Interaction of the Sympathetic Nerve with Pancreatic Cancer Cells Promotes Perineural Invasion through the Activation of STAT3 Signaling

Running title: Role of Sympathetic Nerve in Perineural Invasion

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Key Words: Perineural invasion, sympathetic nerve, Norepinephrine, STAT3

Acknowledgments
The authors thank the staff of the Institution of Genetic Disease Research, Xi’ an Jiaotong University for their technical assistance. Supported in part by grants from the China Scholarship Council (to K. Guo); and grants from Kwang-Hua Education Foundation (to K. Guo).

Conflict of interest disclosures: no conflict of interest to disclose for all authors.
Abstract

Perineural invasion (PNI) is one of the most important causes of local recurrence and poor survival in pancreatic cancer. However, the exact mechanism of PNI is still not clear. In this study, we sought to identify the reciprocal signaling interactions between sympathetic nerves and pancreatic cancer cells and the underlying mechanisms. We used mouse dorsal root ganglia and pancreatic cancer cells co-cultured in vitro, cellular and molecular biology and animal models to evaluate the function of the sympathetic neurotransmitter norepinephrine (NE) in PNI progression and pathogenesis. NE promoted PNI of pancreatic cancer cells and increased levels of phosphorylated STAT3 in a concentration-dependent manner. NE-mediated activation of STAT3 was inhibited by blocking β-adrenergic receptors (AR) and by blocking protein kinase A but not through blocking α-AR. Blocking STAT3 could inhibit NE-induced NGF, MMP2 and MMP9 expression and attenuate the migratory, invasive ability and PNI of pancreatic cancer cells. Furthermore, PNI of pancreatic cancer cells was blocked by treatment with a STAT3 phosphorylation inhibitor in vivo. These studies show that NE plays a critical role in pancreatic cancer PNI development and progression through the β-AR/PKA/STAT3 signaling pathway. Reciprocal signaling interactions between the sympathetic nerves and pancreatic cancer cells critically contribute to pancreatic cancer PNI pathogenesis. Inhibition of the activity of sympathetic nerves or STAT3 may be potential strategies for pancreatic cancer PNI therapy.

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States. It is one of the most lethal malignancies, with median survival duration of less than 6 months and a 5-year survival rate of < 5%. According to the American Cancer Society, a total of 43,920 estimated new cases and 37,390 estimated deaths occurred in 2012. Worldwide, pancreatic cancer contributes to more than 260,000 deaths annually (1,
The dismal prognosis of pancreatic cancer is linked to local recurrence, lymph node metastasis, liver metastasis, peritoneal dissemination and perineural invasion (PNI) (3, 4). PNI is a prominent characteristic of pancreatic cancer, which is encountered in nearly 100% of pancreatic cancer cases upon targeted histopathological inspection of surgical specimens (5). PNI is the initial infiltration of tumor cells into the surrounding nerves and along the nerves, thus providing an alternative route for metastatic spread. It is considered the foremost reason for local tumor recurrence after curative resection and correlates with the abdominal pain sensation of pancreatic cancer patients (6, 7). The molecular changes leading to PNI are poorly understood. Identification and validation of associated genes and molecular pathways underlying PNI could potentially improve treatment outcomes for patients with pancreatic cancer.

The pathogenesis of PNI in pancreatic cancer is not clear, although PNI was first described over a century ago. Initially, the hypotheses of PNI research were mainly focused on anatomy, including the lymphatic spread of tumors into nerves (8, 9) and the nerve sheath as a low-resistance path for tumor spread (10). More recently, based on the neurotropism theory, in which tumor cells invade nerves mediated by neurotrophins, a new hypothesis of PNI was raised, which emphasized the reciprocal signaling interactions between the nerves and invading tumor cells (7). A recent study has shown that PNI in pancreatic cancer is associated with a remodeling of intrapancreatic nerves, and the proportion of sympathetic nerve fibers in the invaded nerves in pancreatic cancer was much smaller than in normal pancreatic nerves (11). Additionally, prior studies showed that the sympathetic neurotransmitter norepinephrine (NE) was overexpressed in human pancreatic cancer tissues (12). However, the precise function and underlying mechanisms of sympathetic nerve fibers of PNI in pancreatic cancer remain unclear, and the reciprocal signaling interactions between the sympathetic nerve and pancreatic cancer cells remain to be elucidated.
The pancreas is richly innervated by the autonomic nervous system, primarily through the plexus from the celiac and superior mesenteric artery ganglia (13, 14). The sympathetic nervous system might be involved in tumorigenesis (15). Previous studies have shown that enhanced activity of the sympathetic nervous system stimulates gastric tumorigenesis (16), and chemical sympathectomy with 6-hydroxydopamine decreases the incidence and number of gastric cancers and has protective effects against colon tumorigenesis (17). Moreover, sympathetic nerves might have an important effect on pancreatic tumorigenesis (15). The role of the sympathetic system in tumorigenesis is further supported by studies showing that the sympathetic neurotransmitter NE promotes tumor progression (17). We have shown that NE promotes pancreatic cancer cell invasion by modulating the expression of matrix metalloproteinases (MMPs) and the angiogenic cytokine VEGF (18). Additionally, other studies have shown that NE can activate the migration of carcinoma cells from cancers of the ovary (19, 20), breast (21), colon (22), and prostate (23). Furthermore, NE could induce chemotaxis in breast carcinoma cells (21).

STAT3, a member of the signal transduction and activation of transcription family, is a central cytoplasmic transcription factor that is activated by phosphorylation of a conserved tyrosine residue in response to extracellular signals and oncogenes. It has been shown to participate in numerous processes key to malignant progression (24, 25). Accumulating evidence has demonstrated that elevated STAT3 activity is frequently found in a wide variety of human tumors, including pancreatic cancer (26). A recent study has shown that activation of STAT3 by NE resulted in increased MMP production in ovarian cancer cells (27). However, whether NE and the activation of STAT3 are associated in pancreatic cancer cells and the impact of their signaling on PNI pathogenesis remain unknown.

