NPTX1 inhibits pancreatic cancer cell proliferation and migration and enhances chemotherapy sensitivity by targeting RBM10

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Abstract. Pancreatic cancer (PC), one of the deadliest diseases worldwide, has exhibited an increasing incidence rate in recent years. The present study aimed to explore the biological mechanism of PC. Therefore, the expression levels of neuronal pentraxin 1 (NPTX1) and RNA-binding protein 10 (RBM10) were detected in PC cell lines using reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses prior to or following NPTX1 and RBM10 overexpression. Additionally, the proliferative ability of PANC-1 and BxPC-3 cells treated with or without gemcitabine (GEM) and cisplatin (DDP) was evaluated using Cell Counting Kit-8 assay. Cell apoptosis and the expression levels of apoptosis-related proteins were determined by TUNEL assay and western blot analysis, respectively. Furthermore, wound healing and Transwell assays were performed to measure the migration and invasion abilities of PANC-1 and BxPC-3 cells. The interaction between RBM10 and NPTX1 mRNA was detected by RNA binding protein immunoprecipitation (RIP) assay. Additionally, cells were treated with actinomycin D to verify the regulatory effect of RBM10 on NPTX1 expression. This effect was further confirmed by RT-qPCR analysis. The results showed that NPTX1 was downregulated in PC cell lines. In addition, NPTX1 overexpression inhibited the proliferation and promoted apoptosis in PC cells. The results from the wound healing and Transwell assays revealed that the migration and invasion abilities of PANC-1 and BxPC-3 cells were reduced following NPTX1 overexpression. However, treatment of NPTX1-overexpressing cells with GEM or DDP attenuated PC cell viability. In addition, the results of the RIP assay revealed that RBM10 could bind with NPTX1. Furthermore, RBM10 overexpression could regulate NPTX1 expression, as evidenced by actinomycin D experiments. Overall, the results of the present study suggested that NPTX1 could inhibit PC and enhance the sensitivity of PC cells to chemotherapy. Additionally, NPTX1 was found to interact with RBM10, indicating that NPTX1 could inhibit PC via targeting RBM10.

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Introduction

As one of the deadliest malignant tumors, pancreatic cancer (PC) currently ranks tenth among the most frequent types of cancer in men and the ninth in women in USA (1). In addition, PC is the third highest cause of cancer-related mortality, accounting for ~227,000 deaths annually worldwide (2). Due to its atypical symptoms, PC is difficult to diagnose, thus resulting in increased incidence and mortality rates (3). The most common non-specific symptoms of PC include abdominal pain and weight loss, while the 5-year survival rate of patients remains <5% (4,5). Although significant progress has been made in the diagnosis and management of PC, its causes remain poorly understood (6). Therefore, the present study aimed to uncover the biological mechanisms underlying the development of PC and identify relatively effective therapeutic approaches.

Neuronal pentraxin 1 (NPTX1), also known as neuropilin-1 is a member of the long pentraxin family of proteins. It is mainly expressed in central neurons and displays promotive effects on neurite growth and regulates cellular properties (7,8). NPTX1 was first identified as an epigenetic target in PC by applying genome scanning technology, which provides a global DNA methylation analysis (9). Notably, emerging evidence has suggested that NPTX1 is involved in the development of different types of cancer. For example, NPTX1 is shown to exhibit antiproliferative effects on colon cancer, while NPTX1 overexpression is found to downregulate cyclin A2 and CDK2 in colon cancer cells (10). In addition, Zhou *et al* (11) report that NPTX1 is a novel epigenetic regulation gene in lung cancer and NPTX1 overexpression can attenuate lung cancer progression. Furthermore, NPTX1 is found to be involved in the progression of PC (12,13). In the current study, the mechanism of NPTX1 in PC was further investigated.

Gemcitabine (GEM), the most important cytidine analogue, exhibits antitumor activity in several tumor models (14). It has been also suggested that cisplatin (DDP) has great potential in treating various solid tumors (15). To date, several studies have investigated the effects of GEM and DDP on PC. For example,

Heinemann (16) reported that treatment with single-agent gemcitabine achieved clinical benefit and symptoms improvement in 20-30% of patients with a higher 1-year survival and a median survival. Another study indicated that the combination of gemcitabine and cisplatin significantly improves the quality of life in patients with locally advanced or metastatic PC, prolonging the survival time with tolerable toxicity (17). The aforementioned studies supported the antitumor activity of both GEM and DDP in PC. Therefore, the present study also investigated the effects of GEM and DDP on PC.

RNA-binding protein 10 (RBM10), a member of the RBP family, is located on chromosome Xp11.23 (18). Previous studies demonstrated that RBM10 could cell promote apoptosis and inhibit cell proliferation (19,20). Xiao *et al* (21) demonstrate that RBM10 is downregulated in PC, while its expression is associated with the prognosis of PC. Other studies reveal that AKT, which is possibly inhibited by RBM10, could be involved in the progression of various types of cancer via regulating NPTX1 (22,23). Therefore, the present study aimed to investigate the regulatory effect of RBM10 on NPTX1 expression and to uncover their potential interaction.

