shown to be important regulatory molecules involved in cancer (11). Welch *et al* identified 309 pseudogenes with significant differential expression in breast cancer (12). Several transcribed pseudogenes, such as phosphatase and tensin homolog pseudogene 1 (*PTENP1*), KRAS proto-oncogene, GTPase pseudogene 1 (*KRASP1*)and POU class 5 homeobox 1 (*OCT4*)-pg4, have been known to promote tumor progression (13,14). In addition, zinc finger family genes, such as zinc finger protein (ZFP)185, have been revealed to have a strong inverse correlation with prostate cancer progression (15). It has been reported that *ZFP91* has a role in hematopoietic repopulating cells (16) and cell proliferation or anti-apoptosis (17). Furthermore, *ZFP91* mRNA expression levels have been demonstrated to be upregulated in the gastric tissue of patients with non-alcoholic steatohepatitis (18). However, to the best of our knowledge, no study to date has reported the role of *ZFP91* pseudogenes (*ZFP91*-Ps) in pancreatic cancer and the cellular function of *ZFP91*-P remains elusive.

The RNA interference (RNAi) technique, as a loss-of-function assay, provides a novel approach for investigating the molecular mechanism of cancer occurrence (19,20). In the present study, *ZFP91*-P expression was specifically knocked down in human pancreatic cancer cells by constructing lentivirus-mediated short hairpin RNA (shRNA). Subsequently, cell migration was evaluated using a scratch and Transwell migration assay, cell proliferation was evaluated using an MTT assay, and the molecular mechanism of *ZFP91*-P in pancreatic cancer occurrence was determined.

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Materials and methods

Cell culture. Human pancreatic cancer cell lines (BXPC-3 and BXPC-3-H) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The BXPC-3-H cell line was generated in our laboratory by screening and verifying high metastatic potential BXPC-3 cells by monoclonal and transwell assays. The 293T human embryonic kidney cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (FBS) and were maintained at 37˚C in a 5% CO_2 humidified atmosphere.

Lentiviral vector construction. The shRNA sequence (5'-GGGCTGCAGATCTAGTCTTCACTCGAGTGAAG ACTAGATCTGCAGCCC-3') was designed to target human *ZFP91*-P gene (NR_024380; www.ncbi.nlm.nih.gov). The control shRNA sequence was: 5'-CTAGCCCGGCCAAGG AAGTGCAATTGCATACTCGAGTATGCAATTGCACTT CCTTGGTTTTTTGTTAAT-3'. The shRNA sequences were designed by the present authors, and synthesized by Genewiz, Inc. (Suzhou, China). The target and control shRNAs were annealed, and ligated into the Nhe I/Pac I (NEB, Ipswich, MA, USA)-linearized pFH-L vector (Shanghai Hollybio, Shanghai, China), and termed pFH-L-shZFP91-P and pFH-L-shCon, respectively.

