

# hsa-miR-141 downregulates TM4SF1 to inhibit pancreatic cancer cell invasion and migration

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**Abstract.** Expression of the transmembrane-4-L-six-family-1 (TM4SF1) is high in human pancreatic cancer cells, but the underlying mechanism remains unclear. In this study, we aimed to identify and characterize microRNAs that regulate TM4SF1 expression in PC cells. Western blot analysis and quantitative polymerase chain reaction were used to detect TM4SF1 and hsa-miR-141 levels in four PC cell lines. SW1990 and BxPc-3 cells were transfected with the inhibitor miR-141, the inhibitor negative control, the miR-141 mimic and the mimic negative control; and cell invasion, migration, proliferation, cell cycle progression and apoptosis were detected by Transwell, MTT and flow cytometry assays, respectively. The miR-141 levels negatively correlated with the TM4SF1 protein levels in PC cells. The TM4SF1 protein levels were lower in the 141M group but higher in the 141I group, although the TM4SF1 mRNA levels had no significant changes, compared to the negative controls. Luciferase assays demonstrated that hsa-miR-141 directly targeted the 3'-untranslated region of the TM4SF1 gene. In addition, miR-141 downregulated TM4SF1 expression to inhibit invasion and migration of PC cells but had no effects on cell proliferation, cell cycle progression or apoptosis. TM4SF1 is a direct target of miR-141. Our findings that TM4SF1 expression was inhibited by miR-141 provide new insights into the oncogenic mechanism of TM4SF1 and suggest that miR-141 represents a novel molecular target for PC therapy.

## Introduction

Pancreatic cancer (PC) is one of the most lethal malignant diseases with a poor prognosis. PC is the fourth leading cause of cancer-related deaths in Western countries and has the lowest patient survival rate among all solid cancers. It has been estimated that 43,920 people were newly diagnosed with PC in 2012 (1). Although great efforts have been made in PC treatment using surgery, radiation therapy and chemotherapy, the 5-year survival rate of PC patients is still disappointing. Therefore, it is important to explore the molecular mechanisms that regulate PC development in order to develop effective therapies for PC.

Transmembrane-4-L-six-family-1 (TM4SF1), a small 22 kDa four-transmembrane-domain protein also known as L6-Ag, is a surface protein highly expressed in human lung, breast, colon, ovarian, renal and prostate carcinomas, and it is weakly expressed in normal vascular endothelium (2). Due to its unique expression pattern, TM4SF1 has attracted much attention as a therapeutic target for monoclonal antibody-based cancer therapy (2). TM4SF1 belongs to a distinct family that includes five other structurally similar proteins: TM4SF4/IL-TMP, TM4SF5/L6H, TM4SF18/L6D, TM4SF19/OCTM4 and TM4SF20/TCCE518 (3). TM4SF1 has been shown to be associated with the growth, motility, invasion and metastasis of tumor cells (4-7). In particular, recent studies have shown that TM4SF1 is highly expressed in PC tissues and cell lines and that downregulation of TM4SF1 can decrease migration, invasion and chemoresistance of PC cells *in vitro*; increase the effectiveness of gemcitabine treatment; and inhibit tumor angiogenesis and metastasis in orthotopic tumor models *in vivo* (8). However, the molecular mechanisms that regulate TM4SF1 expression and function in PC remain unclear.

MicroRNAs (miRNAs) are a new class of endogenous, non-coding and short (19-24 nucleotides) single-stranded RNAs. miRNAs regulate gene expression by binding to the 3'-untranslated region (UTR) of the target gene leading to either downregulation of the mRNA transcript or inhibition of the protein translation process (9). miRNAs can regulate many cellular processes, such as apoptosis, cell cycle progression,

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proliferation, differentiation, invasion and migration, and either promote or inhibit tumorigenesis, depending on the genes they target and their differential expression in normal and cancer tissues (10-17).

hsa-miR-141, a member of the miR-200 family, is over-expressed in ovarian cancer, colorectal cancer, thyroid papillary carcinoma, pancreatic ductal adenocarcinoma, nasopharyngeal carcinoma, prostate tumor, cholangiocarcinoma and endometrial carcinoma, but it is downregulated in gastric cancer, renal cell carcinoma and breast cancer (18-28). These results lead to a controversial issue regarding the function of hsa-miR-141 in cancer progression.

In this study, we first investigated the correlation between the expression level of TM4SF1 and miR-141 in PC cells. Next, we demonstrated whether TM4SF1 is a direct target of miR-141. In addition, we determined whether the miR-141 mimic could affect invasion, migration, cell proliferation, cell cycle progression or apoptosis of PC cells.

