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ORIGINAL RESEARCH

RETRACTED ARTICLE: Exosomal Tenascin-c induces proliferation and invasion of pancreatic cancer cells by WNT signaling

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Songying Qian
Xiaodong Tan
Xinlu Liu
Peng Liu
Yunhao Wu

First Department of General Surgery, Shengjing Hospital of China Medical University, Shenyang, Liaoning, People's Republic of China

Background: Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive gastrointestinal malignancies. PDAC has an unfavorable prognosis and a 5-year survival rate of less than 6%. Early diagnosis is difficult and the disease progresses rapidly. Local invasion and distant metastases are the underlying reasons for PDAC patient death.

Materials and Methods: By exosome proteomic analysis of homologous cell lines, we identified several proteins that distinguished highly- from less-invasive pancreatic cancer cells in situ. The third most prominent protein, Tenascin-c (TNC), was chosen to assess effects on the malignant characteristics of pancreatic cancer cells.

Results: Silencing of TNC by short hairpin RNA (shRNA) in the cell lines PC-1.0 and Aspc-1 changed cellular proliferation, apoptosis, migration, and invasion. TNC expression was found to be positively related to proliferation and apoptosis, with each of these two processes reinforcing the other and regulated by the nuclear factor (NF)-κB pathway. TNC was found to promote PDAC cell line epithelial-mesenchymal transition by regulation of the Wnt/β-catenin pathway.

Conclusion: This study demonstrated exosomal TNC to be closely associated with malignant features of pancreatic cancer cells including local invasion and distant metastasis. Hence, TNC is a potential therapeutic target for the treatment of PDAC invasiveness.

Keywords: pancreatic ductal adenocarcinoma, Tenascin-c, exosome, metastasis, Wnt/β-catenin

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human tumors, characterized by rapid progressive growth and an abundant desmoplastic stroma reaction.[1] The disease is the fourth leading cause of cancer related death, with an overall 5-year survival rate of only 6%, due to rapid disease progression and a high metastatic rate. Early stage diagnosis is rare. Further, there are few treatment options and hence prognosis is poor.[2,3] Recurrence and metastasis are the main factors contributing to the poor survival rate of patients suffering from pancreatic cancer. Accumulating evidence has demonstrated a pivotal role for the tumor microenvironment in the initiation and progression of carcinogenesis.[4] Cell-cell communication, achieved by autocrine, paracrine, or direct cell to cell contact is characteristic of the tumor microenvironment. However, recent evidence suggests that cells may also communicate via other mechanisms, such as exosomes. Tumor-secreted exosomes are emerging as critical messengers in tumor progression and

Correspondence: Xiaodong Tan
First Department of General Surgery, Shengjing Hospital of China Medical University, No. 36 Sanhao Street, Heping District, Shenyang, Liaoning, People's Republic of China
Tel +860 249 6615
Email tanxdcum@163.com

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metastasis.[5] Recognition of the major modulating proteins involved in PDAC metastasis is an important step for not only identification of early metastatic disease but also may be an effective means by which to assess the effectiveness of potential therapies.

Tenascin-c (TNC) is an extracellular matrix (ECM) protein with multiple functions and multiple molecular forms due to alternative splicing and protein modification.[6] TNC is rare or absent in fully differentiated normal tissues, with the exceptions of the physiological processes of embryogenesis and neural development.[7] Numerous studies have demonstrated TNC to reappear in various tumor cell types including; breast cancer,[8] lung cancer,[9] gastrointestinal carcinomas,[10] and glioblastoma (GBM).[11] TNC functions as an ECM that promotes cancer progression. Recently, TNC was identified as an exosomal protein, which could be used to distinguish PDAC from intraductal papillary mucinous neoplasm (IPMN), and as well to differentiate primary from metastatic tumors. Zheng J et al[12] found TNC to discriminate malignant from a benign diagnosis. Hong Ji et al[4] conducted proteomic profiling of exosomes derived from human primary and metastatic colorectal cancer cells and found differential expression of key metastatic factors, including TN… Few studies have assessed the contribution of exosoma… TNC to the malignant features and progression of PDAC. Herein, by analysis of exosome proteomics of homologous cell lines, TNC was found to be highly e…vated in those cell lines that were highly invasive. B… in vi… unctional analysis, TNC silencing markedly …duced cell proliferation and accelerated apoptosis, by activ…ion of the nuclear factor (NF)-κB pathway. …rther, exoso…al TNC was found to enhance e…thelial-mesenchymal transition (EMT) of pancreatic c…cer c… lines by activation of the Wnt/β-catenin p…thway. …s stud… provides a better understandin… of sp…ific T… unctions and suggests TNC to be …seful fo… identification of PDAC metastases.

