Small nuclear ribonucleoprotein associated polypeptide N accelerates cell proliferation in pancreatic adenocarcinoma

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Abstract. The spliceosome, the large RNA-protein molecular complex, is crucial for pre-mRNA splicing. Several antitumor drugs have been found to tightly bind to the components of the spliceosome and mutations in the spliceosome have been reported in several types of cancer. However, the involvement of the spliceosome in pancreatic adenocarcinoma remains unclear. In the present study, small nuclear ribonucleoprotein associated polypeptide N (SNRPN), a key constituent of spliceosomes, was disrupted in BxPC-3 pancreatic adenocarcinoma cells using lentivirus-mediated RNA interference (RNAi). It was found that knockdown of SNRPN reduced the proliferation ability of BxPC-3 cells, as determined by an MTT assay. Furthermore, cell colony formation was impaired in SNRPN depleted adenocarcinoma cells and cell cycle analysis showed that depletion of SNRPN led to S phase cell cycle arrest and apoptosis. These results suggest that SNRPN is a key player in pancreatic adenocarcinoma cell growth, and targeted loss of SNRPN may be a potential therapeutic method for pancreatic cancer.

Introduction

Pancreatic cancer is the fourth most common cause of cancer-related mortality worldwide (1). A number of therapeutic methods, such as surgery, radiation and chemotherapy, have been used in pancreatic cancer clinically. However, pancreatic cancer has a high mortality rate and an overall five-year survival of <6% (data cited from American Cancer Society) (2). Novel therapeutic methods are required to improve the treatment of pancreatic cancer. Thus far, a number of gene abnormalities have been found to be involved in pancreatic cancer (3-5). Gene therapy, using DNA or RNA as a pharmaceutical agent to specifically change the endogenous gene expression profile, is one of the most promising therapeutic methods for the treatment of cancer (6,7). Short hairpin RNA (shRNA) is an effective and specific method to suppress endogenous gene expression (7,8). Recent studies have demonstrated that disruption of gene expression, based on lentivirus-mediated RNAi, could inhibit proliferation and induce apoptosis of pancreatic carcinoma cells (8-11).

Spliceosome, the large RNA-protein molecular complex that removes introns from the pre-mRNA, is crucial for the alternative splicing and maturation of mRNA (12-14). Spliceosome mutations have been found in certain types of cancer (15,16). Several antitumor drugs, derived from bacterial fermentation products and their synthetic derivatives, have been shown to bind tightly to the components of the spliceosome (17). Targeted suppression components of the spliceosome were observed to inhibit carcinoma cell proliferation and migration (18,19). SNRPN (small nuclear ribonucleoprotein associated polypeptide N) is a 29-kD spliceosomal protein associated with small nuclear ribonucleoprotein particles. Variable methylation of SNRPN has been found to be correlated with germ cell tumors and acute myeloid leukemia (20,21). However, the function of SNRPN in pancreatic carcinoma remains unknown. The aim of this study was to investigate the role of SNRPN in pancreatic carcinoma progression. Lentivirus-mediated shRNA was employed to silence SNRPN expression in the BxPC-3 human pancreatic adenocarcinoma cell line. The role of SNRPN in cell growth and apoptosis was examined in the BxPC-3 cells subjected to SNRPN knockdown.

Materials and methods

Cell culture. BxPC-3 pancreatic adenocarcinoma cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) containing 2 mM glutamine and 10% fetal bovine serum (FBS). HEK293T human embryonic kidney cells (Cell Bank of the Chinese Academy of Sciences) were cultured in DMEM (Hyclone, Logan, UT,

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USA) plus 10% FBS. Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2.