## Materials and methods

**Cell culture.** The human PC cell lines SW1990, PANC-1, BxPc-3 and CFPAC-1 cells were obtained from the Shanghai Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Wisent, St. Bruno, QC, Canada) supplemented with 10% fetal bovine serum (FBS, Wisent), 2 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin in a humidified chamber at 37°C with 5% CO<sub>2</sub>.

**miRNAs transfection.** Inhibitor miR-141 (141I), the inhibitor negative control (141I-NC), the miR-141 mimic (141M) and the mimic negative control (141M-NC) were designed and synthesized by GenePharma (Shanghai, China). SW1990 and BxPc-3 cells were seeded in 6-well plates at a density of 50%, 24 h later the cells were transfected with miRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

**Quantitative real-time polymerase chain reaction (qRT-PCR).** Total RNA was extracted from SW1990 and BxPc-3 cells by using TRIzol (Invitrogen). Primescript RT Reagent (Takara, Dalian, China) was used to synthesize cDNA. qRT-PCR was performed using SYBR-Green (Takara) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers were as follows: TM4SF1 forward, 5'-ACCACTATG TCTTGATTCCCTC-3'; and reverse, 5'-ATTGTGGCTCTG TCCTGGGT-3'; GAPDH forward, 5'-TCACCCACACTGTG CCCATCTACGA-3'; and reverse, 5'-CAGCGGAACCGC TCATTGCCAATGG-3'; hsa-miR-141 forward, 5'-CGCTAA CACTGTCTGGTAAAG-3'; and reverse, 5'-GTGCAGGT CCGAGGT-3'; U6 forward, 5'-ATTGGAACGATACAGAGA AGATT-3'; and reverse, 5'-GGAACGCTCACGAATTTG-3'. The conditions were as follows: 95°C for 3 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; and 72°C for 5 min. GAPDH mRNA and U6 were used as internal controls for determining the relative expression level of TM4SF1 mRNA and hsa-miR-141, respectively. The comparative ΔΔCt method was used to calculate relative expression levels of mRNAs and miRNAs, and the fold changes were analyzed by 2<sup>-ΔΔCt</sup>.

**Western blot analysis.** Total protein was extracted from SW1990 and BxPc-3 cells using RIPA buffer supplemented with 1% phenylmethylsulfonyl fluoride (PMSF), and the protein concentration was estimated using a BCA kit (Keygen, Nanjing, China). Protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in Tris-buffered saline (TBS) with 5% non-fat dry milk at 4°C for 15 h then incubated with primary antibodies against TM4SF1 (Abcam, Cambridge, MA, USA) or GAPDH (Beyotime, Jiangsu, China) at 4°C overnight. Membranes were incubated with anti-rabbit (or mouse) IgG-horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature, washed three times, developed with an electrochemiluminescence kit (Pierce, Rockford, IL, USA), and exposed to X-ray film to visualize the images. GAPDH served as a loading control.

**Luciferase reporter assay.** Four oligonucleotides corresponding to the 3'UTR of TM4SF1 were synthesized as follows: wild-type forward, 5'-CTAGATAAAGACTGGCATCTTCACAGGAT GTCAGTGTTTAAATTTAGTAGGCCGG-3'; and reverse, 5'-CCTACTAAATTTAAACACTGACATCCTGTGAAGAT GCCAGTCTTTAT-3'; and mutant-type forward, 5'-CTAGAT AAAGACTGGCATCTTCACAGGATGTTGATGCTTAAA TTTAGTAGGCCGG-3'; and reverse, 5'-CCTACTAAATTT AAGCATCAACATCCTGTGAAGATGCCAGTCTTTAT-3'. The oligonucleotides were cloned into the XbaI site of the pGL3 luciferase reporter gene (Promega, Madison, WI, USA) to generate pGL3-TM4SF1-3'UTR and pGL3-TM4SF1-3'UTR-mut vector. SW1990 and BxPc-3 cells were cultured in 24-well plates and co-transfected with 200 ng of pGL3-TM4SF1 or pGL3-TM4SF1-mut and 20 ng of pRL-SV40 (Promega) containing *Renilla* luciferase and 20 pmol of 141M or 141NC. At 48 h after transfection, cells were collected and luciferase activity was measured by using a Dual-Luciferase Reporter assay kit (Promega) following the manufacturer's instructions. All experiments were repeated three times.

