Original Article

Silencing NEDD9 by lentivirus-delivered shRNA inhibits the growth of BxPC-3 cells *in vitro* and *in vivo*

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Background: Neural precursor cell-expressed, developmentally downregulated protein 9 (NEDD9) is an invasion and metastasis-related gene. It has been proven to be highly expressed and closely associated with tumor proliferation and invasion in several types of human cancers including pancreatic adenocarcinoma. The present study was aimed to investigate and characterize the efficacy of silencing NEDD9 by lentivirus-delivered shRNA in pancreatic cancer (PC) BxPC-3 cells *in vivo* and *in vitro*.

Methods: Five kinds of PC cell lines were used to determine the cell line which expressed NEDD9 the most with qRT-PCR and western blotting. Then, we transduced the lentivirus-delivered NEDD9 shRNA into the human PC BxPC-3 cells to obtain a stable cell line expressing shRNA targeting NEDD9. NEDD9 mRNA and protein expression were measured by qRT-PCR and western blotting, respectively. Cell proliferation, migration, and invasion were assessed by cell colony formation, scratch wound healing, and Transwell assays, respectively. Mouse tumor xenografts were established by injecting tumor cells into the right flank of BALB/c nude mice. The effects of silencing NEDD9 on the growth of BxPC-3 cells *in vivo* were also examined.

Results: Among 5 kinds of PC cell lines, BxPC-3 cells were selected as the most suitab to carry out the following experiment. Transduction of lentivirus-delivered NEDD9 shRNA efficiently reduced NEDD9 expression in pancreatic adenocarcinoma BxPC-3 cells. Silencing NEDD9 by RNAi inhibited proliferation, migration, and invasion of BxPC-3 cells in cell culture. Importantly, it significantly reduced the growth of BxPC-3 cells in mouse xenografts.

Conclusions: Silencing NEDD9 by lentivirus-delivered shRNA efficiently inhibited the growth of PC BxPC-3 cells both *in vitro* and *in vivo*, and may prove to be a potential new therapeutic agent for human PC.

Keywords: Pancreatic cancer (PC); BxPC-3; lentivirus-delivered NEDD9 shRNA

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Introduction

Pancreatic cancer (PC) is an aggressive malignant tumor of the digestive system that has high rates of invasion and early metastasis. Due to its rapid invasion and early metastasis, most PCs are resistant to current standard therapies (1). PC prognosis is very poor with a median survival time of fewer than 6 months and a 5-year survival rate below 6% (2). Thus, there is an urgent need to elucidate the underlying molecular mechanisms of PC initiation, progression, and maintenance to identify novel therapeutic strategies.

The neural precursor cell-expressed, developmentally downregulated protein 9 (NEDD9), also known as HEF1

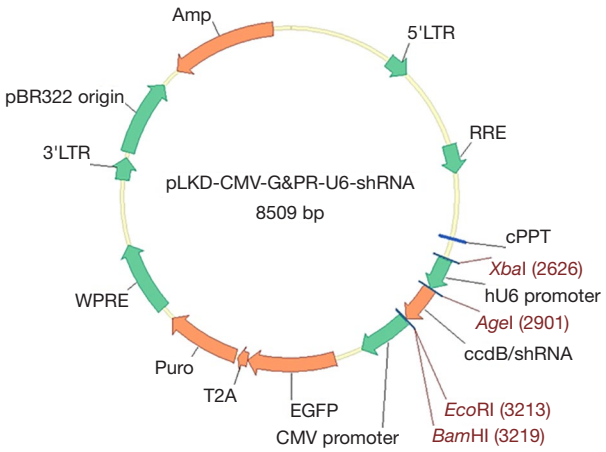

Figure 1 The structure of vector pLKD-CMV-G&PR-U6-NEDD9-shRNA. The vector pLKD-CMV-G&PR-U6-shRNA was cut with Age I and EcoR I enzymes. The shRNA sequence was inserted downstream of the U6 promoter at Age I and EcoR I sites.

and Cas-L, is a non-catalytic scaffolding protein and a member of the Crk-associated substrate (CAS) protein family (3-5). It plays an essential role in the regulation of cell proliferation, adhesion, apoptosis, differentiation, and invasion (6,7). It has been reported that NEDD9 is overexpressed in numerous types of cancer, including breast cancer, lung cancer, gastric cancer, melanoma, and glioblastoma (8-12), suggesting that the abnormal expression of NEDD9 is significantly related to the occurrence and development of cancers. Further studies have indicated that a decrease in the expression of NEDD9 through RNA interference could substantially suppress proliferation, migration, and invasion of melanoma, glioma, and gastric cancer cells (8,13,14).