Lentiviral vector construction and packaging. The sequence of SNRPN was obtained from NCBI (NM_003097). An shRNA sequence for SNRPN (5'-GAATCTTCATTGG CACCTTTACTCGAGTAAAGGTGCCAATGAAGATTCTT TTT-3') and a control shRNA (5'-CTAGCCCGGTTCTCC-GAACGTGTCACGTATCTCGAGATACGTGACACGTTCG GAGAATTTTTTTAAT-3') were designed. The shRNA oligos were cloned into the lentivirus expression plasmid pFH-L (Shanghai Hollybio, Shanghai, China). To generate the shSNRPN and shCon lentivirus, shRNA plasmids along with the envelope plasmid pVSVG-I and packaging plasmid pCMVΔR8.92 (Shanghai Hollybio), were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen Life Technologies). Three days after transfection, media containing recombinant lentivirus were collected and ultra-centrifuged for infection.

Lentiviral infection of BxPC-3 cells. BxPC-3 cells were plated in a 6-well plate at a density of $1.5x10^5$ cells/well. Lentiviral supernatant was added to the culture medium at an MOI of 10. Three days after infection, cells were observed under a microscope (10X objective lens) and the number of green fluorescent protein positive cells was counted to calculate the infection efficiency.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Four days after lentiviral infection, BxPC-3 cells were washed in ice-cold phosphate-buffered saline (PBS) and then collected. Total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNA was then synthesized using M1705 M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). RT-qPCR was then performed to determine the expression level of SNRPN and actin was used as endogenous control. The PCR reaction mixture contained 2X SYBR premix ex taq (10 μl), forward and reverse primers (2.5 μM; 0.8 μl), cDNA (5 μl) and double-distilled H_2O (4.2 μl). The two-step PCR procedure was as follows: Pre-denaturation at 95˚C for 1 min, followed by 40 cycles comprising denaturation at 95˚C for 5 sec and annealing extension at 60˚C for 20 sec. The absorbance values were obtained at the end of every elongation step. The expression levels were analyzed by using the $2^{-\Delta\Delta Ct}$ statistical method. Primers used were as follows: Forward, 5'-GTTTTGGGTCTGGTGTTGCT-3' and reverse, 5'-TCATTACCTGCTGGGATGGT-3' for SNRPN; and forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAACGCAACTA-3' for actin. The experiment was repeated at least three times.

Western blot analysis. Four days after lentivirus infection, BxPC-3 cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation buffer (100 mM Tris-HCl, pH 6.8; 10 mM EDTA, 4% SDS and 10% glycine) for 1 h at 4˚C. After centrifugation at 17,000 xg for 30 min at 4˚C, the supernatants were collected and protein concentration was measured. After combination with 2X sample buffer (100 mM Tris-HCl, pH 6.8; 10 mM EDTA, 4% SDS and 10% glycine), protein samples were denatured at 95˚C for 10 min. Equal quantities of proteins (30 μg) were loaded and separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with primary antibodies, anti-SNRPN (dilution, 1:1,000; HPA003482, Sigma-Aldrich) and anti-GAPDH (dilution, 1:5,000; 10494-1-AP; Proteintech Group, Inc., Chicago, IL, USA), followed by secondary antibodies [horseradish peroxidase-conjugated goat anti-rabbit (dilution, 1:5,000; SC-2054; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Western blot bands were exposed to films using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Amersham, UK).

MTT assay. BxPC-3 cells were seeded in a six-well plate at a density of $3x10^3$ cells/well. After 1, 2, 3, 4 and 5 days of lentiviral infection, 20 μl of 5 mg/ml MTT (Sigma-Aldrich) was added to each well and incubated for 3 h. Then 100 μl acidic isopropanol (10% SDS, 5% isopropanol, 0.01 mol/l HCl), was added into each well to dissolve the formazan precipitate. The absorbance of each well was recorded at a wavelength of 595 nm using an Epoch plate reader (BioTek, Winooski, VT, USA). The experiment was repeated at least three times.

