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Pancreatic Stellate Cells Facilitate Perineural Invasion of Pancreatic Cancer via HGF/c-Met Pathway

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Abstract

Pancreatic cancer (PC) is a highly lethal cancer that has a strong ability for invasion and metastasis, poor prognosis, and a stubbornly high death rate due to late diagnosis and early metastasis. Therefore, a better understanding of the mechanisms of metastasis should provide novel opportunities for therapeutic purposes. As a route of metastasis in PC, perineural invasion (PNI) occurs frequently; however, the molecular mechanism of PNI is still poorly understood. In this study, we show that the hepatocyte growth factor (HGF)/c-Met pathway plays a vital role in the PNI of PC. We found that HGF promotes PC cell migration and invasion by activating the HGF/c-Met pathway, and enhances the expression of nerve growth factor (NGF) and matrix metalloproteinase-9 (MMP9) in vitro. Furthermore, HGF significantly increased PC cell invasion of the dorsal root ganglia (DRG) and promoted the outgrowth of DRG in cocultured models of PC cells and DRG. In contrast, the capacity for invasion and the phenomenon of PNI in PC cells were reduced when the HGF/c-Met pathway was blocked by siRNA. In conclusion, PSCs facilitate PC cell PNI via the HGF/c-Met pathway. Targeting the HGF/c-Met signaling pathway could be a promising therapeutic strategy for PC.

Keywords

pancreatic cancer, perineural invasion, pancreatic stellate cells, HGF/c-Met pathway

Introduction

With continuous improvements in diagnostic techniques and treatment methods, the average survival rate in patients with pancreatic cancer (PC) has increased, and the 5-year survival rate is approximately 8%[1]. However, the mortality rate of patients with PC remains the fourth major cancer. Because the pathogenesis of early PC patients lacks specific symptoms, early diagnosis is difficult. In addition, most patients had metastasis when diagnosed, were too advanced for operation, and had a poor prognosis. Neural invasion is a specific pathological characteristic of PC, and is an independent prognostic factor. A previous study found that the rate of the internal pancreatic nerve infiltration reaches up to 90%, and that external pancreatic nerve infiltration could reach 69%[2].

Perineural invasion (PNI) is the pathological process of neural invasion of cancer cells, and is currently recognized as a transfer channel for cancer cells. Neural invasion is highly correlated with in situ recurrences of PC after radical resection, and seriously affects the prognosis of PC. The high incidence of PNI of pancreatic carcinoma is closely related to the rich nerve distribution in the retroperitoneum. Cancer cells can invade the nerve bundle and disseminate along the nerve bundle to distant locations and cause severe damage[3].

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Hepatocyte growth factor (HGF), which derives mainly from fibroblasts, is known as a platelet-derived mitogen of liver cells. It can promote epithelial cell diffusion, and is currently the only known ligand of the oncogene c-MET, which is activated by HGF to induce complex biological activities[4,5]. HGF induces c-MET phosphorylation to activate downstream signaling pathways and molecules, which include STAT3, Grb2, Gab1, PI3K, and Phospholipase C-γ. Grb2 and Gab1 are key direct receptors, which recruit receptor proteins involved in multiple signaling pathways and induce the biological characteristics of cancer cells and related signal changes[6]. A previous study indicated that c-MET activation leads to increased angiogenesis, cell survival, proliferation and invasiveness, and has been correlated to poor prognosis in PDAC. HGF not only plays a vital role as a chemoattractant and neurotrophic factor, but can also induce directional axon growth and sustain spinal motor neuron and sensory neuron survival in the development of nervous system[7,8]. Many studies have shown that the signal pathway activated by the c-MET receptor is involved in a variety of neurodevelopmental processes, including cell migration and dendrite and axonal regeneration and development[9]. Our study describes the effects and the related mechanism of the HGF/c-MET pathway and the interaction among pancreatic stellate cells, PC cells, and nerves.

Materials and Methods

Cell lines, Culture Conditions and Reagents

Human PC cell lines (AsPC-1, BxPC-3, CFPAC-1, Panc-1, and SW1990) and rat Schwann cells (RSC96) were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS). All cell lines were cultured in the proper medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL ampicillin, and 100 μg/mL streptomycin under a humidified atmosphere at 37°C with 5% CO_2. Recombinant human HGF (rhHGF) was purchased from ProSpec (ProSpec-Tany TechnoGene, Rehovot, Israel). Antibodies were purchased from the following sources: anti-c-Met, anti-p-c-met, and anti-β-actin (Cell Signaling Technology, Danvers, MA, USA); anti-MMP-9 (Bioworld, Minneapolis, MN, USA); and anti-NGF, (Abcam, Cambridge, MA, USA).

Stable Transfection of the c-Met siRNA Vector and Control Vector

The c-Met siRNA1 (sense 5′-GUGCCACUAACUACAU UUATT-3′ and antisense 5′-UAAAUGUAGUUAGUGG CACTT-3′), c-Met siRNA2 (sense 5′-GUCCCGAGA-AUG-GUCAUAATT-3′ and antisense 5′-UUAUGACCAUUC-UCGGGACTT-3′), and the negative control siRNA (sense 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense 5′-ACGUGACACGUUCGGAGAATT-3′) were purchased from GenePharm (Shanghai, China). The c-Met suppressed PC cells and the control PC cells were named si-c-Met and si-Control, respectively. The effect of gene silencing was evaluated by Western blot.