Material and methods

Cell lines and cell culture

The Golden hamster and human pancreatic cancer cell lines PC-1, PC-1.0(granted by the chairman of Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University), Capan-2, and Aspc-1(obtained from Shanghai ATCC Cell Bank) were all maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, CA, USA).The cells were supplemented with 10% fetal bovine serum (FBS) (Gibco), 50 IU/mL penicillin, and 50 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37 °C with 5% CO_2 in a humidified chamber.

Exosome isolation and labeling

Cells were cultured in RPMI 1640 supplemented with 10% exosome-depleted FBS for 48 hrs. Cell-derived exosomes were isolated using ExoQuick-TC (System Bioscience, CA, USA) according to the protocol of the manufacturer. The exosome vesicles were re-susp…in phosphate-buffered saline (PBS). The ultr…tructure o… exosomes was analyzed using a Hitachi …H-7650 Tr…smission Electron Microscope. The …pression …f the re…esentative marker proteins CD63, …D81, an… HSP7… …s detected by western blot.

Cell tran…ec…on

Short hairpin RNAs …(shRNAs) were purchased from Gene…harma (GeneChem …o., Ltd., Shanghai, China). The TN…-shRNA seq…ence in the targeted region of PC-1.0 was 5′-G…TCCGCAA…CACTGGAAGTCTACTGTGATATTC AAGA…ATATG…CAGTAGACTTCCAGTGCTTGTTTTT-…3′. The TNC-shRNA sequence of the targeted region in …pc-1 …as 5′-CCGGCCAGGAATCTTCGACGTGTTTC …TCGAGAAACACGTCGAAGATTCCTGGTTTTTTG-3′. …ells were transfected with shRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA; Thermo Fisher Scientific) in medium without antibiotics according to the manufacturer's instructions. Silencing efficiencies were verified by polymerase chain reaction (PCR).

Western blot

Exosomal proteins and intracellular proteins were extracted using radioimmunoprecipitation assay (RIPA) Lysis Buffer (Beyotime, Jiangsu, China). Similar sample amounts (30 μg) were fractionated by 8% or 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% defatted milk in TBS-T (2.42g Tris, 8 g NaCl, 1ml Tween- 20, pH 7.4) for 2 hrs at room temperature to block nonspecific binding sites. Membranes were incubated for at least 12 hrs at 4 °C with primary antibodies; anti-CD63, anti-CD81, anti-HSP70, anti-alix (each at 1:300; Santa Cruz Biotechnology, Dallas, TX, USA), anti-TNC (1:1000; Abcam, Cambridge, UK), anti-p-β-catenin

(1:300; Santa Cruz Biotechnology, Dallas, TX, USA), anti-CD44, anti-c-jun, anti-c-myc, anti-LEF1, anti-matrix metalloproteinase (MMP)7, anti-LEF1/LEF7, anti-cyclinD 1 (all at 1:1000; Wnt/β-Catenin Activated Targets Antibody Sampler Kit, Cell Signaling Technology, Danvers, MA, USA), anti-E-cadherin, anti-N-cadherin, anti-N-cadherin, anti-Vimentin, anti-Twist, anti-Snail (all at 1:500; Proteintech, Rosemont, IL, USA), anti-GAPDH, anti-β-catenin (each at 1:500; Proteintech, Rosemont, IL, USA), and then washed with TBS-T. The membranes were then incubated with secondary antibodies for 2 hrs at room temperature. The digital signals of the western blots were quantified using an Amersham Imager 600 (GE Healthcare, Little Chalfont, UK).