In our previous study, we found that NEDD9 was overexpressed in PC tissues compared to adjacent noncancerous pancreatic tissues, and that a high expression level of NEDD9 was significantly correlated with clinical staging, lymph node metastasis, and histological differentiation (15). Patients with a higher NEDD9 expression had a significantly shorter survival time than those patients with a lower NEDD9 expression, suggesting that NEDD9 is a potential biomarker for the diagnosis and prognosis of PC, and may be a novel target for anticancer therapies. In the present study, in order to identify whether RNA interference (RNAi) could specifically suppress the expression of target genes and exert distinct biological action in PC tumorigenesis, we investigated NEDD9 expression and its functional roles in human PC BxPC-3 cells *in vitro* and *in vivo* following RNAi-mediated silencing of NEDD9.

Methods

Cell lines selection

Five kinds of cell lines (CFPAC-1, PANC-1, BxPC-3, ASPC-1, and Pacn-02) were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPIM1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, LA, USA) at 37 ℃ in a humidified atmosphere containing 5% CO_2. Quantitative RT-PCR and western blot were used to detect the expression of NEDD9 mRNA and protein levels in the above cell lines. The cell line which expressed NEDD9 the most was selected to carry out the following experiment.

NEDD9 RNAi plasmid construct

To determine the most effective interference target, 3 interference targets were designed and selected. The BxPC-3 cells were divided into 5 groups: blank control (normal BxPC-3 cells), negative control (Y007), NEDD9-shRNA1 (Y2600), NEDD9-shRNA2 (Y2601), and NEDD9-shRNA3 (Y2602). The oligonucleotides for the short hairpin (sh)RNA targeting the sequence of NEDD9 (Y007: TTCTCCGAACGTGTCACGT; Y2600: GCTCTCAGAACGACGCATATG; Y2601: GCAGCTGGTCCCTGAATATCT; Y2602: GCAGGAAATGGTGCACCAAGT; NCBI Gene ID: 4739) were chemically synthesized by Obio Technology (Shanghai, China) and cloned into the vector pLKD-CMV-G&PR-U6-shRNA (Obio Technology, Shanghai, China) to get plasmid pLKD-CMV-G&PR-U6-NEDD9-shRNA (*Figure 1*). The sequence of the plasmid was confirmed by direct DNA sequencing.

Establishment of stable NEDD9 shRNA BxPC-3 cells

BxPC-3 cells were plated in 24-well plates (2×10^4 cells/well) in RPIM1640 (Gibco, CA, USA) supplemented with 10% FBS (HyClone, LA, USA). Every well contained 5 μg/mL polybrene (HyClone, LA, USA) and was incubated at 37 ℃ in 5% CO_2. The pLKD-CMV-G&PR-U6-NEDD9 shRNA together with the packaging plasmids were co-

transfected into 293T cells to obtain a retrovirus. The retrovirus was transduced into BxPC-3 cells and then selected with puromycin to obtain stable NEDD9-shRNA-expressing BxPC-3 cells. The same procedures obtained control BxPC-3 cells stably transduced with pLKD-CMV-G&PR-U6-shRNA. Green fluorescent protein (GFP) expression was observed by fluorescence microscopy 14 d after transduction, and cells were harvested at 14 d after transduction for western blot analysis.

Western blot

Cells were lysed in NP-40 lysis buffer (Beyotime, Shanghai, China) on ice for 20 min. Protein concentrations were determined using a BCA protein assay kit (HyClone-Pierce, LA, USA). Protein samples (36 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline and Tween 20 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.05% Tween, TBS-T). The membranes were then probed with mouse anti-NEDD9 antibody (1:2,000) (Abcam, MA, USA) or anti-GAPDH antibody (1:10,000) (Beyotime, Shanghai, China) at 4 ℃ overnight. Membranes were washed 3 times with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:3,000) (Santa Cruz Biotechnology, USA) for 2 h at room temperature. The immunohistochemical reaction of bound antibodies was visualized using ECL PlusTM western blotting system (Amersham Pharmacia Biotech, UK).