Immunofluorescence

PC cell lines were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, and 3% hydrogen peroxide was added to quench the endogenous peroxidase activity. First, the specimens were permeabilized with 0.2% Triton X-100 containing 1% normal goat serum (NGS) in PBS for 20 min on ice. Bovine serum albumin (BSA) was then used to block for 30 min at 37°C. The specimens were then incubated with primary antibody overnight at 4°C. Finally, staining was detected with fluorescein-conjugated secondary antibodies (Jackson ImmunoResearch, Ely, UK). Nuclei were stained for 5 min using DAPI. The cells were imaged using the appropriate excitation wavelength with a fluorescent microscope (Nikon Eclipse Ti-s, Tokyo, Japan).

Cell Migration and Invasion Assay

The transwell chamber (pore size, 8.0 μm; Millipore, Billerica, MA, USA) with Matrigel coating was inserted into a 24-well culture plate. Approximately 5×10^4 cells suspended in Dulbecco's modified Eagle's medium (DMEM) containing 1% FBS with or without 100 ng/mL rh-HGF were seeded in the upper chamber, and 500 μL of DMEM medium containing 10% FBS was added to the lower chamber. The transwell chamber was incubated for 48 h. The cells on the bottom surface of the filter were fixed in methanol and stained with crystal violet (Boster Biological Technology Ltd., Wuhan, China). The cell number was determined by counting the stained cells under a light microscope in 10 randomly selected fields.

Western Blot Analysis

The DRG and cells were lysed using cell lysis buffer with protease inhibitors (Roche, Penzberg, Germany). Equivalent amounts of proteins were resolved on a denaturing SDS polyacrylamide gel by electrophoresis and electro-transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 h, and then probed with antibodies against c-Met, p-c-Met, NGF, MMP-9, and β-actin at 4°C overnight. After incubation with the primary antibodies, the membranes were hybridized to the appropriate goat anti-mouse or anti-rabbit secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Equal protein sample loading was monitored using an anti-β-actin antibody. The probed proteins were detected by enhanced chemiluminescence (Millipore, Billerica, MA, USA).

Real-Time PCR Assay

Total RNA was extracted using the Fastgen200 RNA isolation system (Fastgen, Shanghai, China). The Prime Script RT reagent kit (TaKaRa, Dalian, China) was used to

reverse-transcribe total RNA into cDNA. According to a previous report[10,11], real-time PCR was used to quantitatively examine the expression of c-Met at the mRNA level. The PCR primer sequences for c-Met and β-actin were as follows: β-actin, F: 5′-GACTTAGTTGCGTTACACCC TTTCT-3′ and R: 5′-GAACGGTGAAGGTGACAGCAGT-3′; c-MET, F: 5′-GACAGCTGACTTGCTGAGAGGA-3′ and R: 5′-TCTCAGAAGTGTCTTTCGGTGC-3′. The thermal profile for c-Met of RT-PCR was an RT step at 37°C for 15 min and at 85°C for 5 s, followed by the PCR step of initial denaturation at 95°C for 30 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The thermal profile for β-actin of RT-PCR was an RT step at 37°C for 15 min and at 85°C for 5 s, followed by the PCR step of initial denaturation at 95°C for 30 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The comparative C(T) method was used to quantitate the expression of each target gene using β-actin as the normalization control.

PNI Model for Assessment of Nerve-Cancer Cell Interactions

The PNI model imitated the microenvironment of the tumor and peripheral nerves as described previously[12–15]. Newborn rats were purchased from the laboratory animal center of Xi'an Jiaotong University, sacrificed with CO_2 and sterilized with 75% ethanol. DRG were dissected from the lumbar areas, stripped of meninges and neural axons, and then implanted into a drop of growth factor-depleted liquid Matrigel (BD Biosciences, Oxford, UK) with the help of a microscope. DRG were stored on ice in DMEM/F12. Next, 25 μL of Matrigel was seeded in 24-well culture plates alongside approximately 1 mm carcinoma cells under an anatomical microscope. To analyze the interaction of cancer cells and DRG, another 25 μL of Matrigel containing the si-c-met cells and DRG was seeded in 24-well culture plates. The 24-well culture plates were placed in an incubator under a humidified atmosphere at 37°C with 5% CO_2 for 30–40 min to make the Matrigel coacervate. After solidification, medium (DMEM/F12 containing HGF or not) was added carefully and replenished every 2 days. Photographic documentation of the cell suspensions on the two adjacent sides was performed with an inverted light microscope imaging system (Ti-E; Nikon Instruments Inc, Shanghai, China) and a Nikon Instruments confocal microscope. To conduct quantitative analysis on the coculture model, we defined the minimum distance between the edge of the cancer cells and the edge of DRG as parameter γ, the migration distance of the cancer cells towards the DRG as parameter α, and the DRG outgrowth length towards the cancer cells as parameter β. The invasion index = α/γ, and the DRG outgrowth index = β/γ (Fig. S1). The migration distance was measured by the image analysis software of the microscope imaging system (NIS-Elements, Nikon Instruments Inc, Shanghai, China). These studies were approved by the relevant Ethical Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, China.