Lentiviral packaging and cell infection. The reconstructed plasmids were transfected into 293T cells with pCMVΔR8.92 and pVSVG-I helper plasmids (Shanghai Hollybio) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection of 72 h at 37˚C in a humidified incubator containing 5% CO_2, supernatants containing Lv-shZFP91-P or Lv-shCon were harvested by purification and precipitation. Then, BXPC-3-H cells (50,000 cells/well) were seeded in 6-well plates and transduced with Lv-shZFP91-P or Lv-shCon at a multiplicity of infection of 20. After 4 days infection, cells were observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan). As the pFH-L lentiviral vector carries a green fluorescence protein (GFP) reporter, the infection efficiency was determined by counting the number of GFP-positive cells compared to total cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and synthesized into cDNA with random primers, according to the manufacturer's protocol (Fermantas; Thermo Fisher Scientific, Inc.). The reaction system of reverse transcription was as follows: 2 μg Total RNA, 1 μl oligo dT (0.5 μg/μl), 4 μl M-MLV buffer, 1.25 μl dNTPs, 0.5 μl RNasin, 0.75 μl M-MLV-RTase and nuclease-free water. RT-qPCR were performed on a BioRad Real-Time PCR platform using the following components: 10 μl 2X SYBR Premix Ex-Taq, 0.8 μl primers, 5 μl cDNA and 4.2 μl ddH_2O. The primers used were as follows: Forward, 5'-ACCTGGGGAACAAAGGCT AC-3' and reverse, 5'-TAGGACCGAGAGGCAAAGAC-3' for *ZFP91*-P; forward, 5'-ATTCCACTTTGCGTTCAAGG-3' and reverse, 5'-CTTCAGAGAGAGGAAAGCCGA-3' for vimentin; forward, 5'-AGCTACTGCCTCCGGTCTTC-3' and reverse; 5'-GTGGTCAACAGCCAGCTCA-3' for β-catenin; and forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAACGCAACTA-3' for β-actin. The reaction conditions were an initial denaturation step at 95˚C for 5 sec and 40 cycles of denaturation at 95˚C for 5 sec followed by elongation at 60˚C for 20 sec. Absorbance values obtained at the end of every elongation step were used to analyze fluorescence. Experiments were repeated at least three times and the comparative quantification cycle ($2^{-\Delta\Delta Cq}$) method (21) was used to analyze the relative mRNA expression levels of *ZFP91*-P.

MTT assay. Infected BXPC-3-H (3,000 cells/well) were reseeded in 96-well plates 4 days after lentivirus infection. The number of viable cells was measured at daily intervals (days 1, 2, 3, 4, and 5). At each time point, 10 μl of 5 mg/ml MTT was added to the cells. After incubation for 4 h, 150 μl acidic isopropanol (5% isopropanol, 10% SDS and 0.01 mol/l HCl) was added to dissolve the formazan crystals. The absorbance of each well was recorded at a wavelength of 595 nm using microplate reader.

Scratch migration assay. The scratch assay is a convenient and inexpensive method to analyze cell migration *in vitro* (22). Infected BXPC-3-H cells (1.0x104 cells/well) were seeded on 96-well plate at 37˚C in a humidified incubator containing 5% CO2 and, when cells were >90% confluent, a scratch was made using the tip of 10 µl sterile pipette. The initial scratch area and the degree of healing after scratching was observed under a binocular light microscope (Olympus CH-2, Olympus Corp., Tokyo, Japan)after 12 and 48 h.

Transwell migration assay. A Transwell® chamber (Corning, Inc, New York, NY, USA) was used to determine the migration ability of BXPC-3-H cells infected with target and control shRNA over 96 h. sh*ZFP91*-P and shCon cells ($3.0x10^4$ cells/well) were seeded into the upper chamber in 100 μl medium containing 0.1% FBS. Subsequently, 1 ml medium, containing 10% FBS as a chemoattractant, was added to the lower chamber. Then the migration system was placed in an incubator for 6 h at 37˚C in 5% CO_2. Finally, the migrated cells were fixed with 4% paraformaldehyde and stained with crystal violet (0.5%). Cell numbers were counted under a binocular light microscope in five random fields (magnification, x100) per filter and detected by the spectrometric absorbance at 570 nm.

Western analysis. BXPC-3-H cells were collected 72 h post-infection with recombinant lentiviruses, washed in ice-cold phosphate-buffered saline and lysed in 2X SDS sample buffer. Total protein (30 μg) was separated by electrophoresis using 10% SDS-PAGE at 80 V for 30 min followed by 150 V for 1 h. Subsequently, the separated protein samples were transferred onto a PVDF transmembrane (Millipore, Bedford, MA, USA) under 300 mA and blocked in 5% non-fat milk with Tris-buffered saline and 0.05% Tween 20 (Sigma, St. Louis, MO, USA) for 2 h at room temperature. The membrane was then incubated with following primary antibodies: Rabbit anti-vimentin (1:1,000 dilution; cat no. #5741;