Materials and methods

Cell culture, treatment and transfection. The normal human pancreatic ductal epithelial cell line HPDE6-C7 and the PC cell lines PANC-1, CAPAN-1, SW1990 and BxPC-3 were obtained from ATCC. The cells were cultured in DMEM supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO_2. Subsequently, cells were treated with 100 μM GEM for 72 h or 1 μM DDP for 24 h. To overexpress NPTX1 and RBM10, PANC-1 and BxPC-3 cells were transfected with NPTX1 and RBM10 overexpression plasmids (ov-NPTX1 and ov-RBM10; Hunan Fenghui Biotechnology Co., Ltd.) at 37˚C for 48 h using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Transfected cells were used for subsequent experiments 48 h later.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from PANC-1 and BxPC-3 cells ($5x10^6$ cells/ml) using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, the extracted RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. To analyze gene expression, qPCR was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The following thermocycling conditions were used: Initial denaturation at 95˚C for 7 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec, and then a final extension at 72˚C for 30 sec. The primer sequences for PCR are presented as below: NPTX1: 5'-ACCGAGGAGAGGGTC AAGAT-3' (forward) and 5'-GTGGGAATGTGAGCTGGA AC-3' (reverse); RBM10: 5'-AGGGCAAGCATGACTATGA-3' (forward) and 5'-GTGGAGAGCTGGATGAAGG-3' (reverse); GAPDH: 5'-GGGAAACTGTGGCGTGAT-3' (forward) and 5'-GAGTGGGTGTCGCTGTTGA-3' (reverse). The $2^{-\Delta\Delta Cq}$ method (24) was employed to determine the relative gene expression levels, which normalized to GAPDH level. The experiments were repeated at least 3 times.

Western blot analysis. Total proteins were extracted from PANC-1 and BxPC-3 cells using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). The protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology). The protein samples (30 μg/lane) were separated by 10% SDS-PAGE and were then transferred onto PVDF membranes. Following blocking with 5% non-fat milk at room temperature for 2 h, the membranes were incubated with the appropriate primary antibodies against NPTX1 (catalog no. bs-4893R; 1:500; Bioss), Bcl-2 (catalog no. ab32124; 1:1,000), Bax (catalog no. ab32503; 1:1,000), cleaved PARP (catalog no. ab32064; 1:1,000), MMP12 (catalog no. ab52897; 1:1,000), ZEB1 (catalog no. ab203829; 1:500), RBM10 (catalog no. ab72423; 1:2,000) and GAPDH (catalog no. ab8245; 1:500) (all Abcam) at 4˚C overnight. Subsequently, the membranes were incubated with the corresponding HRP-conjugated Goat Anti-Rabbit IgG secondary antibodies (catalog no. ab205718; 1:2,000; Abcam) for 2 h at room temperature. Finally, the protein bands were visualized by using an ECL reagent (Merck KGaA). The protein intensity was quantified with ImageJ software v1.8.0 (National Institutes of Health).

Cell counting kit-8 (CCK-8) assay. PANC-1 and BxPC-3 cells were seeded into 96-well plates for 24 h. Subsequently, 10 μl CCK-8 reagent (Beyotime Institute of Biotechnology) was added into each well and the cells were incubated for 24, 48 and 72 h at 37˚C in a humidified incubator with 5% CO_2. The absorbance of each well was detected at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

TUNEL assay. The apoptosis rate of PANC-1 and BxPC-3 cells was determined using a TUNEL assay kit (Beyotime Institute of Biotechnology). Briefly, PANC-1 and BxPC-3 cells were fixed with 4% paraformaldehyde for 15 min and were then permeabilized with 0.25% Triton-X 100 for 20 min at room temperature. Subsequently, cells were incubated with TUNEL reaction solution at 37˚C for 1 h followed by staining with DAPI for 30 min at room temperature. Finally, images of the TUNEL-positive cells randomly selected from 5 fields of view were captured under a fluorescence microscope (magnification, x200).

Wound healing assay. PANC-1 and BxPC-3 cells were inoculated into 6-well plates at a density of $6x10^4$ cells/well and cultured in serum-free DMEM until reaching 90-100% confluence. Subsequently, a straight linear wound was created across the cell monolayer using a 200-μl pipette tip. The cell debris was removed by washing with PBS three times and the cells were then incubated at 37˚C with 5% CO_2. Wound closure was measured using randomly selected cells from 3 fields of view at 0 and 24 h post-treatment with a light microscope (magnification, x200). Finally, ImageJ software v1.8.0 was used to determine cell migration.