Statistical Analyses

SPSS 13.0 software was used for statistical analyses. All the data are expressed as the mean ± standard deviation (SD). The significance of the data was determined using the Pearson correlation coefficient or Fisher's exact test. The differences between the groups were determined by analysis of variance (ANOVA), followed by Bonferroni's correction for multiple comparisons. $p < 0.05$ was considered significant. All experiments were repeated at least three times independently.

Results

HGF Can Increase the Expression of NGF

We first detected the expression of HGF and c-Met in PC cell lines. We found different protein and mRNA levels of c-Met in all five PC cell lines. We found that the expression of c-Met is higher in the BxPc-3 and CFPac-1 cell lines and lower in Panc-1 cell lines (Fig. 1A and B). HGF expression was rarely detected in the PC cell lines and non-active PSCs. In addition, HGF expression was detected by Western blot in active PSCs, DRG, and RSC96 cells (Fig. 1C). We chose the BxPc-3 and Panc-1 cell lines to do further research. Immunofluorescence showed that c-Met is localized at the membrane of PC cell lines (Fig. 1D). We also found that expression of NGF increased with increasing rh-HGF concentration in PC cell lines (BxPc-3 and Panc-1), and, interestingly, when the concentration of rh-HGF reached 100 ng/mL, the NGF levels in both cell lines were no longer elevated (Fig. 1E and F). We showed that HGF secreted by PSCs can stimulate PC cells to produce NGF. HGF is a potentially vital factor for PNI in PC. The effect of HGF may occur through activation of the HGF/c-Met pathway.

HGF Enhances PC Cell Migration, Invasion and Affinity to Nerves Through Activation of the HGF/c-Met Pathway

To determine the effects of HGF/c-Met signaling on cell migration, invasion, and affinity to nerves, PC cells were treated with rh-HGF (100 ng/mL). The expression of c-met, p-met, MMP9, and NGF was significantly increased in the group with rh-HGF compared with that in the vehicle group in BxPc-3 and Panc-1 cells (Fig. 2A). By immunofluorescence, we also found that NGF and c-met were upregulated in both cell lines after treatment with rh-HGF (Fig. 2B). We also found that rh-HGF could intensify the invasiveness of PC cells through transwell chambers (Fig. 2C and D). These data indicate that the activation of HGF/c-Met signaling enhances the invasive ability and affinity to nerves of PC cells through upregulating the expression of invasion-related genes (MMP-9) and NGF.