Transmission electron microscopy (TEM)

Isolated exosomes (2.5 μL) were dried onto freshly glow discharged 300 mesh formvar/carbon-coated TEM grids (Ted Pella, Redding California, USA), negatively stained with 2% aqueous uranyl acetate, and imaged with a Hitachi H7650 TEM (Hitachi High-Technologies Corp., Tokyo, Japan) operated at 80 kV.[13] Images were captured with a side mounted 1K AMT Advantage digital camera (Advanced Microscopy Techniques, Corp. Woburn, MA, USA).

Cell proliferation assay

Cells were uniformly distributed into 96-well plates. To each well was added 10 μL of CCK-8 reagent (Cell Counting Kit-8; Dojindo, Japan). After incubation for 4 hrs at 37 °C in 5% CO_2, the OD at 450 nm for each well was measured with a microplate reader at 24, 48, 72, and 96 hrs.

Cell apoptosis analysis

Stably transfected TNC knockdown cells (PC-1.0) and shRNA-TNC transfected cells (AsPC-1) were harvested and incubated with 1×Annexin V binding solution (Dojindo, Kumamoto, Japan). Then the cells were stained with propidium iodide (PI) and Annexin V. Cellular apoptosis was detected and analyzed with a FACS Calibur flow cytometer using CellQuestPro software (Becton Dickinson, Franklin Lakes, NJ, USA).

Wound healing assay

Cells were cultured until they reached an approximate 95% confluency in 6-well plates. A straight wound was scratched with a 100 μL pipette tip on the monolayer of cells. The cells were washed twice with serum-free RPMI 1640 medium. The cells were observed and photographed at 0, 24, and 48 hrs to identify the migration of cells. Experiments were performed in triplicate with an original magnification: ×10.

Cell invasion and migration assays

Stably transfected TNC knockdown cells (PC-1.0) and shRNA-TNC transfected cells (AsPC-1) were seeded onto filters of a 24-well Transwell chamber (Millipore, Burlington, MA, USA). For the migration assay, 20,000 cells in 200 μL of serum-free RPMI 1640 medium were loaded into the upper chambers and 600 μL of RPMI 1640 medium with 10% FBS was placed into the lower chambers. For the invasion assay, 20,000 cells were inoculated into the upper chambers using Matrigel-coated Transwell inserts (BD Biosciences, San Jose CA, USA). After 24 hrs of incubation, the Transwell plates were washed three times with 1×PBS. Cells beneath the membrane were fixed with 95% ethanol and stained with crystal violet for 20 min at room temperature. The cells passing through the membrane were counted with a microscope.

Statistical analysis

Results are presented as means ± standard error of the means (SEM) for three independent experiments. Statistical analyses were performed with SPSS 17.0 software (Abbott Laboratories, Chicago, IL) and graphics performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Comparisons between experimental and control groups were analyzed by independent *t*-test. The relationships between TNC and clinicopathological features were analyzed using the Chi-squared test. $P<0.05$ was defined as statistically significant. $P>0.05$ was considered not significant (NS).

Results

In pancreatic cancer cells, TNC functions as an exosomal protein

TNC is well known as a functional ECM protein synthesized by pancreatic stellate cells and cancer cells. As an ECM protein, TNC interacts with other ECM components, such as collagen and fibronectin, to alter biological properties that reduce cellular adhesion as well as increase motility and invasiveness. TNC also functions as a ligand that binds to cell surface receptors, such as integrins and syndecans, activating cell signaling pathways that alter cell biological behaviors.[14,15] In this study, we found TNC to be an exosomal protein involved in intercellular communication. To

determine whether TNC is an exosomal protein released by pancreatic cancer cells, four cell lines (PC-1, PC-1.0, AsPC-1, and Capan-2) were incubated in exosome-free medium supplemented with exosome-free FBS. The exosomes in the conditioned media were isolated using SBI ExoQuick-TC, harvested, resuspended in PBS, and examined by TEM for morphology. As shown in Figure 1A, the exosomes isolated from PC-1 cells were concordantly cup-shaped and their size was within 40–120 nm, which is the characteristic diameter of exosomes. As shown in Figure 1B, exosomes isolated from PC-1.0 expressed the exosome-specific markers CD63, CD81, and HSP70. These results fully confirm the vesicles isolated from the pancreatic cancer cell lines to be exosomes based on their size and marker proteins.