**Cell invasion and migration assay.** Invasion and migration of cells were measured by a Matrigel invasion chamber assay, using a chamber of 6.5 mm in diameter with 8-µm pore size Transwell chambers (Corning, Corning, NY, USA). SW1990 and BxPc-3 cells were seeded into the upper chamber (1.0x10<sup>5</sup> cells per Transwell) pre-coated with 1 mg/ml Matrigel for the invasion assay or without Matrigel for the migration assay, and the bottom wells were filled with 500 µl of 10% FBS-DMEM. After incubation for 24 h at 37°C, non-invading or non-migrating cells were removed with cotton swabs, and cells that had invaded or migrated to the underside of the membrane were stained with 0.1% crystal violet for 15 min at 37°C. Then, the membranes were washed with phosphate-buffered saline (PBS), and the invaded or migrated cells were counted under an inverted microscope. All experiments were carried out in triplicate.

**Cell proliferation assay.** SW1990 and BxPc-3 cells were seeded in 96-well culture plates (Costar, Cambridge, UK) at a density of 2x10<sup>3</sup> cells/well, 24 h later the cells were transfected with miRNAs, and 48 h later cell proliferation was detected by using

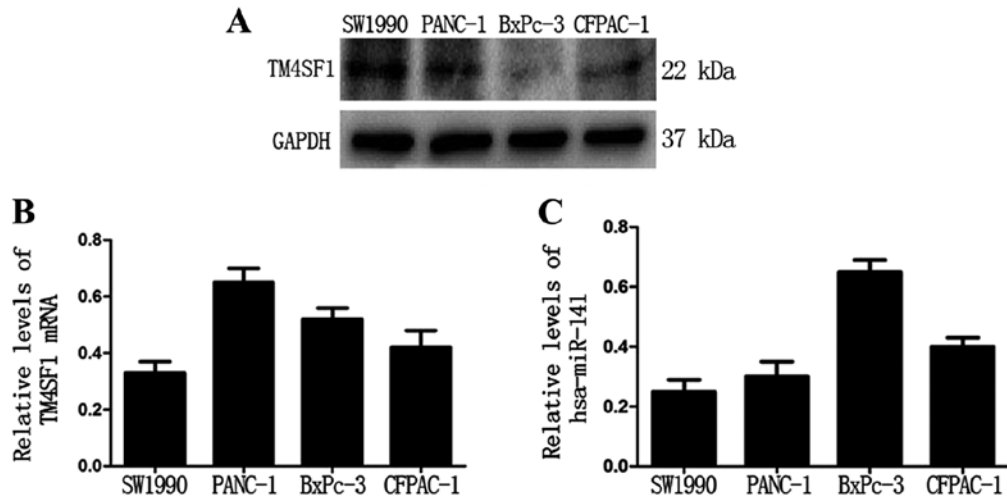


Figure 1. Relative expression levels of miR-141 and TM4SF1 in four PC cell lines. (A) Western blot analysis of the TM4SF1 protein level. Shown are representative blots from three independent experiments. GAPDH was used as a loading control. (B) The TM4SF1 mRNA level was detected by qPCR and normalized by GAPDH mRNA. (C) The miR-203 level was detected by qPCR and normalized to U6.