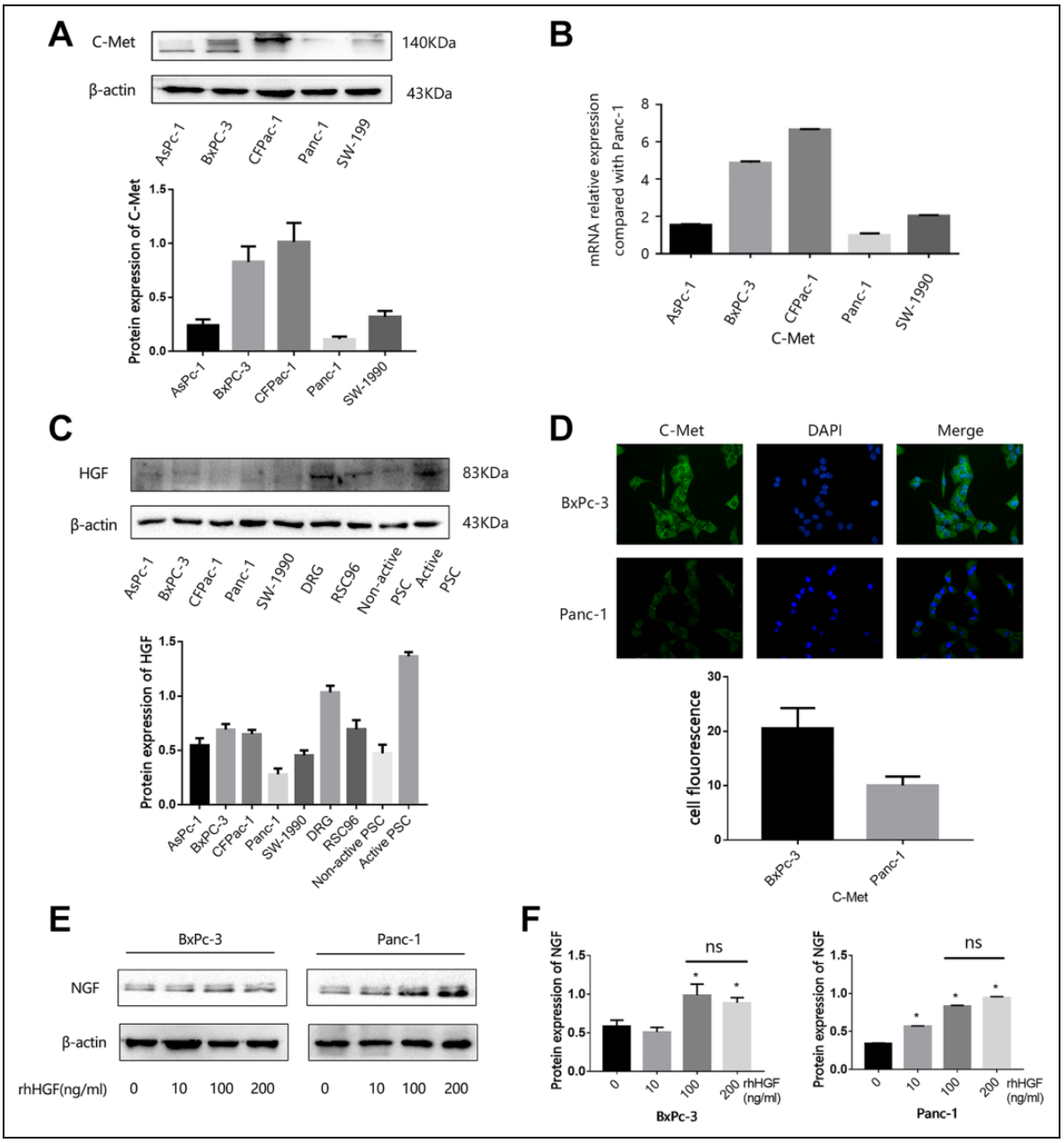

Fig. 1. Expression of HGF and c-Met in PC cell lines and pancreatic stellate cells, and HGF-increased expression of NGF. (A, B) c-Met expression was tested using Western blots and RT-PCR in five pancreatic cancer cell lines: AsPC-1, BxPC-3, CFPAC-1, Panc-1, and SW-1990 cells. (C) HGF expression was examined using Western blots in five pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPAC-1, Panc-1, and SW-1990), DRG, RSC96 and pancreatic stroma cells (non-active PSCs and active PSCs). (D) c-Met is localized at the membrane of PC cell lines. (E, F) Expression of NGF increased with increasing rh-HGF concentration in pancreatic cancer cell lines (BxPc-3 and Panc-1), and when the concentration of rh-HGF reached 100 ng/mL, the NGF levels in both cell lines were no longer elevated.

Inhibition of c-Met by siRNA Reduces Metastasis and Affinity to Nerves in PC Cells

To further verify the above results, we knocked down c-Met expression with siRNA technology in BxPc-3 cells and Panc-1 cells. We found that knockdown of c-Met reduced the expression of MMP-9 and NGF in both cell lines (Fig. 3A). By immunofluorescence, we also found that NGF is downregulated in both cell lines (Fig. 3B). At the protein level, we demonstrated that knockdown of c-Met significantly decreased the expression of MMP-9 and NGF. We also found that knockdown of c-Met could reduce the invasiveness of PC cells through transwell chambers (Fig. 3C and D). Taken together, these data indicate that the HGF/c-Met pathway might participate in the metastatic process and affinity to nerves of PC cells.