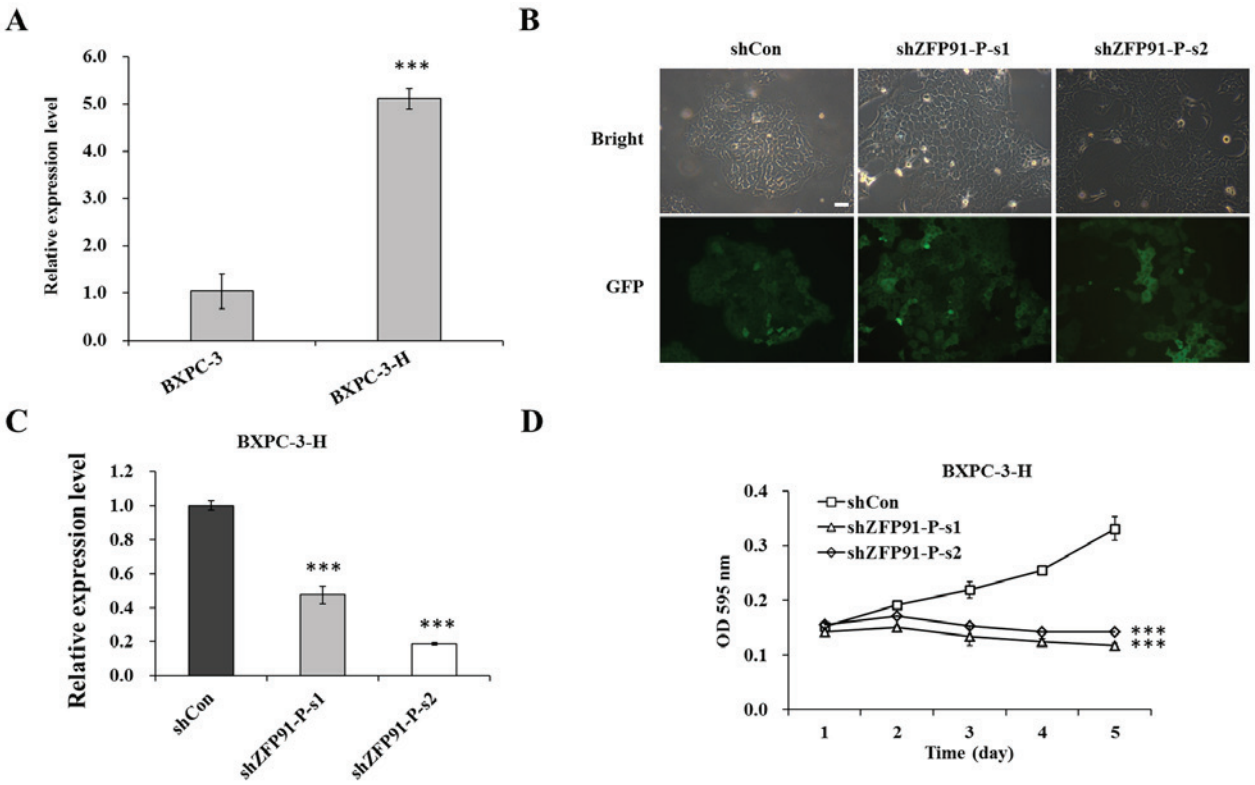

Figure 1. Expression of *ZFP91*-P in pancreatic cancer cells. (A) mRNA expression levels of *ZFP91*-P in BXPC-3 and BXPC-3-H cells were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). β-actin was used as an internal control gene. ***P<0.001 vs. BXPC-3. (B) Fluorescence photomicrographs of BXPC-3-H cells infected by lentivirus. Multiplicity of infection, 20. Magnification, x100. Scale bar, 10 μm. (C) RT-qPCR analysis of *ZFP91*-P mRNA expression in BXPC-3-H cells with three treatments. β-actin was used as an internal control gene. ***P<0.001 vs. shCon. (D) BXPC-3-H cell proliferation after *ZFP91*-P silencing, as determined by an MTT assay. Cells with three treatments including shCon, shZFP91-P-s1, and shZFP91-P-s2 groups. shCon, BXPC-3-H cells infected with control shRNA; shZFP91-P-s1, BXPC-3-H cells infected with ZFP91-P shRNA 1; shZFP91-P-s2, BXPC-3-H cells infected with ZFP91-P shRNA2. ***P<0.001 vs. shCon. Data are presented as mean ± standard error of the mean. sh, shRNA; Con, control; ZFP91-P, zinc finger protein 91 pseudogene; GFP, green fluorescent protein; OD, optical density.