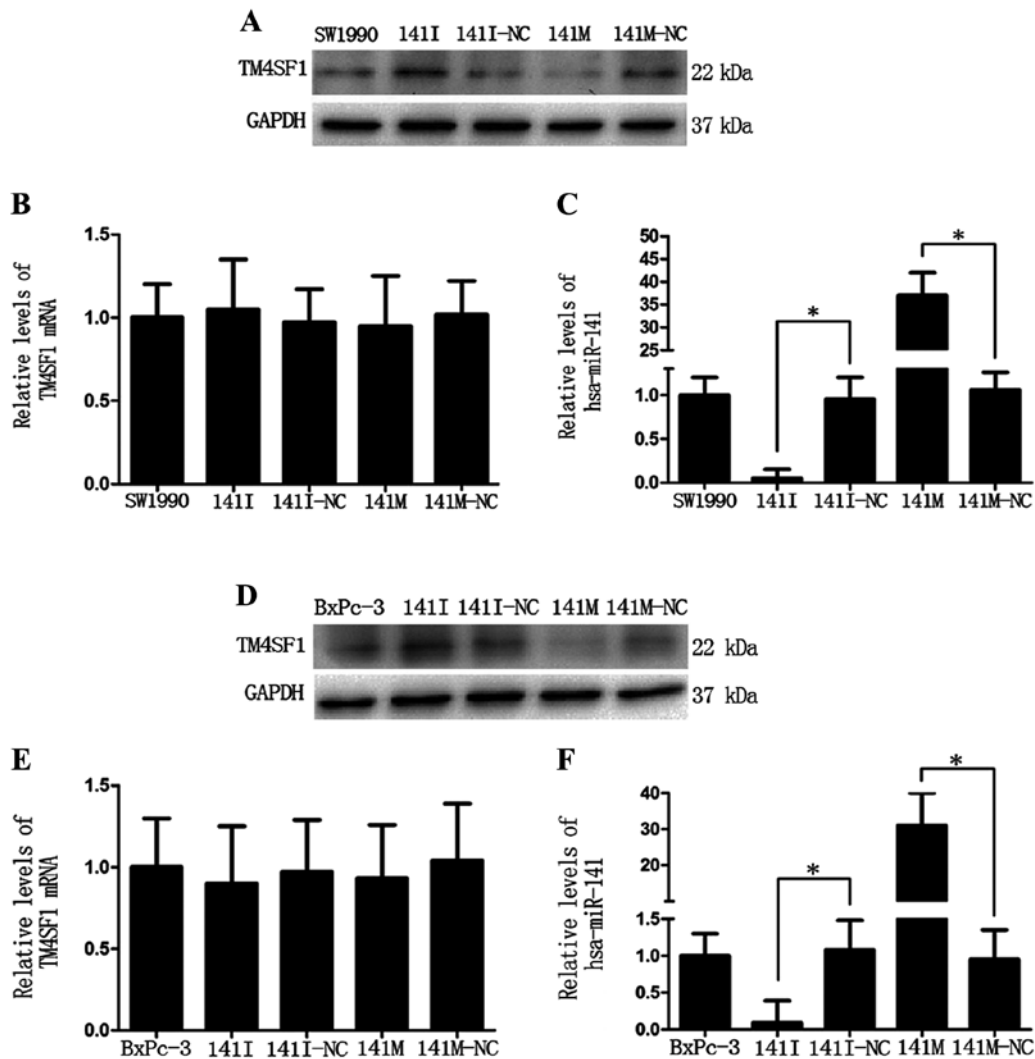


Figure 2. miR-141 inhibits TM4SF1 protein expression. Cells were transfected as indicated, 48 h later the cells were collected and subjected to western blot and qPCR analyses. (A and D) Western blot analysis of the TM4SF1 protein level. Shown are representative blots from three independent experiments. GAPDH was used as a loading control. (B and E) The TM4SF1 mRNA level was analyzed by qRT-PCR and normalized to GAPDH mRNA. (C and F) The hsa-miR-141 level was detected by qPCR and normalized to U6. \* $P < 0.05$ .

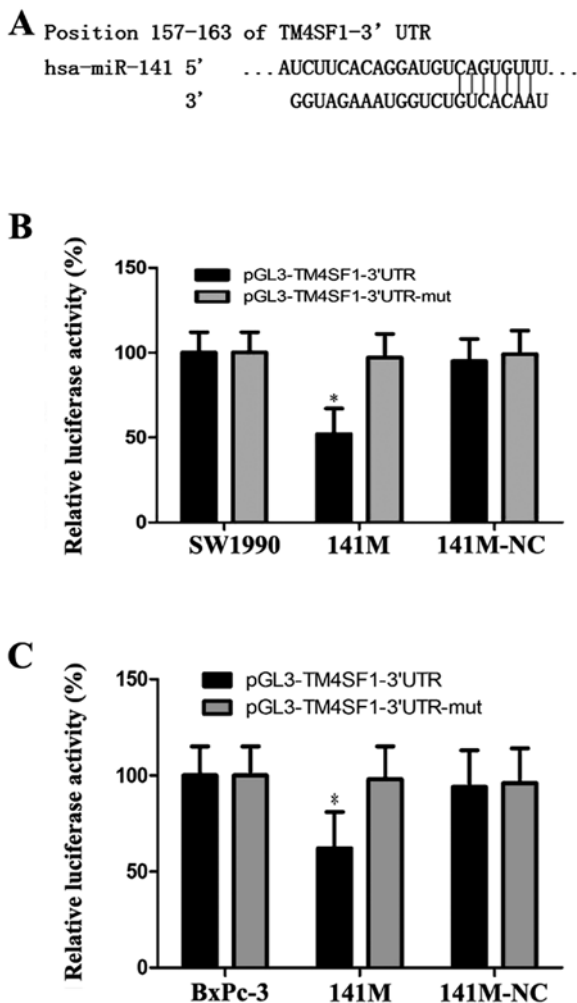


Figure 3. hsa-miR-141 directly targets TM4SF1. (A) The binding site of miR-141 in the 3'UTR region of TM4SF1 was predicted by TargetScan. (B and C) SW1990 and BxPc-3 cells transfected with the pGL3-TM4SF1 reporter vector together with 141M led to significantly reduced luciferase activity. The data were normalized by the ratio of *Renilla* luciferase activities. \* $P < 0.05$ .

an MTT kit (Keygen) following the manufacturer's instructions daily for 5 days. Briefly, 20  $\mu$ l of MTT solution (5 mg/ml) was added to each well, cells were incubated for 4 h at 37°C, then the medium in each well was replaced with 150  $\mu$ l of dimethyl sulfoxide (DMSO), and the plate was oscillated for 10 min. The optical density (OD) was detected by a microplate reader (Tecan, Shanghai, China) at 490 nm with 650 nm as a reference wavelength. Each assay was performed in triplicate and repeated independently three times.