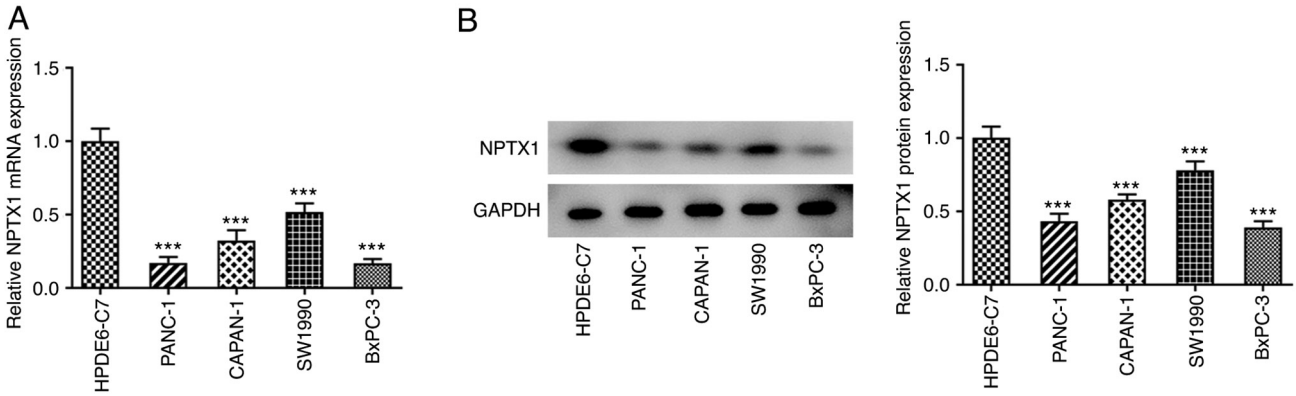

Figure 1. NPTX1 expression is decreased in pancreatic cancer cells. (A) The relative mRNA expression of NPTX1 in pancreatic cancer cells was detected using reverse transcription-quantitative PCR. (B) The protein expression of NPTX1 in pancreatic cancer cells was measured using western blot. Results are the mean ± standard deviation. ***P<0.001 vs. control. NPTX1, neuronal pentraxin 1.

Transwell assay. The invasive ability of PANC-1 and BxPC-3 cells was evaluated by using a 24-well Transwell (CLS3396; Corning, Inc.) assay. Briefly, PANC-1 and BxPC-3 cells ($1x10^5$ cells/well) were added into the upper chamber of the Transwell inserts, while the lower compartment of the Transwell chamber was filled with medium supplemented with 10% FBS. Following incubation at 37˚C for 24 h, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet solution for 30 min at 37˚C. Finally, images of the invading cells randomly selected from 5 fields of view were captured under a light microscope (magnification, x400).

RNA binding protein immunoprecipitation (RIP) assay. Cells were scraped from culture dishes and incubated with glycine after fixing by formaldehyde (0.3%) at room temperature for 10 min. Then the cells were transferred into 1.5-ml tubes and lysed with RIP buffer. Subsequently, the cells were incubated with the anti-RBM10 antibody (catalog no. ab72423; 1:20; Abcam) at 37˚C overnight. Precipitated RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and analyzed by PCR amplification.

Bioinformatics analysis. StarBase (http://starbase.sysu.edu.cn/) predicted the relationship between RBM10 and NPTX1. The RNA-Protein Interaction Prediction (RPISeq; http://pridb.gdcb.iastate.edu/RPISeq) database predicted the interaction probabilities of RBM10 and NPTX1.

Statistical analysis. The data are expressed as mean ± standard deviation. All data were analyzed by using SPSS software (version 20.0; IBM Corp.). Differences between two groups were compared using Student's t-test, while those among multiple groups using ANOVA and Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

NPTX1 is downregulated in PC cells. To determine the expression levels of NPTX1 in PC cell lines, RT-qPCR and western blot analyses were performed. As shown in Fig. 1A and B, the relative mRNA and protein expression levels of NPTX1 were significantly decreased in PC cell lines, particularly in PANC-1 and BxPC-3 cells. Therefore, PANC-1 and BxPC-3 cells were used for the subsequent experiments.

NPTX1 overexpression inhibits proliferation and promotes apoptosis in PC cells. As shown in Fig. 2A-D, NPTX1 was notably upregulated in PANC-1 and BxPC-3 cells following cell transfection with ov-NPTX1. CCK-8 assays revealed that PANC-1 and BxPC-3 cell viability was significantly reduced in NPTX1-overexpressing cells, suggesting that NPTX1 overexpression exerted antiproliferative effects on PC cells (Fig. 2E and F). Furthermore, the apoptosis rate was significantly increased in PANC-1 and BxPC-3 cells overexpressing NPTX1 (Fig. 2G and H). Additionally, the protein level of Bcl-2 was decreased, while the contents of Bax and cleaved poly ADP-ribose polymerase (PARP) were significantly increased in PANC-1 and BxPC-3 cells after transfection with ov-NPTX1 (Fig. 2I and J).

NPTX1 overexpression attenuates the migration and invasion ability of PC cells. The results showed that NPTX1 overexpression reduced the migration and invasion abilities of PANC-1 cells compared with the untransfected cells (Fig. 3A). Accordingly, NPTX1 overexpression attenuated the migration and invasion abilities of BxPC-3 cells (Fig. 3B). As shown in Fig. 3C and D, MMP12 and zinc finger E-box-binding homeobox 1 were significantly downregulated in PC cells overexpressing NPTX1. The aforementioned findings indicated that NPTX1 overexpression exerted an inhibitory effect on PC cell migration and invasion.