Quantitative RT-PCR

Total RNA from BxPC-3cells was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. cDNA was synthesized with M-MLV retroviridase (Promega, WI, USA). Primers were synthesized by Obio Technology (Shanghai, China). The sequences of the used primers were as follows: NEDD9, upstream 5'-TTACGTCCACCTACAGGGTA-3', downstream 5'-CGGGCTTTGTAATCTCTTG-3'; β-actin, upstream 5'-TTCTACAATGAGCTGCGTG-3', downstream 5'-CTCAAACATGATCTGGGTC-3'. qRT-PCR was performed on the ABI PRISM 7500 Sequence Detection System (Biosystems, CA, USA). β-actin was used as an endogenous control. PCR reactions were performed in triplicate, and data were analyzed through the comparative threshold cycle (C_T) method.

Colony formation assay

Stably transfected cells were cultured in RPIM1640 (Gibco, CA, USA) supplemented with 10% FBS (HyClone, LA, USA) in 6-well plates. The cells were plated at an initial density of 1,000 cells/well. The medium was replaced with 10 mL fresh medium every 3 d. After 14 d of culture, the cells were fixed with methanol and stained with 0.5% crystal violet (Beyotime, Shanghai, China) for 30 min. Colonies with more than 50 cells were counted, and photos were taken using inverted fluorescence microscopy.

Cell migration and invasion assays

The migration and invasion of BxPC-3 cells were quantified by wound healing and Transwell assays, respectively. For wound healing assay, cells (5×10^5 cells/well) were plated in 6-well plates. After cells became 80–90% confluent, the "wound" was scratched manually with a 200-μL pipette tip. Images were taken at ×100 magnification fields from each 6-well plate at 0, 16, and 24 h. For Transwell assay, cells were placed in the upper chamber of Transwells (8 μmol/L pore size polycarbonate membranes) (Corning, CA, USA) with serum-free RPIM1640 medium. Lower chambers were loaded with 500 μL RPIM1640 containing 10% FBS. After 24 h of incubation at 37 ℃ in 5% CO_2, non-migratory cells were removed. Invasive cells were fixed with 4% paraformaldehyde for 15 min and stained with 500 μL crystal violet. After 3 washes with ddH_2O, 3 fields per well were photographed under phase-contrast microscopy. The number of cells per field was counted.

In vivo study of pancreatic xenograft tumor models in nude mice

Animal experiments were approved by the Third Affiliated Hospital of Nantong University Medical Ethics Committee. Six-week-old male BALB/c nude mice (N=18) were purchased from Shanghai SLAC Laboratory Animal (Shanghai, China). The mice were randomly divided into 3 groups (n=6 per group): BxPC-3 control (blank), negative-shRNA (negative), and NEDD9-shRNA (shRNA) groups. Cells (1×10^7 in 0.2 mL RPIM1640 supplemented with 10% FBS) were injected into the right flank of the mice. The tumor volume was measured every 3–4 d. Tumor volume was calculated using the formula: width^2 (mm^2) × length

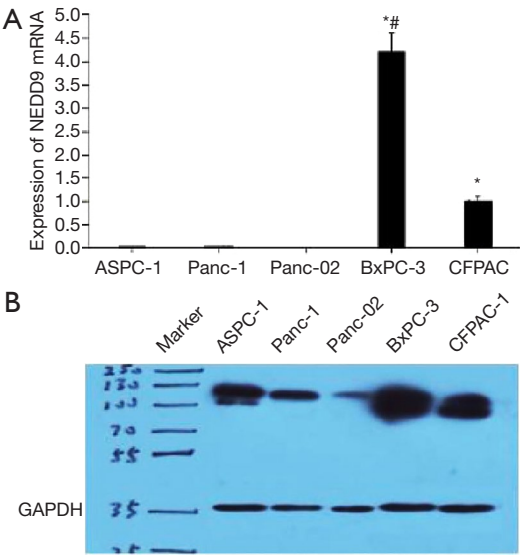

Figure 2 Cell line selection. qRT-PCR and western blot were used to detect the expression of NEDD9 in 5 kinds of cell lines (A,B). No NEDD9 expression was detected in Panc-02 by qRT-PCR. *, P<0.05, compared with the ASPC-1 and Panc-1 group; #, P<0.05, compared with the CFPAC-1 group.