Colony formation assay. After lentivirus infection, BxPC-3 cell suspension was seeded into six-well plates at an initial density of 900 cells/well. Cell culture medium was changed every 3 days. After the majority of single clones contained >50 cells, images were captured by brightfield/fluorescence microscopy (BX50; Olympus Corp., Tokyo, Japan). The clones were fixed using 4% paraformaldehyde (Sangon Biotech, Shanghai, China) and stained with crystal violet according to the manufacturer's instructions. The experiment was repeated at least three times. The number of colonies (>50 cells/colony) was counted using Colony Counter software (Image-Pro Plus 6.0; Media Cybernetics Inc., Bethesda, MD, USA). The morphology and size of the colonies was examined under a microscope (4X objective lens).

Flow cytometric analysis. BxPC-3 cells were seeded at a density of $6x10^4$ cells/well and infected by lentivirus. When the confluence of BxPC-3 cells was ~70%, cells were harvested and washed with ice-cold PBS. Cells were then fixed overnight at 75% ethanol at 4˚C and labeled with propidium iodide (PI) following the instruction of kit (C1052; Beyotime Institute of Biotechnology, Inc., Shanghai, China). The fluorescence of PI in the cells was measured using Cell Lab Quanta Beckman Coulter (Miami, FL, USA). The experiment was repeated at least three times.

Statistical analysis. All data are presented as the mean ± standard error with at least three repeats. Statistical analysis was performed based on Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Lentivirus-mediated RNAi significantly reduces the SNRPN level in BxPC-3 cells. To investigate the function of SNRPN in pancreatic adenocarcinoma cells, the lentivirus-mediated