Cell Signaling Technology, Danvers, MA, USA); rabbit anti-β-catenin (1:1,000 dilution; cat no. #8480; Cell Signaling Technology) and rabbit anti-GAPDH (1:500,000 dilution; cat no. 10494-1-AP; Proteintech Group, Inc., Chicago, IL, USA) at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat no. SC-2054; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h at room temperature. An ECL kit (Amersham; GE Healthcare Life Sciences) was used to visualize the blot.

Statistical analysis. All statistical data are expressed as mean ± standard error of three independent experiments. Student's t-test was used to compare differences between groups. P<0.05 was considered to indicate a statistically significant. Comparisons were carried out by Student's t-test and one-way ANOVA analysis using SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA).

Results

Expression of ZFP91-P is downregulated by Lv-shZFP91-P in pancreatic cancer cells. The expression of *ZFP91*-P was determined in two pancreatic cancer cell lines, BXPC-3 and BXPC-3-H. As shown in Fig. 1A, *ZFP91*-P mRNA levels were significantly upregulated in BXPC-3-H cells compared with in BXPC-3 cells (P<0.001). Thus, only BXPC-3-H cells were used in subsequent experiments. To clarify the biological function of *ZFP91*-P in pancreatic cancer cells, a lentivirus-mediated RNAi system was used to suppress *ZFP91*-P expression in BXPC-3-H cells. Fluorescence microscopy showed that >80% of cells were GFP-positive in the Lv-shCon and Lv-sh*ZFP91*-P groups 3 days after lentivirus infection (Fig. 1B). Subsequently, RT-qPCR was used to analyze the knockdown efficiency of *ZFP91*-P. As shown in Fig. 1C, expression of ZFP91 was significantly inhibited by 52.5% in the Lv-shZFP91-P-s1 group and 81.4% in the Lv-shZFP91-P-s2 group compared with the Lv-shCon group, respectively (P=0.0006; P=0.0002).

Knockdown of ZFP91-P significantly inhibits BXPC-3-H cell proliferation. To evaluate the effect of sh*ZFP91*-P on pancreatic cancer cell proliferation, an MTT assay was conducted in BXPC-3-H cells. The results showed that the growth curves of the Lv-sh*ZFP91*-P group were significantly lower than those of the Lv-shCon group (P<0.001; Fig. 1D), suggesting that knockdown of *ZFP91*-P could significantly inhibit pancreatic cancer cell proliferation.

Depletion of ZFP91-P significantly inhibits the migratory ability of metastatic pancreatic cancer cells. A scratch migration assay was performed to evaluate the migration ability of