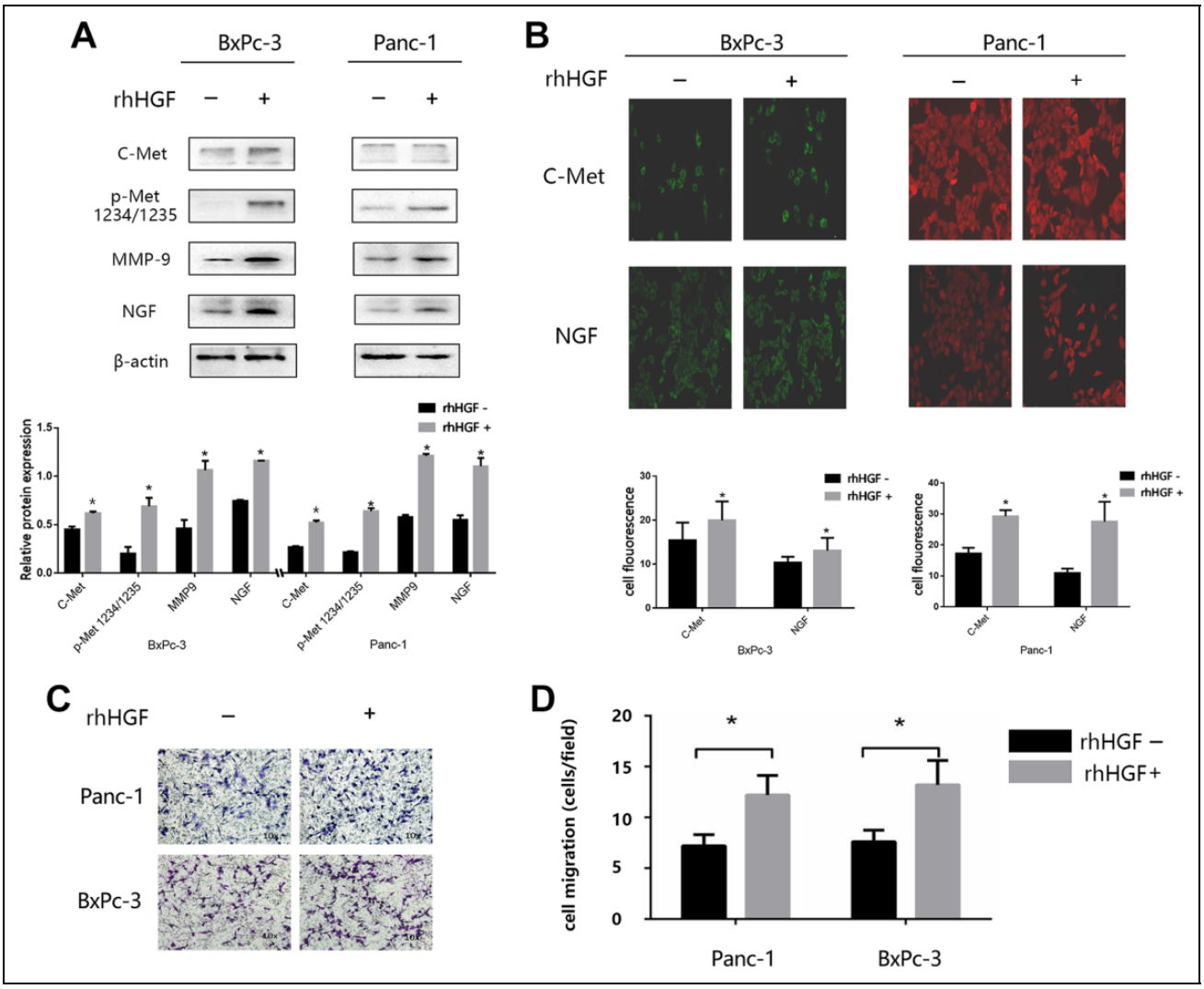

Fig. 2. HGF enhances pancreatic cancer cell migration, invasion, and affinity to nerves through activating the HGF/c-Met pathway. (A) Expression of c-Met, p-Met, MMP9, and NGF was significantly increased in the group with rh-HGF compared with that in the vehicle group in BxPc-3 cells and Panc-1 cells as analyzed by Western blotting. (B) NGF and c-Met were upregulated in both cell lines as analyzed by immunofluorescence. (C) The effect of the PC cell invasion capability was assessed using a Matrigel invasion assay. (D) Invasion capability was significantly increased in the group with rh-HGF compared with that in the vehicle group in BxPc-3 cells and Panc-1 cells.

The Capacity of PNI of PC Cells is Increased by Activation of the HGF/c-Met Pathway

We wanted to show further that reciprocity between PC cells and nerves involves the HGF/c-Met pathway, which accelerates the PNI of PC cells. An in vitro neural invasion model was created using BxPc-3 cells and Panc-1 cells with newborn rat DRG in Matrigel.

At 4 days after coculture of PC cells and DRG, we found that PC cells tend to grow towards DRG neurites. The side of the PC cells facing the DRG formed peak-like clusters and gradually moved to the DRG. Simultaneously, the side of DRG neurites facing the PC cells had directional divergence toward the cancer cell mass. This phenomenon is evident in the groups with the addition of exogenous rh-HGF, and we observed accelerated migration of the cancer cells towards to the neurites of DRG (Fig. 4A and B). The invasion index and DRG outgrowth index is also significantly higher than those in the control group (Fig. 4C and D). To further investigate the role of the HGF/c-Met pathway in the interaction between PC cells and DRG, si-c-Met cells were used in the coculture system. The results demonstrated that the migration ability and the mutual growth trend between the PC cells and neurites in the si-c-Met group were significantly suppressed compared with those in the control group (Fig. 5A–D). These data indicate that the HGF/c-Met pathway accelerates the PNI of PC cells in vitro.