TNC levels are higher in highly-invasive cell lines than in less-invasive cell lines

Our previous proteomics data showed TNC levels in PC-1.0 to be multiples of PC-1.To confirm that TNC expression differs between highly- and less-invasive pancreatic cell lines, exosomes were isolated from four cell lines and levels of exosomal proteins assessed by western blot. Results demonstrated expression levels of TNC to be higher with highly-invasive than less-invasive pancreatic cancer cells (Figure 2).

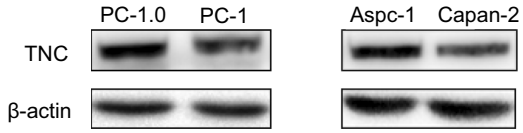

Figure 2 Differences in exosomal TNC (Tenascin-c) among PC-1, PC-1.0, Capan-2, and Aspc-1.

TNC knockdown inhibits the malignant behaviors of PC-1.0 and Aspc-1 cell lines

The expression of TNC after transfection with TNC shRNA was assessed by quantitative real-time polymerase chain reaction (RT-PCR). TNC mRNA levels were significantly decreased in the PC-1.0 and Aspc-1 cells after transfection with shRNA1, shRNA2, or shRNA3 when compared to controls, Figure 3A. CCK-8 and cell apoptosis analyses of Aspc-1 and PC-1.0 cell lines were performed 24 hrs after TNC silencing by shRNA. Results demonstrated TNC silencing to decrease proliferation of pancreatic cancer cells at 72 and 96 hrs, Figure 3B. Silencing TNC significantly affected cell apoptosis (Figure 3C) and reduced migration of PC-1.0 and Aspc-1, as judged by the wound healing assay (Figure 3D).

TNC promotes EMT through activation of the Wnt/β-catenin pathway

To verify a role for TNC in migration and invasion by PC-1.0 and Aspc-1 cells, we performed Transwell analyses with or without Matrigel. The analyses were performed using Aspc-1 cell with TNC silenced for 48 hrs or by use of a stably transfected TNC knockdown of the PC-1.0 cell line. Silencing TNC decreased pancreatic cancer cell migration and invasion (>60%), Figure 4A. Since TNC affected the migration and invasion of pancreatic cancer cell lines, TNC may promote EMT. Western blot of several EMT proteins demonstrated knockdown of TNC increased the levels of E-cadherin, but decreased levels of N-cadherin, Vimentin, Snail, and Twist (Figure 4B). It is well-known that the Wnt/β-catenin pathway is crucial to EMT modulation. Hence, western blot was used to assess whether TNC promoted EMT through this classical pathway. Results showed TNC silencing to increase phosphorylation of β-catenin and to decrease pathway components; MMP-7, C-myc, CD44, LEF-1, Met, TCF/LEF, and cyclin

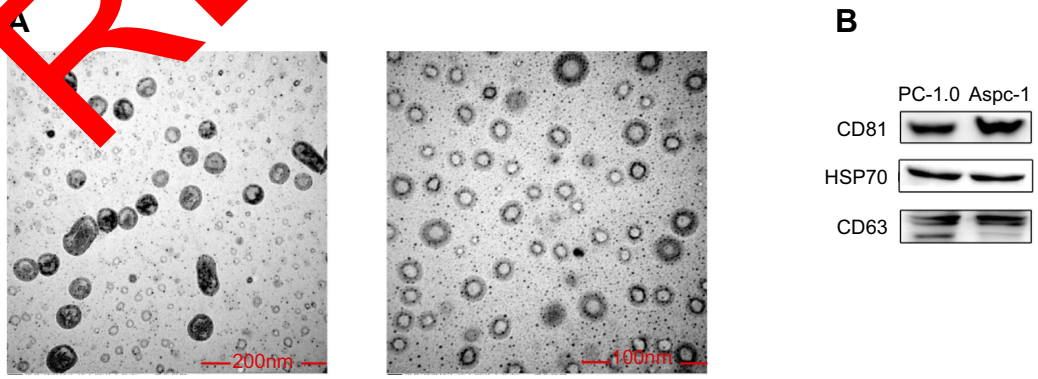

Figure 1 Characterization of exosomes. (**A**) Electron micrograph of exosomes isolated from PC-1.0 conditioned medium, revealing typical morphology and size (40–120 nm). (**B**) Western blot analysis of three markers, CD63, CD81, and HSP70, for exosomes from PC-1.0 and Aspc-1.