NPTX1 overexpression enhances the sensitivity of PANC-1 and BxPC-3 cells to GEM and DDP. CCK-8 assays revealed that the viability of PANC-1 and BxPC-3 cells treated with GEM was decreased in a dose-dependent manner (Fig. 4A and B). In addition, NPTX1 overexpression further decreased the viability of PANC-1 and BxPC-3 cells treated with GEM compared with cells treated with negative control overexpression plasmid (ov-NC). This finding supported that NPTX1 overexpression could enhance the sensitivity of PC cells to GEM. Furthermore, NPTX1 overexpression notably decreased the viability of DDP-treated PANC-1 and BxPC-3 cells compared with the ov-NC group. Similarly, NPTX1

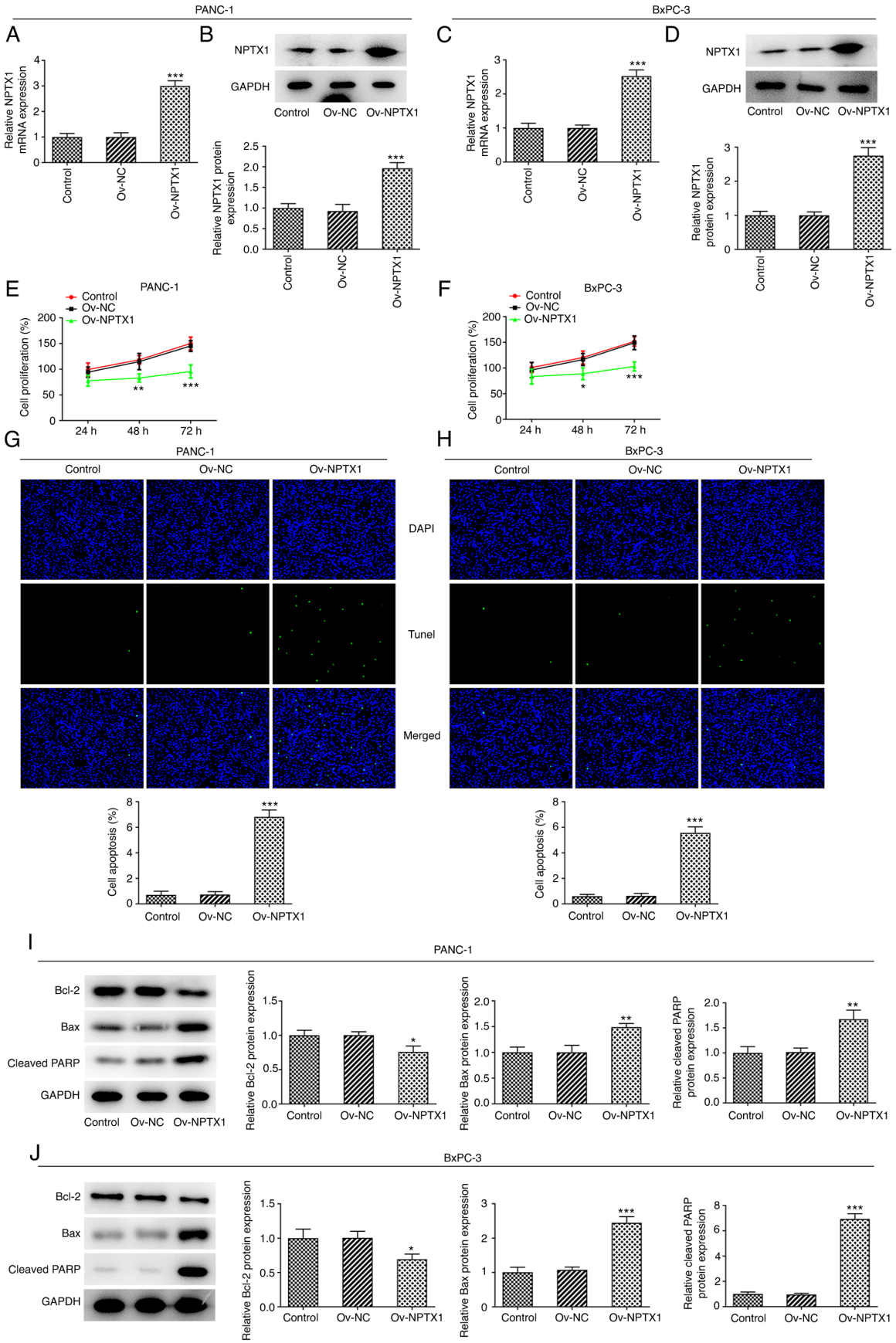

Figure 2. NPTX1 overexpression inhibits proliferation and promotes apoptosis in pancreatic cancer cells. The relative (A) mRNA expression and (B) protein level of NPTX1 in PANC-1 cells were detected using RT-qPCR and western blotting. The relative (C) mRNA expression and (D) protein level of NPTX1 in BxPC-3 cells were detected using RT-qPCR and western blotting. The cell viability of (E) PANC-1 and (F) BxPC-3 cells was detected using CCK-8. (G and H) The apoptosis level was detected using TUNEL. Original magnification, x200. (I and J) The expressions of Bcl2, Bax and cleaved PARP were measured using western blotting. Results are the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. Ov-NC. NPTX1, neuronal pentraxin 1; RT-qPCR, reverse transcription-quantitative PCR; PARP, poly ADP-ribose polymerase; Ov, overexpression plasmid; NC, negative control.