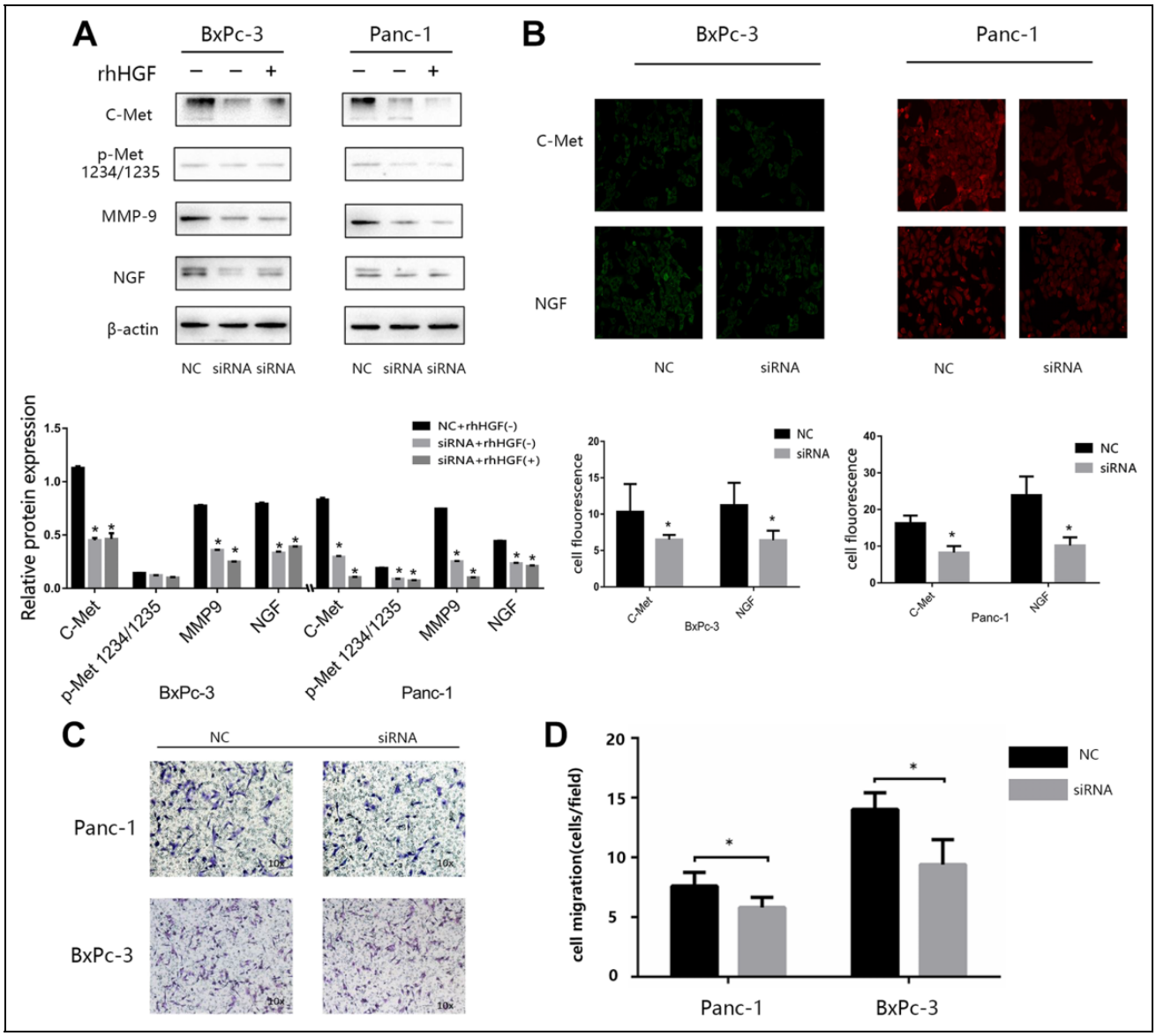

Fig. 3. Inhibiting c-Met by siRNA reduces metastasis and the affinity to nerves in pancreatic cancer cells. (A) Knockdown of c-Met reduced the expression of MMP-9 and NGF in both cell lines as analyzed by Western blotting. (B) NGF is downregulated in both c-Met knockdown cell lines when analyzed by immunofluorescence. (C) The effect of the PC cell invasion capability was assessed using a Matrigel invasion assay. (D) The invasion capability was significantly decreased in the group treated with siRNA compared with that in the group treated with vehicle in BxPc-3 cells and Panc-1 cells.

Discussion

PC has high mortality because of the invasive and metastatic phenotype of the cancer cells. Accordingly, PC patients have a poor prognosis, which is related to its local recurrence, lymph node and liver metastases, peritoneal dissemination, and PNI[16,17]. In this study, we focused on the PNI of PC, and investigated the mechanism for this type of metastasis. Although there are many studies that explore PNI progression, we still do not understand it well.

Previous studies revealed that c-Met is detectable only at low levels in the normal human exocrine pancreas, but it is upregulated in PC more than in the normal human pancreas. In addition, the c-MET-encoded HGF receptor is also overexpressed in a proportion of human PC cell lines examined[18–20]. In our study, we also found that c-MET is overexpressed in PC cells. Meanwhile, we found that HGF is highly expressed in stromal cells (PSCs, RSC96) of PC, but is not expressed in PC cells, which showed that HGF is a paracrine mediator produced by nonparenchymal and stromal cells[21]. Furthermore, our study shows HGF is more highly expressed when PSCs are activated from a quiescent state.