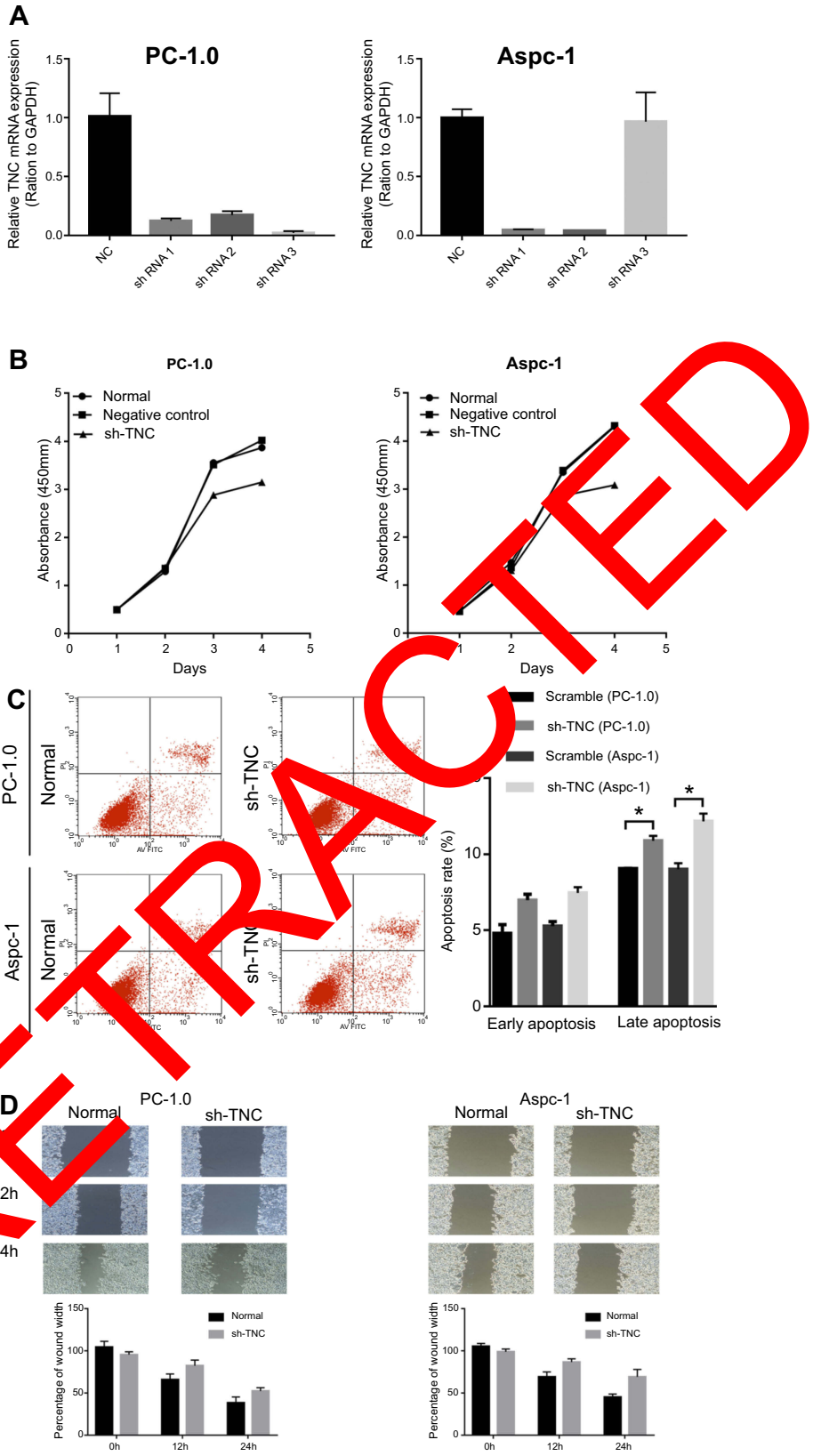

Figure 3 Silencing TNC decreased proliferation of PC-1.0 and Aspc-1 cells, and influencing cell apoptosis. (**A**) Transfection efficiency detected by quantitative RT-PCR in PC-1.0 and Aspc-1. (**B**) Silencing TNC with shRNA decreased the proliferation of PC-1.0 and Aspc-1 cells as measured by the CCK-8 proliferation assay (Invalid shRNA was used as a negative control). (**C**) Flow cytometry revealed that silencing TNC promoted late apoptosis of PC-1.0 and Aspc-1 cells. (**D**) The wound healing assay revealed that silencing TNC weakened the migration of PC-1.0 and Aspc-1 cells. Data are presented as the mean ± SEM (n=3 per group), **P*<0.05, ***P*<0.01, ****P*<0.0001, vs the NC group.