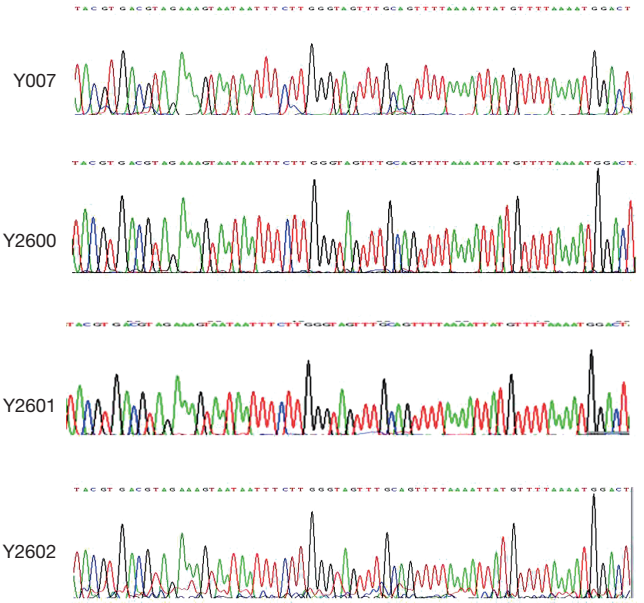

Figure 3 Transfection of cells under fluorescence and white light microscope. The blank control (normal BxPC-3 cells); Y007, negative control; Y2600, NEDD9-shRNA1 BxPC-3 cells; Y2601, NEDD9-shRNA 2 BxPC-3 cells; Y2602, NEDD9-shRNA 3 BxPC-3 cells. Original magnifications, ×100; all the cells were observed 14 d after transduction.

(mm)/2 every 3–4 d using a caliper. After 5 weeks, the mice were sacrificed, and tumors were harvested and weighed.

Statistical analysis

Each experiment was conducted at least 3 times. Values are presented as mean ± standard deviation (SD). Statistical analysis of the data was performed using a Student's t-test with IBM SPSS Statistic v19.0 (IBM Co., Armonk, NY, USA). P<0.05 indicates a significant difference, and P<0.01 indicates a highly significant difference.

Results

In vitro study

Cell line selection

Five kinds of pancreatic tumor cells (CFPAC-1, PANC-1, BxPC-3, ASPC-1 and Panc-02 cell lines) were selected to detect the expression of NEDD9. According to the result of qPCR, the BxPC-3 cells expressed NEDD9 the most while no expression was detected with Panc-02 (*Figure 2A*). From the western blot result, 2 phosphorylated isoforms of NEDD9, migrating at 105 kDa (p105) and 115 kDa (p115), were detected (*Figure 2B*) while NEDD9 was expressed the most in the BxPC-3 cells which conformed with the qPCR result. Consequently, BxPC-3 cells were selected to carry out the following experiment.

Establishment of stable BxPC-3 cells expressing NEDD9 shRNA

Four shRNA sequences (Y007, Y2600, Y2601, and Y2602) in plasmid pLKD-CMV-G&PR-U6-NEDD9-shRNA and the cloning sites in vector pLKD-CMV-G&PR-U6-shRNA were confirmed by DNA sequencing (*Figure 3*). The plasmids were transfected into 293T cells to pack into lentivirus NEDD9-RNAi and negative control shRNA, respectively. The titer of viruses was 3.04×10^8 titer U/mL. After NEDD9-shRNA and nonspecific shRNA, marked by fluorescence, were transfected into BxPC-3 cells, the cells were selected with puromycin for 14 d to obtain stable BxPC-3 cells expressing NEDD9 shRNA. Cells showing green fluorescence were considered successfully transduced. Significant GFP was observed in the shRNA transfection group and the negative control group while