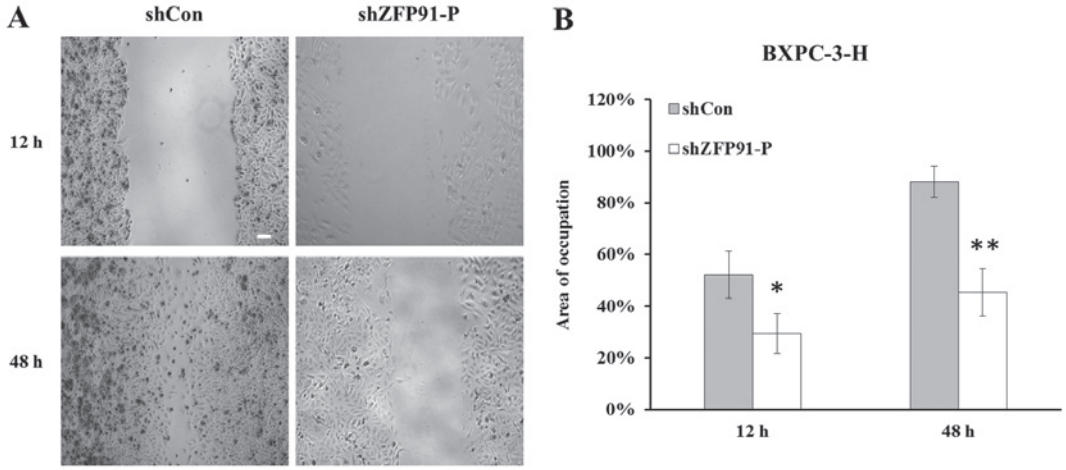

Figure 2. Scratch migration assay of the migratory ability of BXPC-3-H cells. (A) Images of the migration of BXPC-3-H cells when treated with shCon and shZFP91-P. Scale bar, 10 μm. Magnification, x40. (B) Percentage of wound confluence when pancreatic cancer cells were treated with different types of lentivirus. Data are presented as mean ± standard error of the mean. *P<0.05, **P<0.01 vs. shCon. sh, shRNA; Con, control; ZFP91-P, zinc finger protein 91 pseudogene.

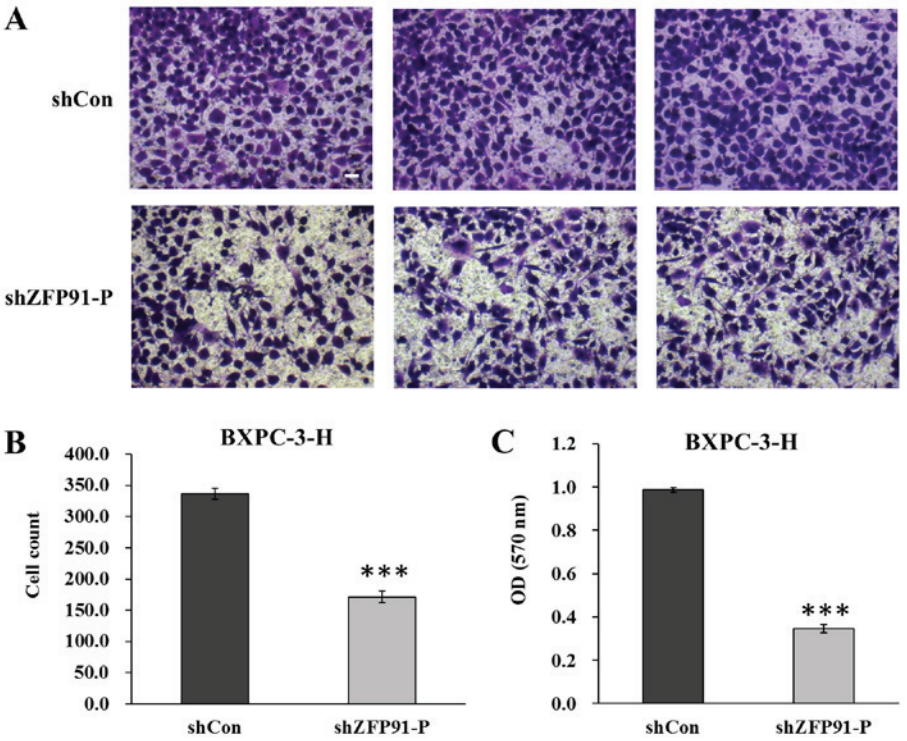

Figure 3. Transwell migration assay revealed that depletion of *ZFP91*-P resulted in decreased cell migration potential. (A) Microscopic images of migrated BXPC-3-H cells at 6 h (3 replicates). Crystal violet staining. Scale bar, 10 μm. Magnification, x40. (B) Number of migrated cells. (C) Quantitative analysis of stained migrated cancer cells using a microplate reading at an OD at 570 nm. Data are presented as mean ± standard error of the mean. ***P<0.001 vs. shCon. sh, shRNA; Con, control; ZFP91-P, zinc finger protein 91 pseudogene; OD, optical density.