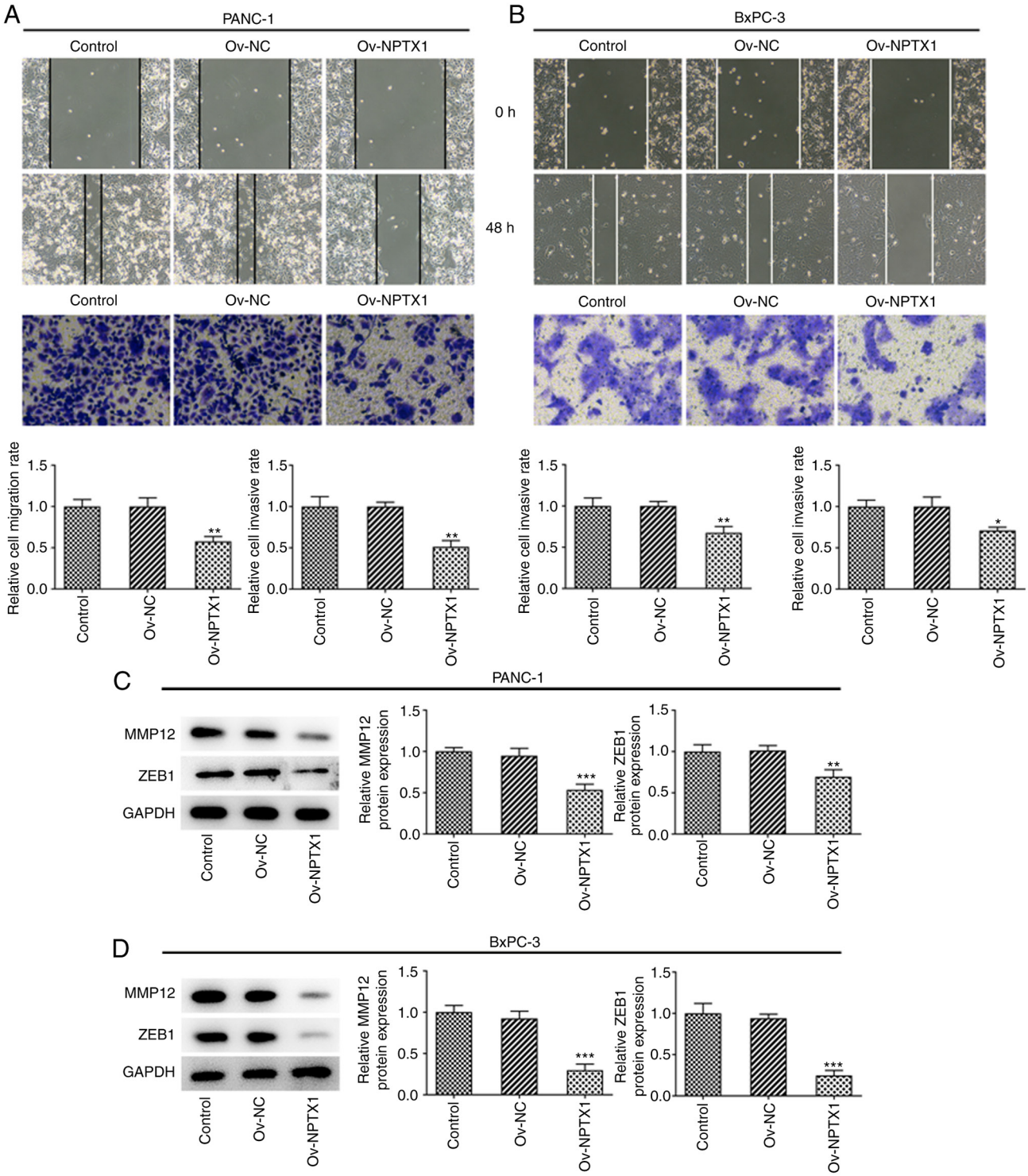

Figure 3. NPTX1 overexpression inhibits migration and invasiveness in pancreatic cancer cells. (A) The migration and invasiveness of PANC-1 cells was detected using wound healing and Transwell. Original magnification, x100. (B) The migration and invasiveness of BxPC-3 cells was detected using wound healing and Transwell. Original magnification, x100. (C) The expressions of MMP12 and ZEB1 in PANC-1 cells were measured by western blot. (D) The expressions of MMP12 and ZEB1 in BxPC-3 cells were measured by western blotting. Results are the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. Ov-NC. NPTX1, neuronal pentraxin 1; ZEB1, zinc finger E-box-binding homeobox 1; Ov, overexpression plasmid; NC, negative control.

overexpression exerted the same effect on the sensitivity of PC cells to DDP (Fig. 4C and D).