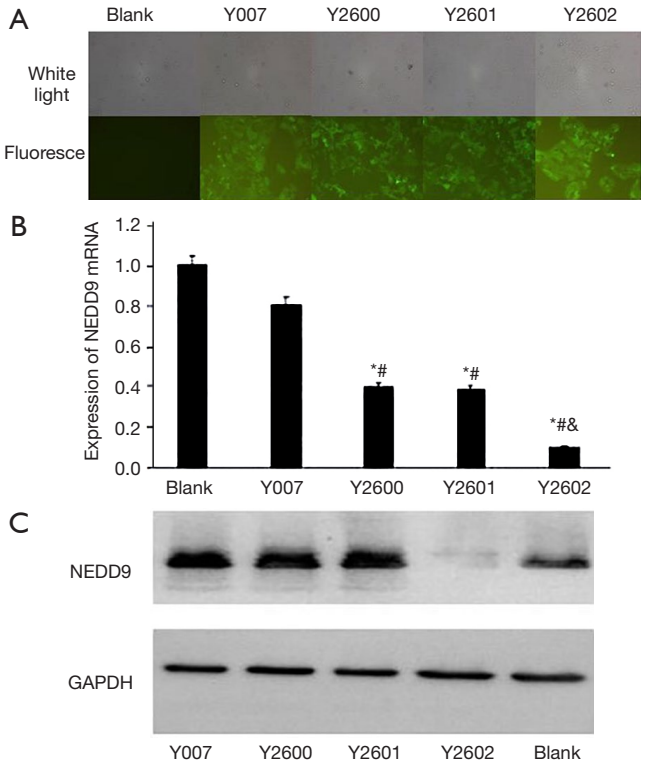

Figure 4 The construction and selection of NEDD9 RNAi plasmid and stable BxPC-3 cells expressing NEDD9 shRNA. Four shRNA sequences (Y007, Y2600, Y2601, and Y2602) in plasmid pLKD-CMV-G&PR-U6-NEDD9-shRNA and the cloning sites in vector pLKD-CMV-G&PR-U6-shRNA were confirmed by DNA sequencing (A). NEDD9 mRNA and protein level in 5 group cells were assessed by qRT-PCR and western blot analysis (B,C). *, P<0.05, compared with the blank control group; #, P<0.05, compared with the negative control group (Y007); &, P<0.05, compared with the Y2600 and Y2601 group. Blank control group, normal BxPC-3 cells group; Y007, the negative control group; Y2600, NEDD9-shRNA1 BxPC-3 group; Y2601, NEDD9-shRNA 2 BxPC-3 group; Y2602, NEDD9-3shRNA BxPC-3 group.

there was no fluorescence in the blank control group. This indicated that NEDD9 shRNA had been successfully transfected into BxPC-3 cells (*Figure 4A*). The stably transduced cell lines were confirmed by qRT-PCR and western blot for detecting the expression of NEDD9 mRNA and protein levels, respectively. We found that NEDD9-Y2602 shRNA inhibited the expression of NEDD9 significantly (*Figure 4B,C*); therefore, it was selected as the best shRNA with which to carry out the interference experiment.

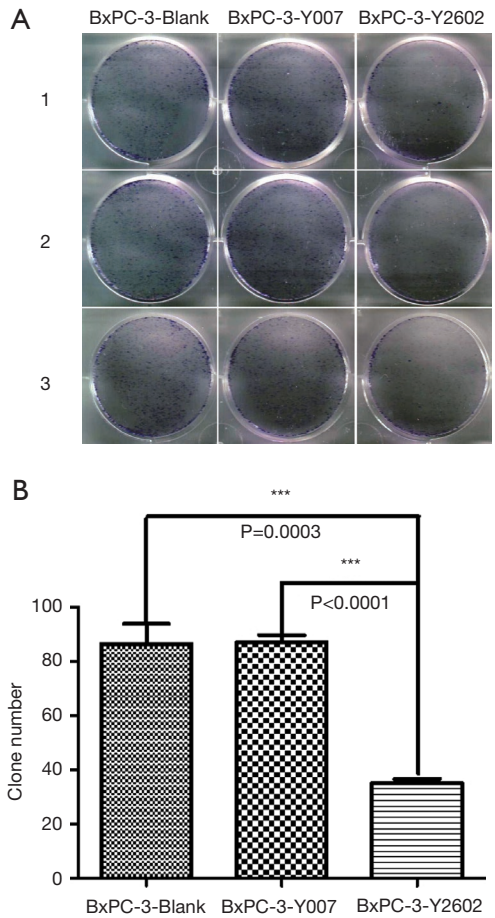

Figure 5 Effects of siNEDD9 on cell proliferation *in vitro*. (A) Images of colony formation of BxPC-3 cells in the blank, negative control (Y007), and RNAi groups (Y2602). Every group were repeated three times. (B) Quantification of the colony formation for the 3 groups. ***, P<0.001.