**Flow cytometry analysis of cell cycle progression and apoptosis.** Cell cycle progression and apoptosis were assessed by flow cytometry (Becton-Dickinson, San Jose, CA, USA). SW1990 and BxPc-3 cells were grown and transfected with miRNAs. For cell cycle analysis, cells were collected and washed twice with PBS and fixed with 70% ethanol at -20°C overnight. Cells were washed twice with PBS and resuspended in 500  $\mu$ l of PBS with 0.2% Triton X-100, 10 mM EDTA, 100  $\mu$ g/ml RNase A, and 50  $\mu$ g/ml propidium iodide (PI). The samples were incubated at room temperature for 30 min. For apoptosis, cells were collected and washed twice with PBS,

suspended in 100  $\mu$ l of 1X binding buffer, and stained with 5  $\mu$ l of Annexin V fluorescein isothiocyanate (FITC) and 5  $\mu$ l of PI at room temperature for 15 min in the dark. Then, the samples were analyzed by flow cytometry (Becton-Dickinson). All experiments were carried out in triplicate.

**Statistical analysis.** Each experiment was conducted at least three times. All data were expressed as mean  $\pm$  standard deviation (SD). Differences between each group were analyzed by a Student's t-test. Statistical analysis was performed with SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA).  $P < 0.05$  was considered statistically significant.

## Results

**The TM4SF1 protein level negatively correlates with the hsa-miR-141 level in PC cell lines.** First, we examined the protein level of TM4SF1 in four PC cell lines (SW1990, PANC-1, BxPC-3 and CFPAC-1). Western blot analysis showed that the protein level of TM4SF1 was the highest in SW1990 cells and the lowest in BxPC-3 cells (Fig. 1A). However, qRT-PCR analysis showed that there was no significant relationship between TM4SF1 protein and mRNA levels (Fig. 1B). Notably, qRT-PCR analysis showed that the expression level of miR-141 was the highest in BxPc-3 cells and the lowest in SW1990 cells (Fig. 1C). These data suggest that the TM4SF1 protein level is negatively correlated with the hsa-miR-141 level in PC cells.

**TM4SF1 is a target of hsa-miR-141 in PC cells.** Next, we detected protein and mRNA expression levels of TM4SF1 in SW1990 and BxPc-3 cells transfected with miR-141 mimic (141M), miR-141 inhibitor (141I), or the corresponding negative control (141M-NC or 141I-NC). Western blot analysis showed that the TM4SF1 protein level was lower in the 141M group and higher in the 141I group (Fig. 2A and D), compared to the negative controls, respectively. In addition, the TM4SF1 protein level negatively correlated with the hsa-miR-141 level ( $P < 0.05$ , Fig. 2C and F). qRT-PCR analysis showed that there was no obvious change in the TM4SF1 mRNA level (Fig. 2B and E). These data indicate that miR-141 post-transcriptionally inhibits TM4SF1 expression.

To confirm that hsa-miR-141 directly targets the 3'UTR of the TM4SF1 gene, we used TargetScan to predict the 3'UTR of TM4SF1 and the binding site of miR-141 (Fig. 3A). Based on this program, we generated pGL3-TM4SF1 and pGL3-TM4SF1-mut vectors as the luciferase reporter and control, respectively, and transfected them into SW1990 and BxPc-3 cells, together with 141M or 141M-NC. The luciferase assay showed that luciferase activity was approximately 48% and 43% less in the 141M group compared with the control ( $P < 0.05$ , Fig. 3B and C). These results suggest that miR-141 directly targets TM4SF1 via the binding site in its 3'UTR region.