RBM10 overexpression stabilizes the mRNA and protein expression levels of NPTX1. Bioinformatics analysis using the StarBase (http://starbase.sysu.edu.cn/) and RNA-Protein Interaction Prediction (RPISeq; http://pridb.gdcb.iastate.edu/RPISeq) databases predicted that RBP could interact with NPTX1. In addition, the mRNA expression and protein level of RBM10 in PC cells were significantly downregulated compared with normal pancreatic ductal epithelial cell line HPDE6-C7 cells (Fig. 5A and B). RT-qPCR and western blot

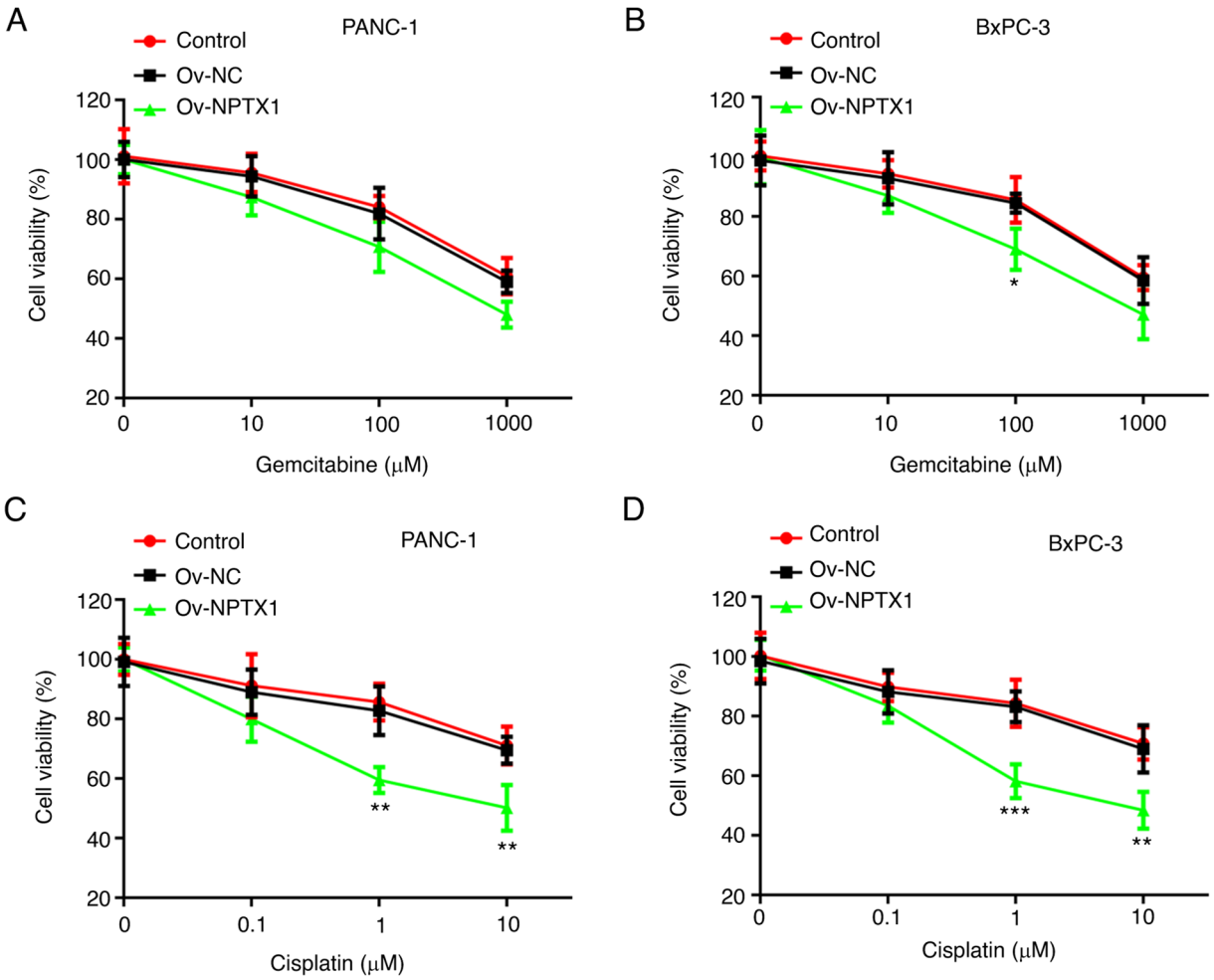

Figure 4. NPTX1 overexpression enhances the sensibility of gemcitabine and cisplatin. The cell viability of (A) PANC-1 and (B) BxPC-3 cells was detected using CCK-8 after the treatment of gemcitabine. The cell viability of (C) PANC-1 and (D) BxPC-3 cells was detected using CCK-8 after the treatment of cisplatin. Results are the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. Ov-NC. NPTX1, neuronal pentraxin 1; Ov, overexpression plasmid; NC, negative control.