Silencing of NEDD9 inhibits proliferation, migration, and invasion of BxPC-3 cells

To further characterize the role of NEDD9-shRNA in BxPC-3 cells, we performed colony formation assays to assess the effect of NEDD9-shRNA on the proliferation of BxPC-3 cells. After culturing for 14 d, the NEDD9-RNAi group showed significantly fewer clones compared to the blank and negative control groups (P<0.001). No significant difference was found between the blank and negative control group (P>0.05) (*Figure 5A,B*). Moreover, the wound-healing assay showed that cell migration was significantly decreased in the RNAi group compared to the blank and negative control groups (P<0.05; *Figure 6A,B*). The Transwell assay demonstrated that there were significantly fewer invasive

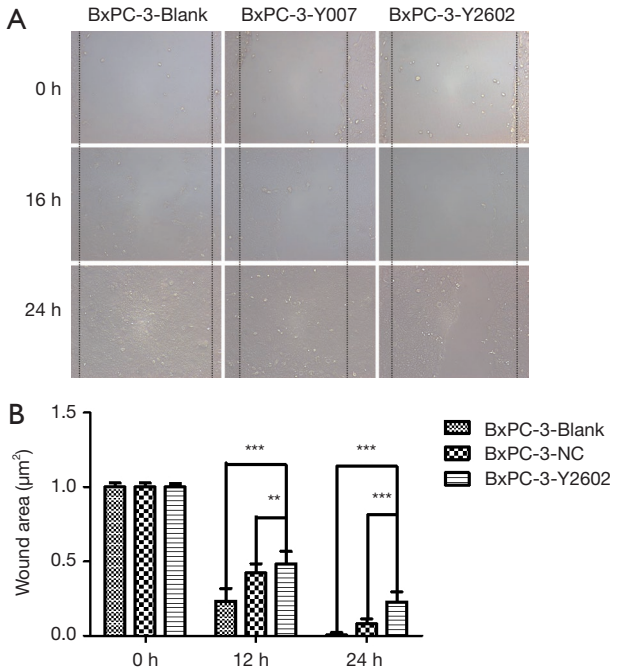

Figure 6 Effects of siNEDD9 on cell migration *in vitro*. (A) Images of wound healing assay at the indicated time points in the blank, negative control (Y007), and RNAi groups (Y2602); (B) quantification of the wound healing assay for the 3 groups. **, P<0.01; ***, P<0.001.

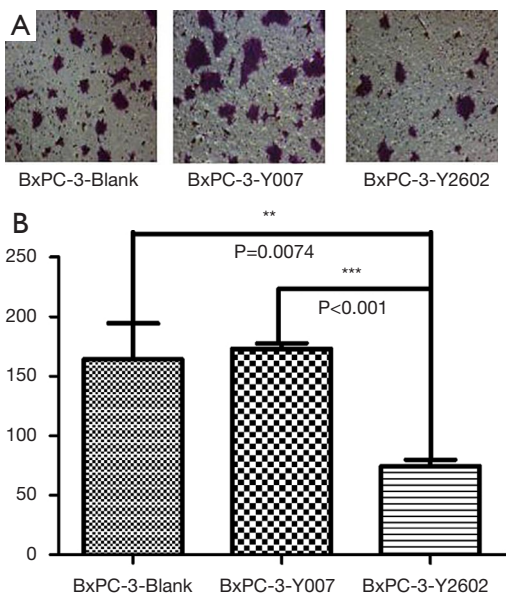

Figure 7 Effects of siNEDD9 on cell invasion *in vitro*. Images of Transwell assay in the blank, negative control (Y007), and RNAi groups (Y2602) (A). Quantification of the Transwell assay for the 3 groups (B). **, P<0.01; ***, P<0.001.

cells in the NEDD9-RNAi group compared to the blank and negative control groups (*Figure 7A,B*, P<0.01). These results demonstrate that silencing NEDD9 by RNAi reduced the proliferation, migration, and invasion of BxPC-3 cells.