**hsa-miR-141 inhibits invasion and migration of PC cells in vitro.** Matrigel invasion and Transwell assays were used to detect the effects of hsa-miR-141 on the invasion and migration of SW1990 and BxPc-3 cells *in vitro*. As shown in Fig. 4A, B, E and F, transfection with 141I could promote invasion, while 141M inhibited invasion of SW1990 and BxPc-3 cells ( $P < 0.05$ ). Similarly, as shown in Fig. 4C, D, G and H, transfection with

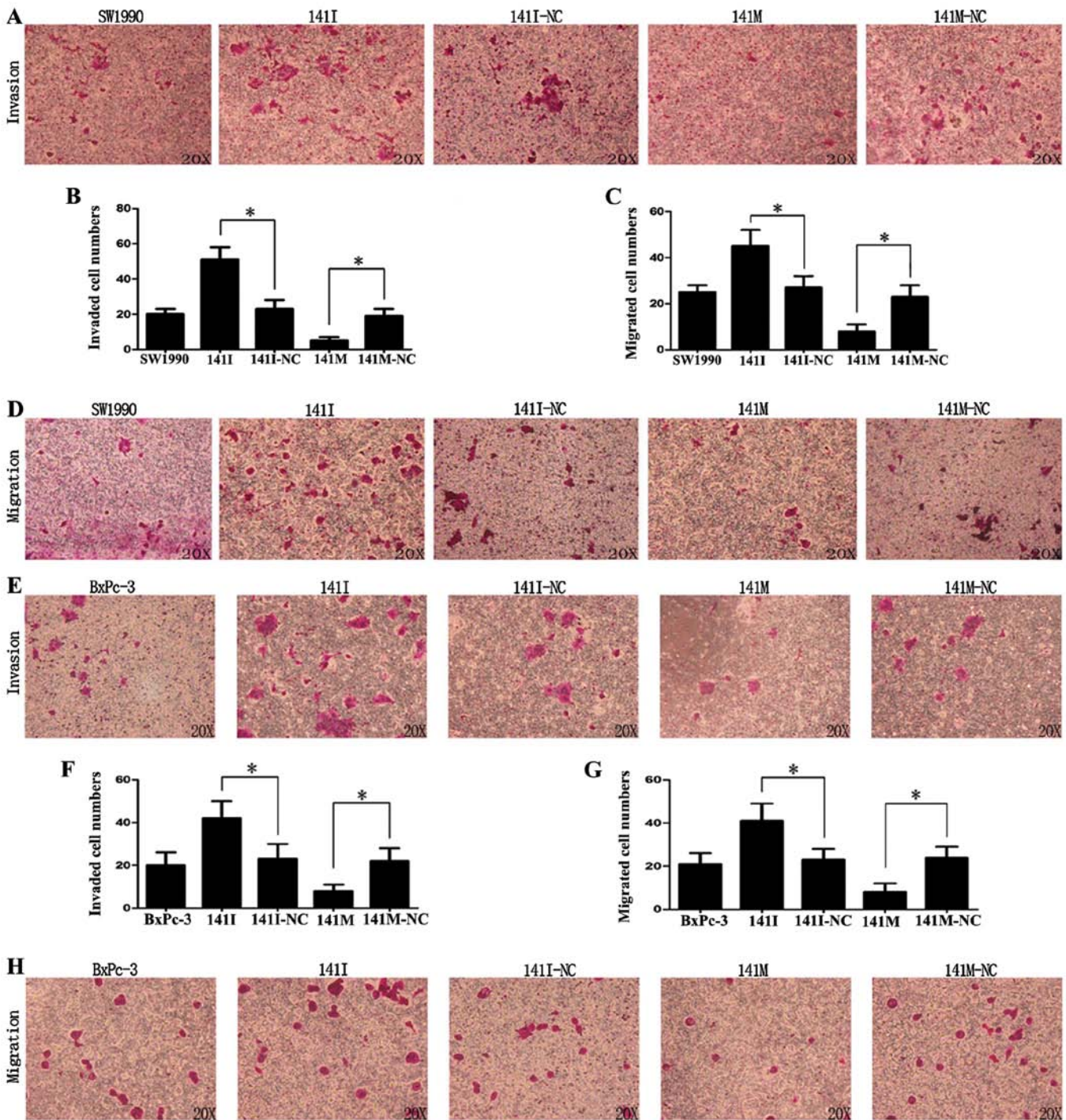


Figure 4. miR-141 inhibits the invasion and migration of SW1990 and BxPc-3 cells. (A, D, E and H) Representative photographs of invading or migrating cells. (B and F) The number of invading cells from three independent experiments ( $P<0.05$ ). (C and G) The number of migrating cells from three independent experiments ( $P<0.05$ ).

141I could promote migration, while 141M could inhibit migration of SW1990 and BxPc-3 cells ( $P<0.05$ ). These data suggest that hsa-miR-141 was able to inhibit invasion and migration of PC cells *in vitro*.