analyses demonstrated that the expression of RBM10 was markedly increased in PANC-1 and BxPC-3 cells following cell transfection with ov-RBM10 (Fig. 5C-F). As shown in Fig. 5G-J, NPTX1 was significantly upregulated in PANC-1 and BxPC-3 cells overexpressing RBM10. Furthermore, RIP assays showed that RBM10 could bind with NPTX1 mRNA (Fig. 5K). To further verify that the expression of RBM10 could stabilize the expression of NPTX1, a mRNA stability assay was performed using actinomycin D. As shown in Fig. 5L, RBM10 overexpression enhanced the expression of NPTX1 in PANC-1 and BxPC-3 cells.

Discussion

PC, an aggressive type of cancer of the digestive system, has become a severe health problem globally (25). The lack of diagnostic and prognostic biomarkers allowing the early diagnosis and prognosis of patients with PC have contributed to the poor survival rate in these patients (26). Therefore, PC screening and treatment have come to represent a major challenge in the clinical setting (27). The present study demonstrated that NPTX1 was downregulated in PC cell lines. NPTX1 overexpression suppressed the cell proliferation of PANC-1 and BxPC-3 cells by CCK-8 assay and promoted cell apoptosis by Tunel assay and the detection of levels of Bcl2, Bax and cleaved PARP. In addition, NPTX1 overexpression also was found to inhibit the invasion and migration of PANC-1 and BxPC-3 cells with decreased levels of MMP12 and zinc finger E-box-binding homeobox 1. Moreover, upregulated NPTX1 enhanced the sensitivity of 0-1 μM of GEM and DDP in PANC-1 and BxPC-3 cells. Mechanistic investigations showed that NPTX1 was combined with RBM10 and overexpression of RBM10 increased the stability of the mRNA and protein levels of NPTX1.

It has been reported that NPTX1, which was first identified in the central nervous system, serves a key role in regulating neural lineage specification (28). In addition, it has been suggested that NPTX1 is involved in cancer progression. For example, a previous study demonstrated that NPTX1 silencing promoted cell proliferation, migration and EMT process in head and neck squamous cell (29). In addition, Zhao *et al* (22) found that NPTX1 suppresses the growth ability of HCC cells and contributes to mitochondria-related apoptosis by an AKT-mediated signaling mechanism.

As a pyrimidine nucleoside analog and anticancer drug, GEM shows high efficacy against several types of solid tumors (30). DDP is considered as one of the most effective