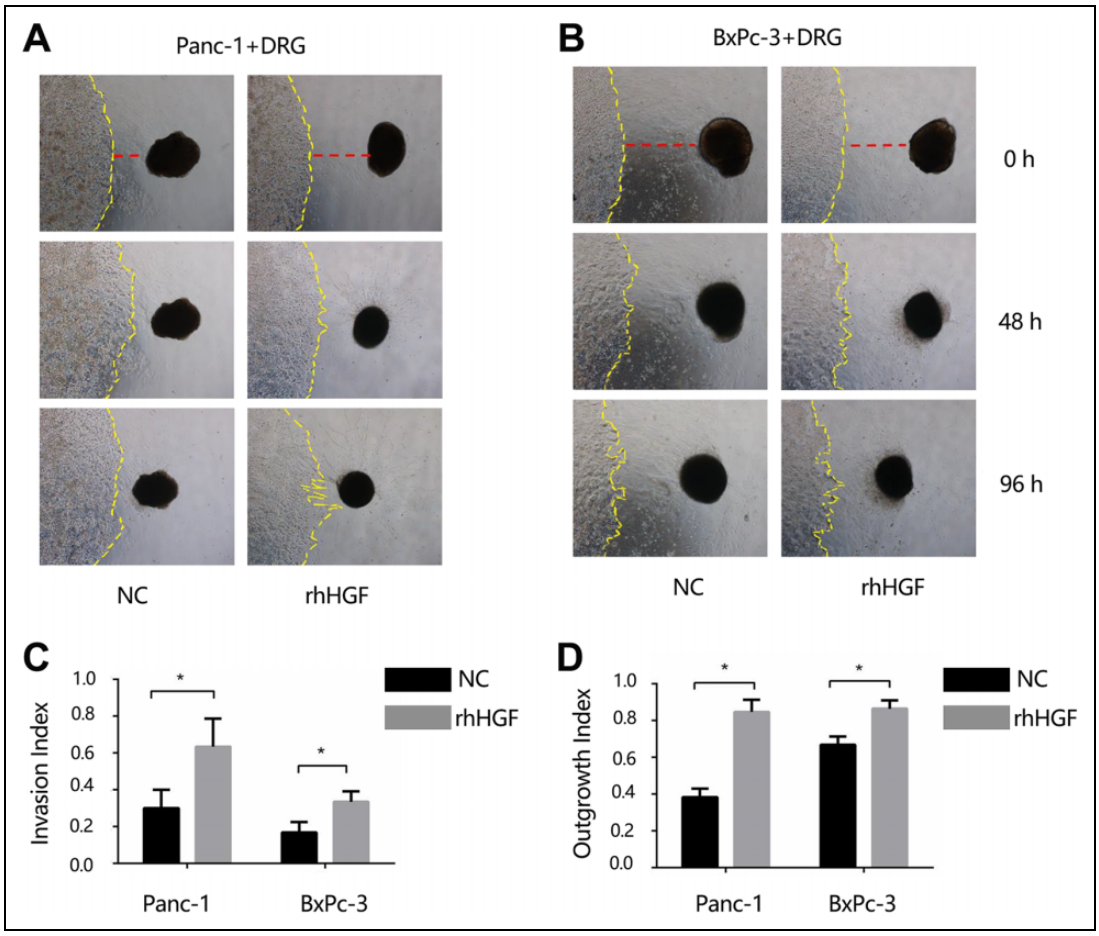

Fig. 4. rh-HGF accelerated the migration of the cancer cells towards DRG neurites. (A, B) The side of the PC cells facing the DRG formed peak-like clusters and gradually moved toward the DRG. The side of the DRG neurites facing the PC cells had directional divergence toward the cancer cell mass. (C, D) The invasion index and DRG outgrowth index, when treated with rh-HGF, are higher than those in the control group in both PC cell lines.

Many studies have researched the role in PC of the HGF/c-Met pathway, which has been shown to regulate proliferation, invasion, and metastasis of human PC[4,22–24]. Previously, autocrine IL-1a, neuromedin U, and Neuropilin-1 were shown to promote HGF secretion by stimulating stromal cells, and then to enhance the metastatic potential of PC cells[24–26]. The HGF/c-Met pathway can activate intracellular signaling cascades such as PI3K/AKT, MAPK/ERK[27,28] in PC models, leading to cancer cell invasiveness, motility, and resistance to gemcitabine therapy. We discovered the activation of the HGF/c-Met pathway by PC cell upregulation of MMP-9, which is confirmed to function in extracellular matrix (ECM) degradation, invasive growth, and angiogenesis[29].

The pivotal role of PNI in the recurrence and metastasis of PC has been gradually noticed. PNI is now considered another important PC metastatic pathway. There are two prominent theories about PNI: one is the "route of low resistance", which means PC cells can invade into local nerves through undermining the integrity of the nerve bundles, resulting in more cancer cells invading the perineurium from the damaged nerve[3]. The other is reciprocal signal interactions between cancer cells and peripheral nerves, namely, cancer cells or neural cells can promote mutual growth by secreting neurotrophic factors and related receptors or chemokines, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neural cell adhesion molecules (NCAM), glial cell line-derived neurotrophic factor (GDNF), matrix metalloproteinases (MMPs) and CXCR12[2,10,16,30–32]. L1CAM also play an important role in the PC PNI. L1CAM secreted from SCs acts as a strong chemoattractant to cancer cells, through activation of MAP kinase signaling. L1CAM also upregulated expression of metalloproteinase-2 (MMP-2) and MMP-9 by PDAC cells, through STAT3 activation[33]. Proteomic analysis also highlighted an increased expression of SCG2 and VGF in invaded compared to non-invaded nerves[34]. Previous research indicated that, in addition to functioning as a mitogen and a morphogen in non-neural systems, HGF can function as a guide and survival factor in the developing nervous