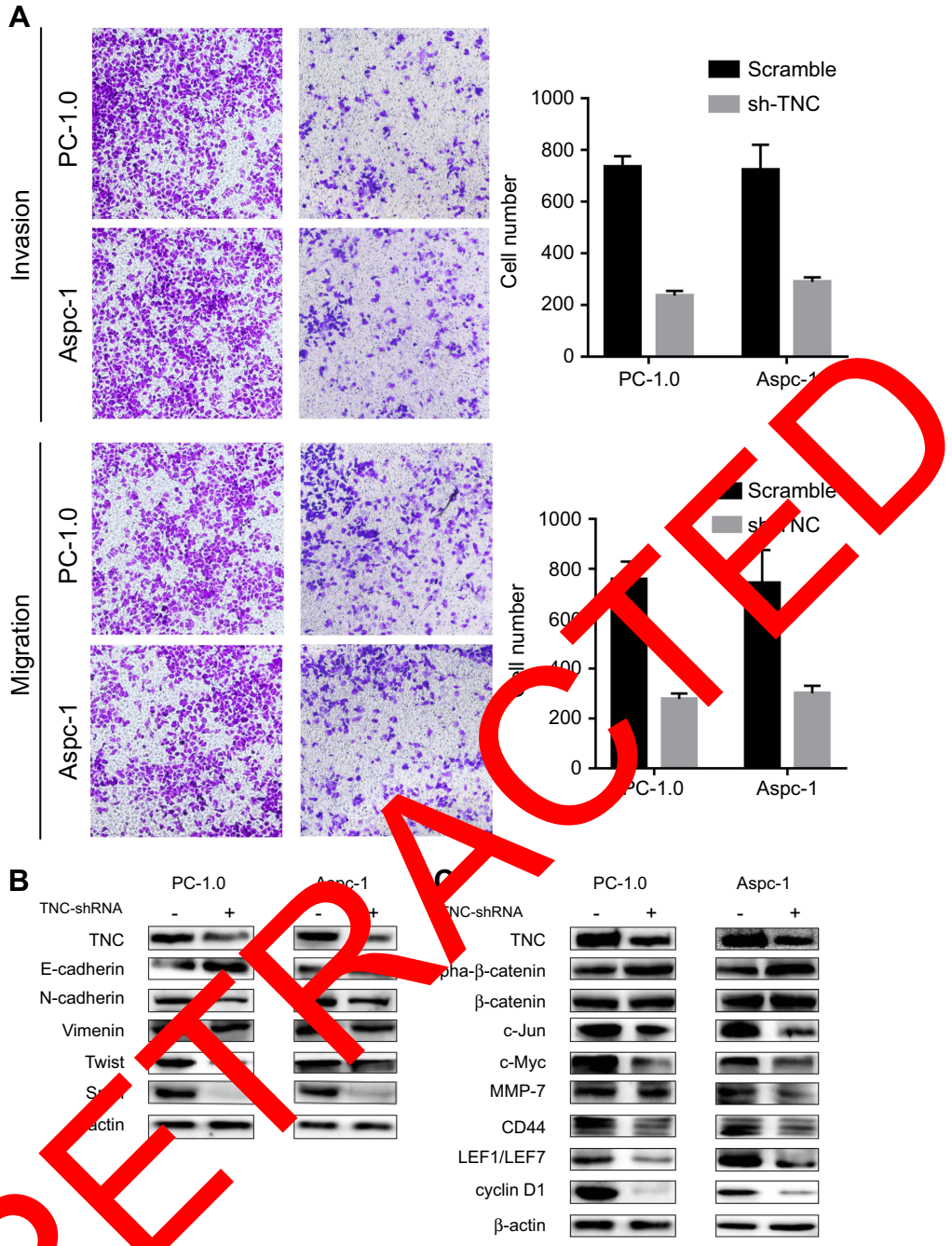

Figure 4 Silenc TNC d igration and invasion of Aspc-1 and Panc-1 cells, and inhibited cell EMT through the Wnt/β-catenin pathway. (**A**) Silencing TNC inhibited the migrat invasion of PC-1.0 and Aspc-1 cells as measured by the Transwell assay. (**B**) Silencing TNC increased the expression of E-cadherin, and decreased the expression of N-ca rin, Vimentin, Twist, and Snail in PC-1.0 and Aspc-1 cells as shown by western blot. (**C**) The interaction between TNC and the Wnt/β-catenin pathway. Cells were treat with shRNA for TNC (TNC-shRNA) or untreated (Scramble). (**$P<0.01$).

D_1 (Figure 4C). As such, the Wnt/β-catenin pathway was clearly inhibited.

TNC promotes proliferation through activation of the NF/κB pathway

Since the NF/κB pathway can activate similar targets (eg c-myc and cyclin D_1) and can function as a classical pathway involved in cell proliferation and apoptosis, we assessed TNC effects on the proliferation of pancreatic cells through the NF/κB pathway. Western blot was performed for P-NFκB p65 and total P65 after Aspc-1 was transfected with TNC shRNA. Results showed P-NFκB p6k5 levels to be lower in transfected cells than in untreated cells, with no change in P65 (Figure 5). Hence, TNC can activate

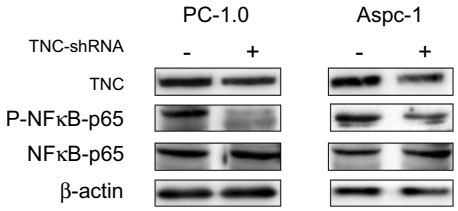

Figure 5 TNC (Tenascin-c) facilitated proliferation of PC-1.0 and Aspc-1 through the NF-κB pathway.

the NF/κB pathway and may promote proliferation of pancreatic cells through the NF/κB pathway.

Discussion