BXPC-3-H cells *in vitro*. At the selected time points of 12 and 48 h, cell migration distance was observed under a microscope (magnification, x100) (Fig. 2A). Cell migration ability was significantly inhibited in the Lv-sh*ZFP91*-P group compared with the Lv-shCon group, with control BXPC-3-H cells showing a better ability to heal the scratch (P<0.05) (Fig. 2B). Subsequently, a Transwell assay was used to determine the effect of *ZFP91*-P knockdown in regulating pancreatic cancer cell migration (Fig. 3A). As indicated in Fig. 3B, significantly fewer cells in the Lv-sh*ZFP91*-P group migrated to the lower surface of the membrane, compared with cells in the Lv-shCon group (P<0.001). In addition, the crystal violet staining intensity was significantly lower in the Lv-sh*ZFP91*-P group than in the Lv-shCon group (P<0.001) (Fig. 3C). These results suggest that *ZFP91*-P may have a key role in pancreatic cancer metastasis.

Downregulation of ZFP91-P elevates the expression of β-catenin and inhibits the expression of vimentin via epithelial-mesenchymal transition (EMT) signaling. To

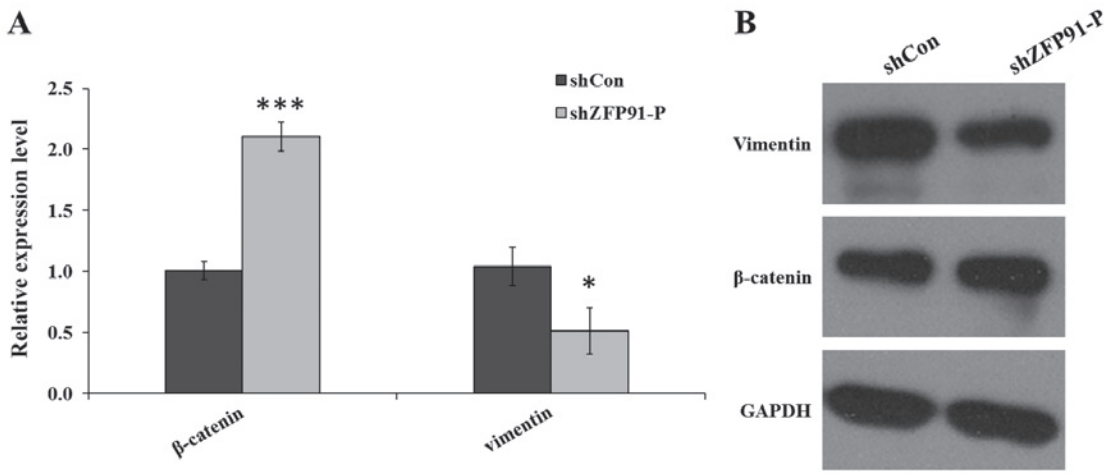

Figure 4. Relative mRNA and protein expression level of β-catenin and vimentin. (A) mRNA expression levels of β-catenin and vimentin in BXPC-3-H cells were determined by reverse transcription-quantitative polymerase chain reaction. Data are presented as mean ± standard error of the mean. *P<0.05, ***P<0.001 vs. shCon. (B) Protein expression levels of β-catenin and vimentin in BXPC-3-H cells were determined by western blotting. sh, shRNA; Con, control; ZFP91-P, zinc finger protein 91 pseudogene.

evaluate the regulatory role of *ZFP91*-P in EMT signaling in human pancreatic cancer, the effect of *ZFP91*-P silencing on the expression of β-catenin and vimentin, which are critical for tumor invasion and metastasis, was examined. RT-qPCR results showed that the mRNA expression levels of β-catenin were significantly elevated (P<0.001) while vimentin was significantly reduced (P<0.05) in the Lv-sh*ZFP91*-P group compared with the Lv-shCon group in BXPC-3-H cells (Fig. 4A). Analysis of protein expression levels revealed the same pattern (Fig. 4B).