*miR-141 has no effects on proliferation, cell cycle progression, and apoptosis of PC cells in vitro.* To clarify whether hsa-miR-141 could affect cell proliferation, cell cycle progression, or apoptosis in SW1990 and BxPc-3 cells, we performed an MTT assay and found that transfection of 141M or 141I caused

no significant difference in cell proliferation in each group (Fig. 5A and F). Flow cytometry analysis of cell cycle progression and apoptosis showed that the percentage of cells in the S+G2 phase and the total apoptosis rate were not significantly different in each group (Fig. 5D, I, E and J).

## Discussion

TM4SF1 is a member of the tetraspanin superfamily and was first described as a tumor-specific antigen in many human epithe-

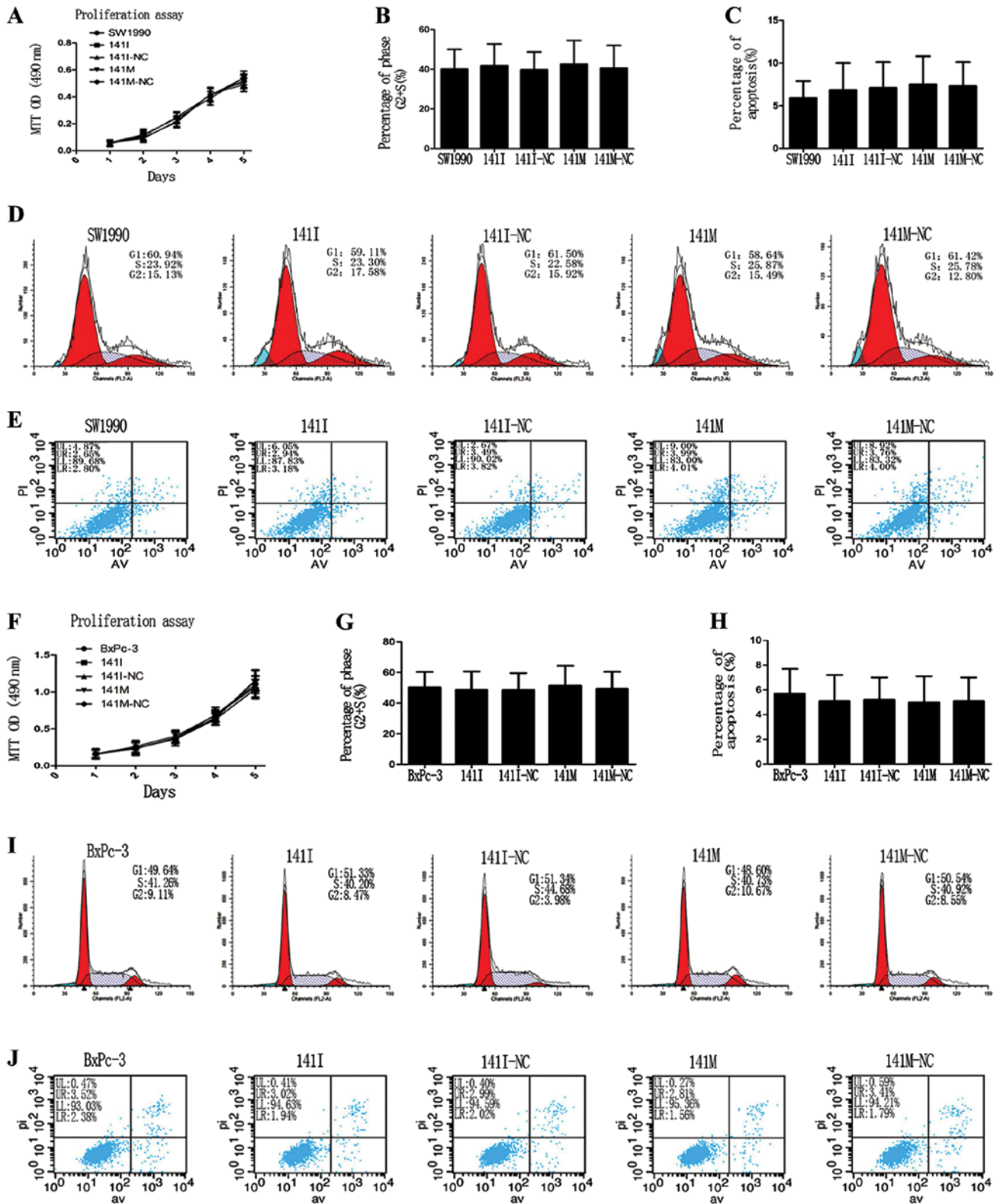


Figure 5. miR-141 has no obvious effects on proliferation, cell cycle progression, or apoptosis of SW1990 or BxPc-3 cells. SW1990 and BxPc-3 cells were transfected as indicated and then collected for the MTT assay and flow cytometry analysis. (A and F) The proliferation ability of SW1990 and BxPc-3 cells was detected by an MTT assay. (B, D, G and I) Cell cycle progression of SW1990 or BxPc-3 cells was measured by flow cytometry. (C, E, H and J) Apoptosis of SW1990 or BxPc-3 cells was monitored by flow cytometry. LR+UR is the total apoptosis rate.