PC-1 and PC-1.0 are isologous cell lines that are respectively, less-invasive and highly-invasive. Analysis of cellular supernatant exosomes from these two cell lines may provide insight into the invasive properties of cancer cells. In this study, we selected one protein (TNC) that was expressed significantly higher in PC-1.0 than in PC-1 based on proteomic analysis (unpublished data). Differential expression was confirmed in Golden hamster and human pancreatic cancer cell lines. The influence of TNC on malignant behavior was assessed in a series of functional tests that demonstrated exosomal TNC to be not only an initiating factor for invasion and metastasis but also a key element that modulated cell proliferation and apoptosis.

TNC is a hexameric ECM protein with several functional domains that interact with other extracellular matrix proteins, exocrine proteins, and cell surface receptors. Since TNC is an exocrine protein, most investigations have assessed its role as an extracellular matrix for tumor progression. Park et al[11] demonstrated that exogenous TNC downregulated expression of the Wnt inhibitor, Dick-kopf-1 (DKK1), by blockade of actin stress fiber formation, which subsequently activated Wnt signaling and induced Wnt target genes in tumor and endothelial cells. By induction of a proangiogenic tumor microenvironment, DKK1 downregulation promoted tumor progression. In breast cancer cells, Katoh et al[16] demonstrated TNC binding to avb1 and avb6 integrins to induce EMT-like changes. Recently, TNC was reported to function as an exosomal protein in breast cancer, colorectal cancer, and glioblastoma.[10,17,18] Based on the initial work of Zheng et al[12] that demonstrated TNC in exosomes from pancreatic duct fluid, we further investigated the malignant characteristics of TNC and demonstrated differential levels in high- and low-grade malignant tumors.