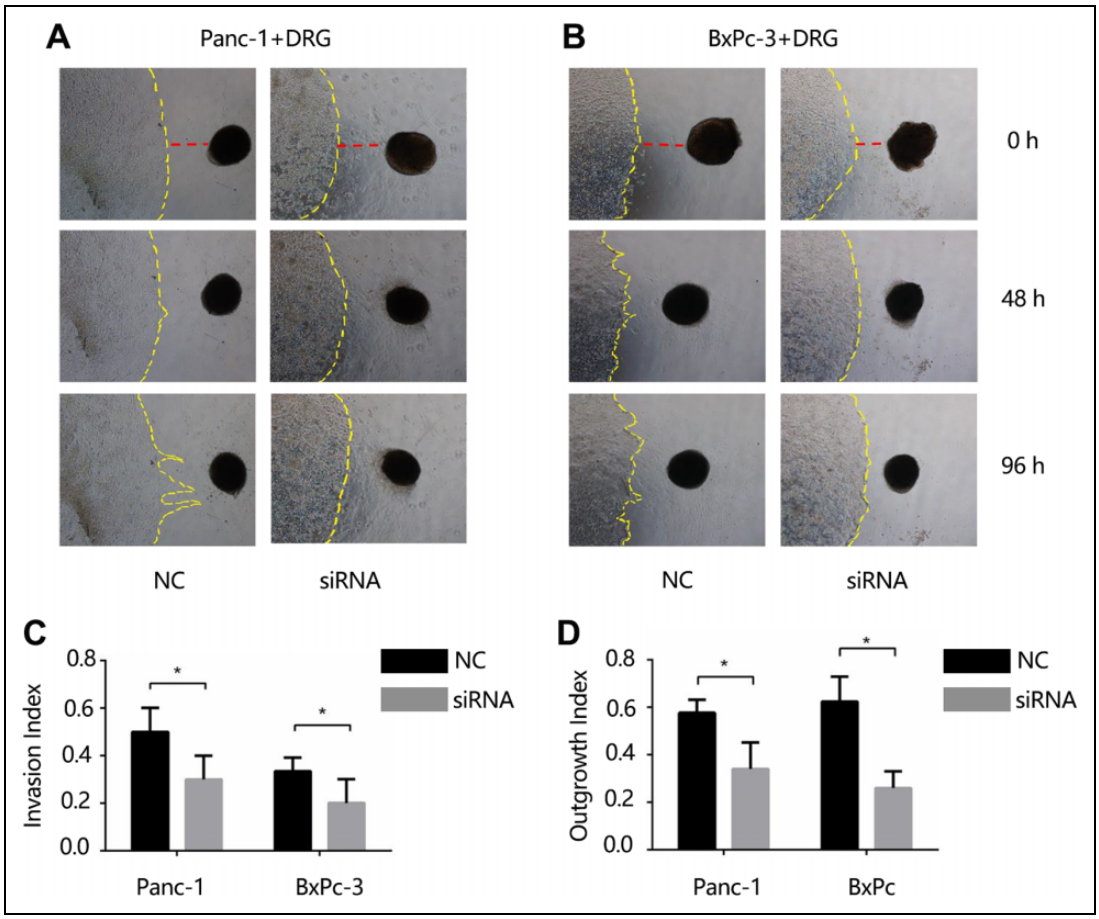

Fig. 5. si-c-Met significantly suppressed migration of cancer cells toward DRG neurites. (A, B) The side of the PC cells facing the DRG formed peak-like clusters and gradually moved toward the DRG. The side of the DRG neurites facing the PC cells had directional divergence toward the cancer cell mass. (C, D) The invasion index and DRG outgrowth index in si-c-Met are significantly lower than those in the control group in both PC cell lines.

system[7,8]. Our study showed that rh-HGF could accelerate the mutual growth of cancer cells and neurites of DRG in the coculture model of PNI in vitro. Furthermore, we found that HGF could upregulate the expression of NGF, which is a representative neurotrophic factor that can promote nerve growth. In contrast, the neural tropism of PC cells was significantly decreased when blocking the HGF/c-Met pathway by silencing c-Met. These results illustrate the interaction between neural cells, PSCs, and cancer cells: PSCs and neural cells provide an appropriate condition for cancer invasion via secreting HGF, and the interaction improves the growth of both the nerves and cancer cells. Taken together, HGF derived from PSCs enhanced the invasion and metastasis of c-Met-positive PC cells. In addition, HGF-induced tropism of PC cells towards the nerves leads to PC cell dissemination along an alternative route.

Additional research found that cancer cell-derived TGF-β also promotes cancer cell invasion in an autocrine fashion, but it strongly suppresses HGF secretion from fibroblasts in a paracrine fashion[23]. We predict that when TGF-β is inhibited, it cannot reduce cancer cell invasion, possibly due to upregulation of HGF. This hypothesis needs further investigation to illustrate the mechanism.

Ethical Approval

This study was approved by the relevant Ethical Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, China.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Ethical Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University approved protocols.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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