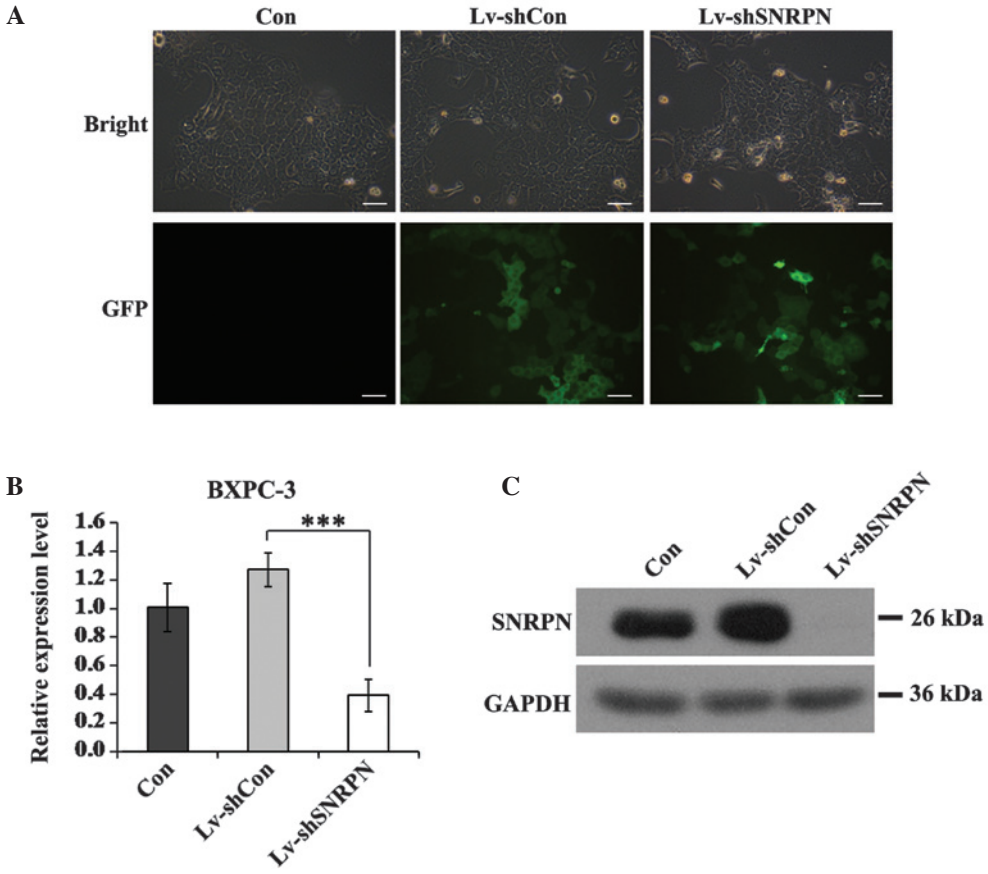

Figure 1. Knockdown of SNRPN using lentivirus-mediated shRNA in pancreatic adenocarcinoma cells. (A) Representative images of BXPC-3 cells after 3 days of lentivirus infection were shown (scale bar, 100 μm). (B) Expression analysis of SNRPN mRNA level in BXPC-3 cells by reverse transcription-quantitative polymerase chain reaction in the uninfected group, Lv-shCon group and Lv-shSNRPN group. The actin gene served as an was the internal control. (C) Expression analysis of SNRPN protein level in BXPC-3 cells by western blot analysis in uninfected, Lv-shCon and Lv-shSNRPN groups. The GAPDH protein was the internal control. Equal quantities of protein samples (30 μg) were loaded in each lane. ***P<0.001 vs. Lv-shCon group. SNRPN, small nuclear ribonucleoprotein associated polypeptide N; sh, small hairpin.

RNAi technique was used. Three days after lentivirus infection, infection rate was determined by evaluating the Lv-shSNRPN expressed GFP level. More than 90% cells were infected by Lv-shSNRPN (Fig. 1A), which indicates that lentivirus could efficiently deliver the shRNA into the adenocarcinoma cells. To determine the knockdown efficiency of shRNA for endogenous SNRPN, the mRNA level was measured by RT-qPCR and the protein level was measured by western blot analysis. It was demonstrated that following Lv-shSNRPN infection, the mRNA and protein levels of SNRPN were markedly decreased (Fig. 1B and C). These results suggest that lentivirus mediated RNAi could strongly inhibit endogenous SNRPN expression in BxPC-3 cells.

Suppression of SNRPN inhibits BxPC-3 cell proliferation and colony formation. In order to investigate the function of SNRPN in adenocarcinoma cells, the proliferation and colony formation of BxPC-3 cells were measured after the suppression of SNRPN. As shown by MTT cell proliferation assays, inhibiting SNRPN expression was observed to significantly reduce cell viability, compared with uninfected or Lv-shCon-infected BxPC-3 cells (Fig. 2A). A colony formation assay was then conducted to determine the function of SNRPN in adenocarcinoma tumorigenesis *in vitro*. As shown in Fig. 2B and C, after Lv-shSNRPN infection, the number of colonies formed was significantly decreased (22.7±5.0 colonies compared with 98.3±4.5 in Lv-shCon infected group and 91.3±2.9 in the uninfected group). These results suggest that SNRPN is important for cell proliferation and tumorigenesis of pancreatic adenocarcinoma. Targeted inhibition of SNRPN may be a potential therapeutic method for the treatment of human pancreatic adenocarcinoma.

SNPRN inhibition leads to cell cycle arrest and apoptosis of BxPC-3 cells. To identify the mechanisms that underly the effect of the SNRPN suppression induced reduction in proliferation ability, flow cytometry was performed. Notably, it was determined that following Lv-shSNRPN infection, more cells were accumulated at the S phase of the cell cycle and the percentage of cells in the G0/G1 phase was reduced (Fig. 3A and B). Further analysis found that cells accumulated in the sub-G1 phase (Fig. 3C), indicating that cell cycle arrest induced apoptosis after SNRPN knockdown.