lial malignancies such as lung, breast, colon, ovarian, renal and prostate carcinomas (2,4). Janes and Watt found that TM4SF1 could interact with integrin family members to form transmem-

brane complexes that affect cell adhesion, migration and tumor metastasis (29). TM4SF1 is overexpressed in the endothelium of human cancers, and it has been proposed that TM4SF1 acts as a

'molecular organizer' to facilitate the gathering of specific cell surface proteins and the formation and stability of functional signaling complexes in tumor angiogenesis (30,31).

The specific mechanism by which TM4SF1 is over-expressed in epithelial tumors remains unclear. By using TargetScan, we predicted that hsa-miR-141 could target TM4SF1 and regulate its expression. First, we detected the levels of TM4SF1 and hsa-miR-141 in four PC cell lines and found that TM4SF1 protein levels negatively correlated with hsa-miR-141 levels in different PC cell lines. Furthermore, western blot analysis showed that the TM4SF1 protein level was lower in the miR-141 mimic group and higher in the miR-141 inhibitor group, compared to the negative controls, respectively. Importantly, in these cells the TM4SF1 protein level but not the TM4SF1 mRNA level negatively correlated with the hsa-miR-141 level. These data suggest that hsa-miR-141 down-regulates TM4SF1 expression at the post-transcriptional level. Next, we performed a luciferase assay and provided evidence that TM4SF1 is a direct target gene of hsa-miR-141.

hsa-miR-141 belongs to the miR-200 family, which consists of the following members: miR-141, miR-200a, miR-200b, miR-200c and miR-429 (32). Overexpression of hsa-miR-141 has been shown to inhibit invasion and migration of breast cancer, colorectal cancer and pancreatic cancer (33-35). Consistent with these previous studies, in this study we employed Matrigel invasion and Transwell migration assays to demonstrate that the miR-141 mimic resulted in a significant decrease of cell invasion and migration, while the miR-141 inhibitor led to a significant increase of cell invasion and migration. Given the crucial role of the cell surface protein TM4SF1 in tumor invasion and metastasis (29), it is reasonable to expect that miR-141 could directly target and downregulate the expression of TM4SF1, leading to loss of oncogenic function of TM4SF1. Our findings satisfactorily explain the downregulation of miR-141 and overexpression of TM4SF1 in PC and support current opinions that TM4SF1 is an oncogene and that miR-141 is a tumor-suppressive miRNA.

Notably, the effects of hsa-miR-141 on cancer cell proliferation have been studied, but the role of hsa-miR-141 in cell proliferation of different cancers is controversial. The miR-200 family has been reported to be overexpressed in pancreatic ductal adenocarcinoma (PDAC) cells and enhance cell proliferation (21). Similar results have been reported in cholangiocarcinoma, ovarian carcinoma and choriocarcinoma (24,36,37). However, overexpression of hsa-miR-141 can significantly inhibit the proliferation of gastric cancer cells (26). In this study, we performed an MTT assay and flow cytometry analysis and found that miR-141 had no obvious effects on cell proliferation, cell cycle progression, or apoptosis in our experimental settings. SW1990 and BxPC-3 cells are derived from pancreatic adenocarcinoma, while CFPAC and PANC-1 cells originate from PDAC. The different sources of PC cells may lead to different results with regard to the role of hsa-miR-141 in the regulation of cell proliferation and apoptosis. Further studies that employ a variety of different PC cell lines and *in vivo* xenograft mouse models will help clarify the controversial results.

In conclusion, in this study we showed that the miR-141 level negatively correlated with TM4SF1 protein in PC cells. By using gain and loss of function approaches, we demonstrated

that miR-141 downregulated TM4SF1 expression to inhibit the metastatic potential of PC cells but had no effects on cell proliferation, cell cycle progression or apoptosis. Furthermore, for the first time, we identified TM4SF1 as a direct target of miR-141. Our findings that TM4SF1 expression is inhibited by miR-141 provide new insight into the oncogenic function mechanism of TM4SF1 and suggest that miR-141 represents a novel molecular target for PC therapy.

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