Discussion

Pancreatic cancer is one of the most fatal neoplastic diseases, as it is typically diagnosed at an advanced stage. The majority of cases of pancreatic cancer are inoperable and metastasized, therefore, it is difficult to overcome this health problem. Thus far, numerous gene abnormalities have been found to be involved in pancreatic cancer (23-25). Gene therapy is designed to deliver a therapeutic gene into a target site to regulate expression of the specific gene (26-28).

It has been reported that *ZFP91* may have an important role in cell proliferation (17) and may be involved in prostate cancer (29). Several transcribed pseudogenes, such as *PTENP1*, *KRASP1* and *OCT4*-pg4, have been known to promote tumor progression (13,14). These findings prompted the present study to investigate the *ZFP91*-P gene as a target site in pancreatic cancer therapy.

In the present study, the association between *ZFP91*-P and characteristics of pancreatic cancer were initially examined. *ZFP91*-P showed high expression in BXPC-3-H and BXPC-3 pancreatic cancer cells. Subsequently, the expression of *ZFP91*-P was knocked down in BXPC-3-H cells using a lentivirus-based shRNA system. The cell proliferation and migration ability were impaired in the absence of *ZFP91*-P, suggesting that *ZFP91*-P may promote the metastatic and motility properties of pancreatic cancer cells.

EMT is a biological process that can enhance cell migratory capacity and invasiveness. It is clear that EMT occurs in three distinct biological settings, including organ development (30), tumor growth and cancer progression (31). Furthermore, it has been reported that EMT acts as a major driver of tumor invasion (32), with a crucial role in the aggressiveness and invasion of pancreatic cancer (33). The expression of mesenchymal makers, such as ZEB1, vimentin, Slug and Snail, are positive for majority of cases of pancreatic cancer (34). To further clarify these results, the present study identified the signaling elements targeted by *ZFP91*-P for the promotion of EMT in pancreatic cancer. *ZFP91*-P silencing appeared to reverse EMT, as shown by increased expression of β-catenin and decreased expression of vimentin. Vimentin, a major component of the intermediate filament family, has been demonstrated to markedly reverse EMT in pancreatic cancer cells, acting as a mesenchymal marker (35). In addition, its overexpression may result in accelerated cell growth, invasion and poor prognosis in numerous types of cancer (36). The current results indicate that *ZFP91*-P silencing can inhibit cell proliferation and migration by reversing the EMT pathway. β-catenin not only has a critical role in cell-cell adhesion by interacting with cadherins at the plasma membrane, but is also involved in a signaling cascade at the center of the Wnt signaling pathway. Several previous studies have demonstrated that β-catenin is essential for normal pancreatic organogenesis (37,38). However, stable expression of β-catenin within the pancreatic epithelium can give rise to the formation of tumors (39,40). By contrast, concurrent activation of β-catenin and Kras prevents the formation of pancreatic intraepithelial neoplasias (40). In addition, increased β-catenin activity by overexpression of OCT-4 can inhibit cell differentiation (40). Based on these points, we propose that β-catenin may be concurrently activated with certain genes during *ZFP91*-P silencing in pancreatic cancer cells. However, further studies are required to investigated the molecular mechanism of β-catenin in pancreatic cancer.

In conclusion, the current study indicates that *ZFP91*-P is an important regulator of pancreatic cancer cell migration and proliferation, and *ZFP91*-P silencing may suppress the migration of pancreatic cancer cells by reversing EMT. Therefore,

ZFP91-P may be a useful target for gene therapy in pancreatic cancer. However, the biological behavior of *ZFP91*-P in pancreatic cancer needs to be thoroughly explored in future studies.

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