Discussion

A number of therapeutic methods have been used in pancreatic cancer clinically. However, pancreatic cancer has a high mortality rate and is the fourth most common cause of

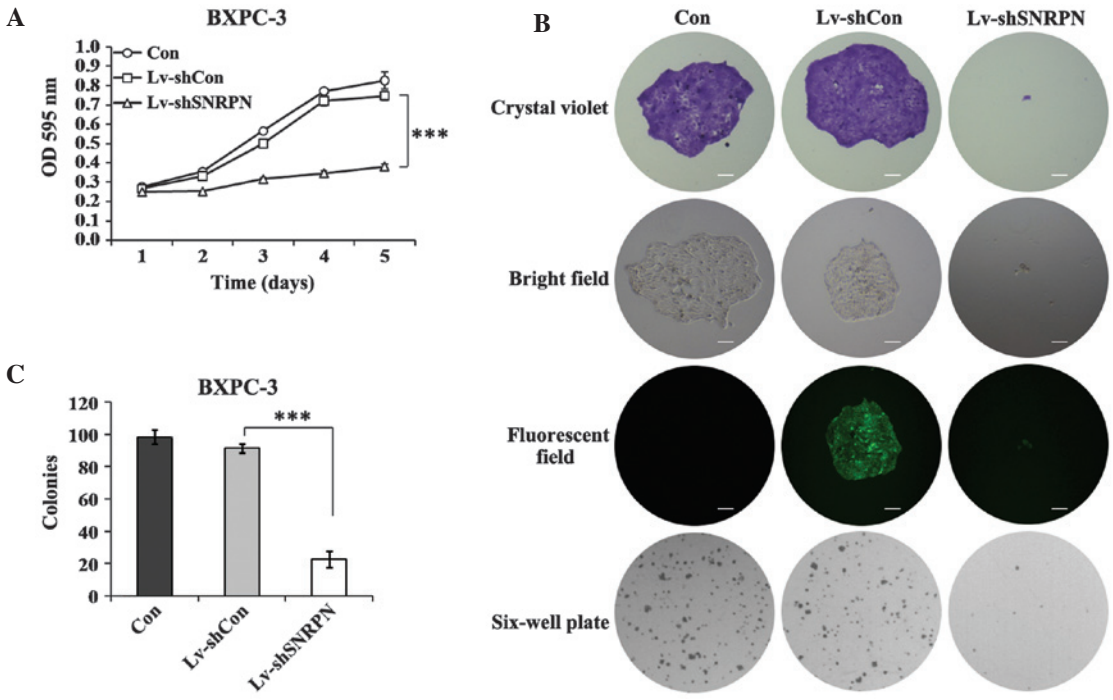

Figure 2. SNRPN depletion inhibits the proliferation and colony formation of BXPC-3 cells. (A) Cell proliferation in the Lv-shSNRPN group was significantly inhibited, as detected by MTT assay. (B) Representative images of colonies under light microscopy and fluorescence microscopy (scale bar, 250 μm). (C) Statistical analysis of the number of colonies. ***P<0.001 vs. Lv-shCon group. SNRPN, small nuclear ribonucleoprotein associated polypeptide N; sh, small hairpin.