In vivo study

NEDD9-RNAi suppresses the growth of xenograft tumor in nude mice

To determine whether knockdown of NEDD9 inhibits pancreatic tumor growth *in vivo*, BxPC-3 control (blank), negative-shRNA (negative), and NEDD9-shRNA (shRNA) cells were injected into the right flank of the BALB/c nude mice. The growth of pancreatic adenocarcinoma tumor xenografts in the 3 treatment groups was compared. Measurement of tumor volumes began once subcutaneous tumors became palpable and continued until day 35, at which point tumors were harvested and weighed (*Figure 8A,B*). The average volume of tumors in the NEDD9-shRNA group was significantly smaller compared to the volume of tumors in the16 negative or blank groups (P<0.05; *Figure 8C*). In addition, xenografts in the NEDD9-RNAi group had markedly lower tumor weights (0.4±0.13 g) on day 35 compared to xenografts of the negative (0.7±0.12 g) (P<0.05) or blank (0.9±0.37 g) (P<0.05) groups (*Figure 8D*). Thus, silencing the expression of NEDD9 by RNAi can inhibit the growth of the BxPC-3 xenografts in nude mice.

Discussion

PC is the 12th most common malignancy and the 7th leading cause of cancer mortality, accounting for more than 330,000 deaths in 2012 (16). Recent studies have suggested that the incidence and mortality of PC are rising in many countries, and some projection studies have estimated that PC will escalate from the 4th to the 2nd leading cause of cancer death in the USA by 2020 (17). It has already induced a substantial globe burden which needs to be taken seriously (18,19). Owing to its extremely aggressive nature and reduced survival rate (20), the underlying molecular mechanisms of PC urgently need to be clarified in order to innovate novel therapeutic strategies.

NEDD9, a skeletal protein belonging to the Crk-associated substrate (CAS) family, acts as a router in the cell signal transduction process and plays a vital role in the regulation of cell cycle-related events such as DNA

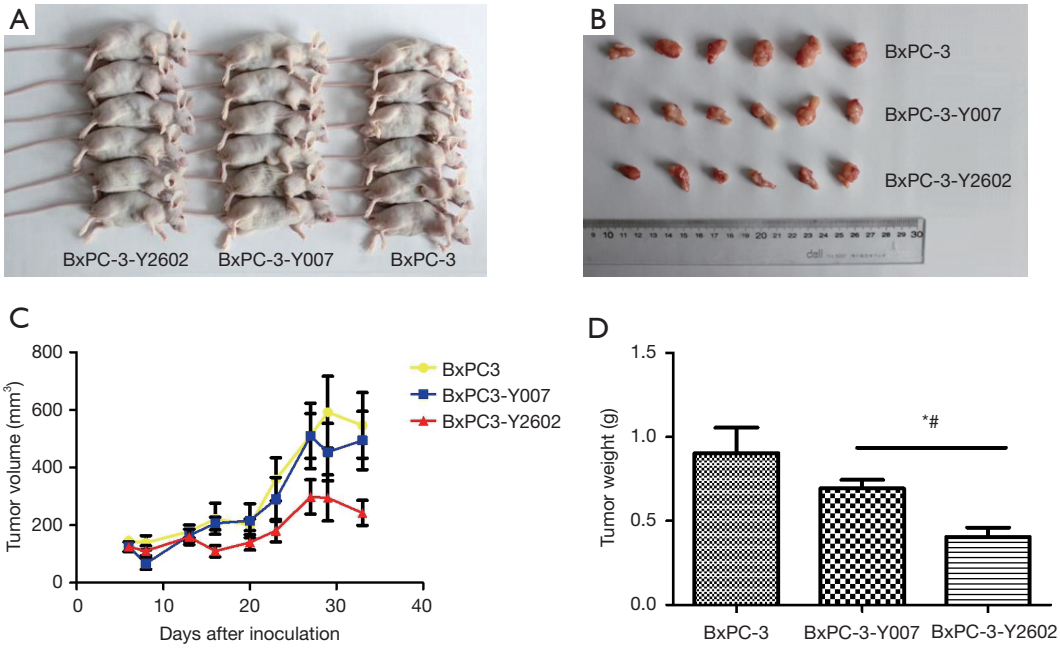

Figure 8 NEDD9-RNAi suppresses the growth of xenograft tumor in nude mice. Tumor-bearing mice were sacrificed at 35 d after BxPC-3 cell injection (A), and the Xenograft tumors were all taken out and measured (B). The volume of tumor xenografts of the 3 groups was measured and recorded at day 10, 20, 30, and 35, and the variation tendency of the tumor volume is shown in (C). (D) The weights of dissected tumors at day 35. Data are means ± SD. *, P<0.05, compared with the blank control group; #, P<0.05, compared with the negative control group (Y007).