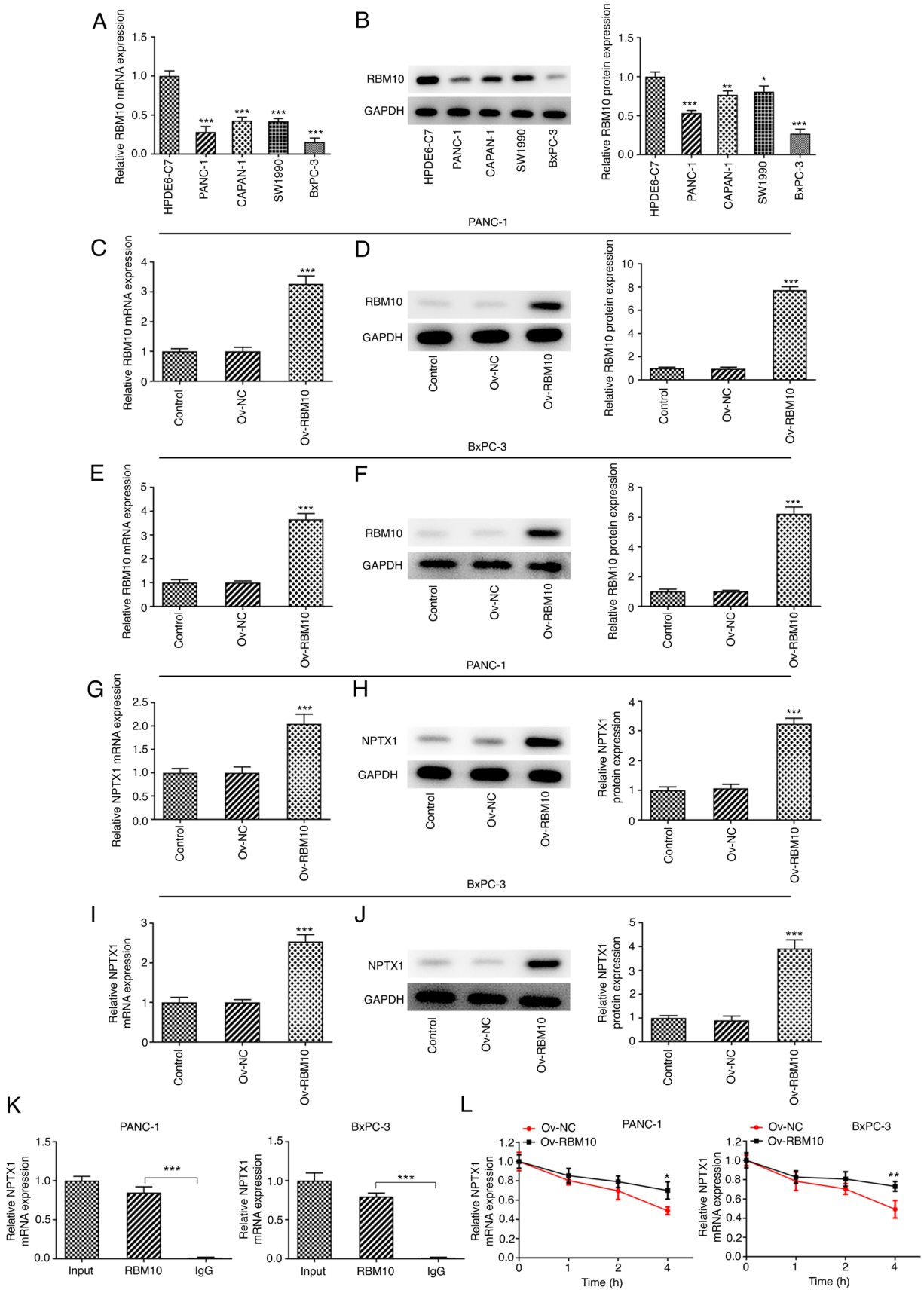

Figure 5. RBM10 overexpression stabilizes the mRNA expression and protein expression of NPTX1. The mRNA expression and protein expression of NPTX1 in pancreatic cancer cells were detected using (A) RT-qPCR and (B) western blotting, respectively. The mRNA expression and protein expression of RBM10 in (C and D) PANC-1 and (E and F) BxPC-3 cells were detected using RT-qPCR and western blotting, respectively. The mRNA expression and protein expression of NPTX1 in (G and H) PANC-1 and (I and J) BxPC-3 cells were detected using RT-qPCR and western blotting, respectively. (K) The binding of RBM10 and NPTX1 mRNA was verified by RNA binding protein immunoprecipitation assay. (L) The mRNA expression of NPTX1 in PANC-1 and BxPC-3 cells was detected using RT-qPCR. Results are the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. HPDE6-C7 or Ov-NC. NPTX1, neuronal pentraxin 1; RT-qPCR, reverse transcription-quantitative PCR; Ov, overexpression plasmid; NC, negative control

anticancer drugs, owing to its ability to activate or silence different genes to activate the cellular self-defense system (31). In addition, GEM and DDP are used to treat several types of cancer, such as lung, ovarian, bladder and breast cancer (32-35). The results of the present study demonstrated that treatment of PANC-1 and BxPC-3 cells with GEM or DDP could decrease cell viability in a dose-dependent manner.

In the present study, the role of NPTX1 in PC was investigated. The results demonstrated that NPTX1 was downregulated in PC cells, while NPTX1 overexpression attenuated the proliferation, migration, invasion and expression of apoptosis-related proteins in these cells. Notably, NPTX1 overexpression could promote cell apoptosis and enhance the sensitivity of PC cells to GEM and DDP.

RBM10 is involved in the repair of damaged tissue as well as in different cellular processes (36,37). Loiselle and Sutherland (37) and Rodor *et al* (38) revealed that RBM10 is involved in cell proliferation and tissue infiltration, thus accelerating the progression of different diseases. Through the StarBase database, it was predicted that RBM10 RBP can be combined with NPTX1. The RNA-Protein Interaction Prediction (RPISeq) database (http://pridb.gdcb.iastate.edu/RPISeq) also predicted that the interaction probability of RBM10 with NPTX1 was 0.85 (>0.5 means combined). The results of the present study showed that RBM10 could interact with NPTX1. Furthermore, RBM10 was downregulated in PC cells, while its overexpression notably upregulated NPTX1, thus suggesting that RBM10 overexpression could regulate the expression of NPTX1. This finding was further verified by mRNA stability assays using actinomycin D. However, there were certain limitations to the present study. For example, the effect of GEM and DDP co-administration on the aforementioned processes was not investigated. Therefore, further studies will be conducted to fully uncover the role of combination use of GEM and DDP in PC. In addition, it has been recently reported that interferon signaling pathways are involved in the occurrence of PC and chronic pancreatitis (39,40). It is therefore hypothesized that interferon signaling pathways are important for the occurrence of PC and chronic pancreatitis and the relationship between NPTX1 and the interferon system in PC will be explored in a study.

Overall, the present study demonstrated that RBM10 could interact with NPTX1, while NPTX1 overexpression could enhance the proliferation, migration, invasion and apoptosis of PC cells via targeting RBM10. Additionally, NPTX1 overexpression could enhance the sensitivity of PC cells to GEM and DDP chemotherapy, which provides a novel biological marker for GEM and DDP-resistant PC patients.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JW and GL designed the study, drafted and revised the manuscript. KA and LS analyzed the data and searched the literature. JW, GL and KA performed the experiments. JW and GL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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