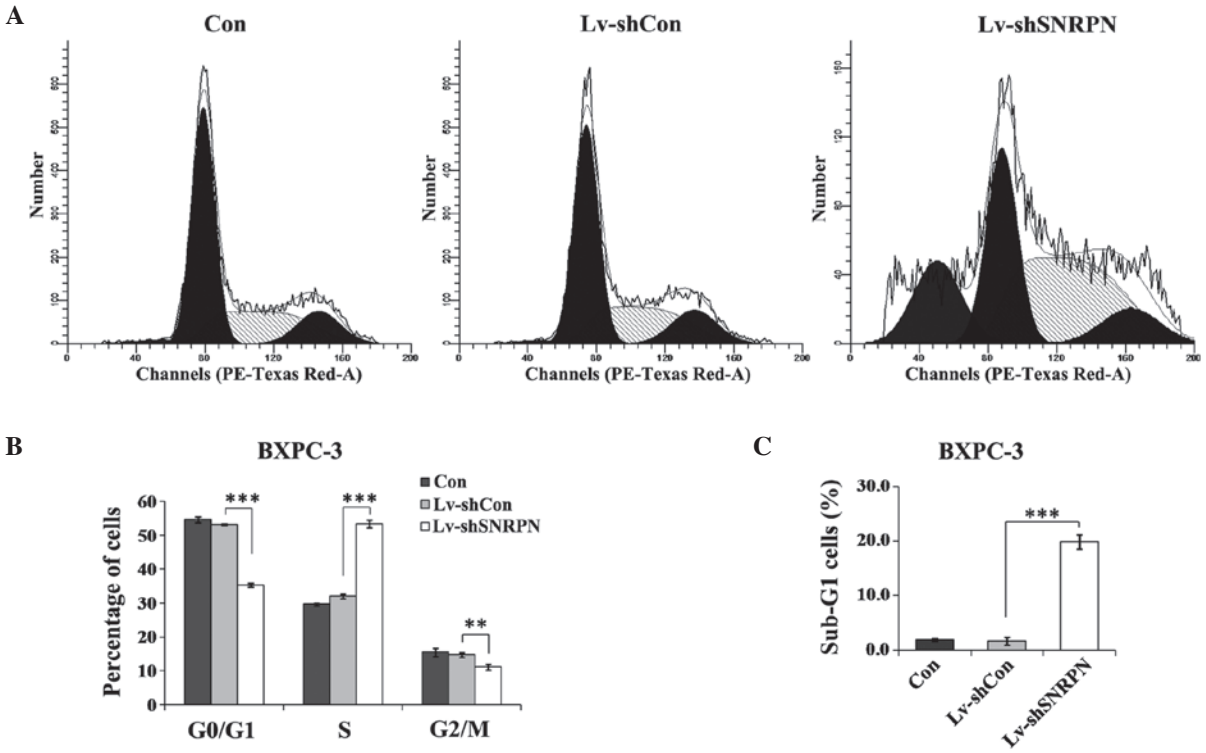

Figure 3. SNRPN knockdown induces cell cycle arrest at S phase and apoptosis in BXPC-3 cells. (A) Cell cycle distribution of BXPC-3 cells was analyzed by flow cytometry. (B) The populations of cells in G0/G1, S and G2/M phases. (C) The population of cells in the sub-G1 phase was increased in the Lv-shSNRPN group. **P<0.01 and ***P<0.001 vs. Lv-shCon group. SNRPN, small nuclear ribonucleoprotein associated polypeptide N; sh, small hairpin.

cancer-related mortality worldwide (1). Novel therapeutic methods are required for the treatment of pancreatic cancer.

Lentivirus-mediated gene delivery is a promising high efficiency and safe therapeutic method. Compared with other methods, it is able to infect replicating and non-replicating cells. Since the first clinical trial approved in 2002, no apparent risk for serious adverse events has been reported with the use of lentivirus vectors (22,23). In the present study,

using lentivirus-mediated RNAi, >90% BxPC-3 cells were infected by lentivirus as indicated by GFP expression. In addition, SNRPN expression was efficiently knocked down at the mRNA and protein levels in cultured pancreatic carcinoma cells.

SNRPN is a spliceosomal protein involved in alternative RNA splicing. Variable methylation of SNRPN has been found to be associated with germ cell tumors and acute myeloid leukemia (20,21). The results indicated that SNRPN depletion could inhibit the proliferation and colony formation of BxPC-3 pancreatic adenocarcinoma cells. Furthermore, cell cycle analysis showed SNRPN depletion led to S phase cell cycle arrest and cell accumulation at the sub G1 phase. One of the possible mechanisms underlying this effect is that SNRPN may alternatively splice certain cell cycle related genes. Aside from its housekeeping role of pre-mRNA splicing, additional functions of SNRPN have been proposed. For instance, SNRPN has a role in axonal RNA metabolism as indicated by its localization in axonal transport granules; furthermore, the interacting protein of SNRPN was not involved in splicing regulation (24). The signaling pathway of SNRPN, involved in the BxPC-3 cell proliferation and tumorigenesis, requires elucidation.

In conclusion, the results suggest that SNRPN may promote pancreatic adenocarcinoma cell growth via regulation of the cell cycle and apoptosis, and lentivirus-mediated SNRPN knockdown may be a potential therapeutic method for pancreatic cancer.

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