replication, chromatin condensing, and segregation (21,22). Accumulating evidence has shown that NEDD9 plays an essential role in multiple steps of tumorigenesis for various cancers, and that deregulation of NEDD9 is closely associated with the pathophysiology of several types of cancers, including breast, lung, and gastric cancers. For instance, NEDD9 overexpression promotes migration and invasion of breast tumor cells through multiple mechanisms including activating the focal adhesion complex (FAK and SRC), mediating effects of TGFβ and integrins, and increasing synthesis of tumor-associated glycocalyx (9,23,24). In lung and gastric cancers, elevated expression of NEDD9 have been closely associated with malignant progression and metastases (10,11). In our previous study, we investigated the expression of NEDD9 in 106 cases of PC and paired adjacent healthy tissues by RT-PCR, western blotting, and immunohistochemistry. We found that NEDD9 was upregulated in PC tissues, but weakly expressed in adjacent healthy tissues.

Moreover, the clinicopathological analysis revealed that NEDD9 protein expression was significantly correlated with TNM stage, tumor differentiation, and metastasis (15). Taken together, this evidence suggests that NEDD9 may act as an oncogene and participates in the invasion and metastasis in PC. We, therefore, studied the effect of inhibiting expression of NEDD9 on pancreatic adenocarcinoma BxPC-3 cells both *in vivo* and *in vitro*.

However, NEDD9 is a scaffold protein with no apparent enzymatic functions, which poses a challenge for developing small molecules that can inhibit the functions of NEDD9. RNA interference technology is a new silencing technology which involves the insertion into cells of endogenous or exogenous double-stranded RNA. It can efficiently suppress the expression of genes and proteins (25). As a method of RNAi, endogenously expressed shRNAs have been completed through various viral systems, including retrovirus, adenovirus, adeno-associated virus (AAV), and lentivirus (26). The viral systems have a clear advantage of high transduction efficiency and stable expression of shRNAs for a prolonged period. So, in this study, we developed a lentivirus-delivered shRNA system to efficiently reduce the expression of NEDD9 in a pancreatic adenocarcinoma BxPC-3 cell model. We demonstrated that lentivirus-delivered shRNA reduced NEDD9 expression

in pancreatic adenocarcinoma BxPC-3 cells efficiently and stably for an extended period. This suggests that lentivirus-delivered shRNA may be a potential agent for inhibiting targeted genes.

In this study, we selected 3 shRNA sequences of siRNA which was targeting NEDD9 and BxPC-3 cells by lentivirus to silence NEDD9 in cells. Expression of mRNA was detected by qTR-PCR while protein was detected by western blot. The results showed that NEDD9-shRNA Y2602 elicited the maximum decrease among the 3 cell lines; therefore, we selected NEDD9-shRNA Y2602 for further experiment.

We also examined cell proliferation, invasion, and migration using cell colony formation, scratch wound healing, and Transwell assays after silencing NEDD9 by shRNA. The results showed that NEDD9-shRNA Y2602 inhibited proliferation, migration, and invasion of BxPC-3 cells *in vitro*. More importantly, we injected (shRNA) cells into the right flank of BALB/c nude mice to determine whether knockdown of NEDD9 inhibits pancreatic tumor growth *in vivo*. We found that silencing NEDD9 by RNAi significantly reduced the growth of BxPC-3 cells in mouse xenografts as well. Given these findings, we propose NEDD9 maybe a direct target in PC treatment.

In summary, NEDD9 plays a vital role in the carcinogenesis of PC. In our study, inhibiting the expression of NEDD9 mRNA and protein by lentivirus-delivered shRNA could not only inhibit proliferation, migration, and invasion of pancreatic adenocarcinoma BxPC-3 cells *in vitro*, but also suppressed the growth of BxPC-3 cells in mouse xenografts. This suggests that NEDD9 may serve as a potential marker, and may act as an effective gene therapy of PC in the future. However, the specific mechanisms underlying the protective effects of NEDD9 RNAi against PC remain unknown, and further experiments should be carried out to investigate the therapeutic role of NEDD9 RNAi in treating PC.

Acknowledgments

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.09.02). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were approved by the Third Affiliated Hospital of Nantong University Medical Ethics Committee.

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