Herein, TNC silencing weakened apoptosis in PC-1.0 and Aspc-1 and impacted cell proliferation. The effect on apoptosis was earlier than on proliferation. Twenty-four hours after silencing TNC with shRNA, apoptosis was markedly suppressed, while effects on proliferation emerged 72 hrs after silencing of TNC. This may due to promotion of proliferation by apoptosis which has been previously reported in solid tumors like breast cancer. Huang[19] demonstrated activated caspase 3, a key contributor to the execution of apoptosis, to play a role in growth stimulation. Moreover, prostaglandin E2, a downstream effector of caspase 3, could potentially stimulate tumor cell growth and may explain earlier apoptosis followed by proliferation with TNC silencing. However, further investigations are necessary to confirm this TNC effect on pancreatic cancer cells and inhibition of cellular apoptosis. Since NF-κB is a classical signaling pathway that regulates apoptosis, we assessed the influence of exosomal TNC on NF-κB pathway activation and subsequent resistance to apoptosis in pancreatic cancer cells. We examined the levels of IKKα, IKKβ, P-IKBα (Ser32 Ser36) and P-NFκB p65 (ser536), finding these proteins to be reduced after silencing TNC silencing. These results demonstrate the NF-κB pathway to play a role in TNC-induced apoptosis in pancreatic cancer cells.

In addition to the impact on cell proliferation and apoptosis, we also found that silencing TNC decreased the migration and invasion of both PC-1.0 and Aspc-1 cells by wound healing and Transwell assays. Exosome TNC has been shown to distinguish primary and metastatic colorectal cancer.[4] PC-1 and PC-1.0 are isologous cell lines derived from an in situ pancreatic cancer and associated hepatic metastasis, after treatment with U0126. We inferred that exosome TNC may differentiate primary from metastatic pancreatic cancer and hence explored the role of TNC in the EMT of PC-1.0 and Aspc-1 cells. EMT is a biological process characterized by the transformation of epithelial cells into mesenchymal cells. In this process, epithelial molecular marker, E-cadherin, is down-regulated and mesenchymal molecular markers N-cadherin, Vimentin, Twist, and Snail are up-regulated.[20] EMT results in loss of cell polarity, weakening of intercellular connections, and restructuring of intracellular cytoskeletal proteins. During EMT, pancreatic cancer cells exhibit malignant behaviors such as migration, invasion, and chemotherapy resistance. For adhesion between epithelial cells, the intracellular domain of E-cadherin connects to β-catenin. With α-catenin, β-catenin interacts with

cytoskeletal proteins stabilizing cell connections between epithelial cells,[21] which reveals the relevance of the Wnt/β-catenin pathway to EMT. Silencing TNC with shRNA elevated levels of phosphorylated β-catenin, which demonstrated activation of the Wnt/β-catenin pathway. Further, Wnt/β-catenin pathway targets, CD44, C-Jun, MMP-7, MET, and LEF1/LEF7 were elevated after TNC silencing. In our previous study, MMP-7 was shown to disrupt tight junctions and to increase invasion of pancreatic cancer cells by activation of the epidermal growth factor receptor (EGFR) mediated MEK–ERK signaling pathway.[22] MMPs are a group of endopeptidases which can degrade the ECM. TNC is an ECM degraded by MMPs that can bind to receptors on pancreatic cells through its specific domains. In this study, we demonstrated TNC to activate the Wnt/β-catenin pathway, which up-regulated its protein targets including MMP-7. It is possible that positive feedback increased TNC activation of the Wnt/β-catenin pathway. This would up-regulate the level of MMP-7, degrading TNC, exposing its binding domains, and accelerating the activation of the Wnt/β-catenin pathway. However, this assertion requires rigorous proof.

Conclusions

In conclusion, our work provides evidence that TNC can be secreted from pancreatic cancer cells facilitating cell to cell communication and changing cell function. Down-regulation of TNC level can reverse malignant features of pancreatic cancer cells. Exosomal TNC was found to promote pancreatic cancer cell proliferation and apoptosis by activation of the NF-κB pathway. Further, exosomal TNC promoted pancreatic cancer cell migration, invasion, and EMT by activation of the Wnt/β-catenin pathway. Based on these results, targeting of exosomal TNC may provide an alternative therapeutic approach for metastatic prevention of pancreatic cancer.

Ethics statement

The use of PC-1 and PC-1.0 cell lines are allowed by the Ethics Committee of the China Medical University.

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Disclosure

The authors report no conflicts of interest for this work.

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