In the present study, we sought to determine the role of the sympathetic neurotransmitter NE in pancreatic cancer PNI and the regulatory effect of NE on
STAT3 activation and function. We discovered that signaling interactions between sympathetic nerves and pancreatic cancer cells promotes PNI in pancreatic cancer and the STAT3 signaling pathway critically regulates pancreatic cancer PNI and is a potential therapeutic target for PNI in pancreatic cancer.

Materials and Methods

Cell lines and culture conditions
The human pancreatic cancer cell lines MIA PaCa-2 and BxPC-3 were from the American Type Culture Collection (ATCC). The cell line was used in fewer than 6 months after receipt or resuscitation. ATCC performs authentication of cell line via the cytochrome C oxidase I gene sequences assay, short tandem repeat profiling and cytogenetic analysis. Only mycoplasma tests were performed for the cell line authentication in our laboratory. We did not carry out additional testing to authenticate cell line, but its morphology and behavior were consistent with ATCC descriptions. The cell lines were maintained at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories).

Western blot analysis
Standard Western blotting was carried out using whole-cell protein lysates and primary antibodies against pSTAT3 and STAT3 (Cell Signaling Technology), NGF (Millipore), MMP2 and MMP9 (Santa Cruz Biotechnology) and a secondary antibody (anti-rabbit IgG or anti-mouse IgG; Santa Cruz Biotechnology). Equal protein sample loading was monitored using an anti-β-actin antibody (Santa Cruz Biotechnology). The probed proteins were detected using an enhanced chemiluminescence system (Amersham Life Sciences) according to the manufacturer’s instructions.

In vitro perineural invasion model
The in vitro perineural Invasion model was performed essentially as described
previously (28, 29). Briefly, mice (BALB/c, 2-4 weeks old) were sacrificed using CO2 euthanasia according to MSKCC institutional guidelines, and their excised dorsal root ganglia (DRG) were implanted approximately 500 µm adjacent to a colony of carcinoma cells in growth factor-depleted Matrigel matrix (BD Biosciences). Cultures were grown in DMEM containing 10% FCS in 37°C and 5% CO2 incubation conditions. The FCS was removed as soon as cancer cells made contact with the DRG neurites. The neural invasion index was calculated as described previously (28).

**Cell scratch-wound assay**

Pancreatic cancer cells were grown in 6-well plates until confluence. A wound was generated by scraping with a 10 µl pipette tip. After 12 hours, the cells in the wounded monolayer were photographed, and cell migration was assessed by measuring the gap sizes in multiple fields.

**Cell migration assay**

Cell migration assays were conducted using a modified 24-well Boyden chamber with a membrane that was not coated with Matrigel (BD Biosciences). Ten percent fetal bovine serum-containing medium was placed in the lower chambers to be used as a chemoattractant. Pancreatic cancer cells \((3\times10^5)\) in a 300 µl volume of serum-free medium were placed in the upper chambers and incubated at 37°C for 24 h. Migrated cells on the bottom surface of the filter were fixed, stained with Crystal Violet (Boster Biological Technology Ltd., Wuhan, China), and counted under a microscope in five randomly selected fields.

**Cell invasion assay**

The invasion assay was performed using a specialized Chemicon invasion chamber that included a 24-well tissue culture plate with 12 cell culture inserts (Millipore). The inserts contained an 8 µm pore size polycarbonate membrane with a precoated thin layer of basement membrane matrix (ECMatrix). Ten percent fetal bovine serum-containing medium was placed in the lower chambers to act as a
chemoattractant. Pancreatic cancer cells (3×10^5) in a 300 µl volume of serum-free medium were placed in the upper chambers and incubated at 37°C for 48 h. Invasive cells on the lower surface of the membrane, which had invaded the ECMMatrix and had migrated through the polycarbonate membrane, were stained with Crystal Violet, and counted under a microscope in five randomly selected fields.

**In vivo model of neural invasion**

The in vivo perineural invasion model was performed essentially as described previously (28, 30). Briefly, nude athymic mice were anesthetized using isoflurane, and their left sciatic nerve was exposed. The nerve could be easily identified deep to the femorococygeous and biceps femoris muscles. Pancreatic cancer cells from the MIA PaCa-2 cell line were microscopically injected into the sciatic nerve, distal to the bifurcation of the tibial and common peroneal nerves. Microinjection of 3 µl of cell suspension at a concentration of 1 × 10^5 cells per microliter was performed using a 5 µl microsyringe over a 2 min period. Sciatic nerve function, which innervates the hind limb paw muscles, was measured weekly for 7 weeks. Functional measures to monitor tumor neural invasion included (a) gross behavior, which was monitored for 10 min weekly for signs of motor weakness or repetitive biting of the hind limb; (b) limb function, which was graded according to the hind limb paw response to manual extension of the body from 4 (normal) to 1 (total paw paralysis); and (c) the sciatic nerve function index, which was calculated as the spread length (in mm) between the first and fifth toes of the mouse’s hind limbs.

**Immunohistochemistry**

Paraffin-embedded nerves were subjected to H&E and immunohistochemistry. After deparaffinization and rehydration, antigen retrieval was performed by boiling with citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide. After rinsing, the specimens were blocked for 30 min with sheep serum at 37 °C, and the sections were incubated at 4°C overnight with the p-STAT3
primary antibody (1:200). A biotinylated secondary antibody was added and visualized with streptavidin-labeled horseradish peroxidase. The reaction products were visualized by incubation with 3,3-diaminobenzidine (DAB). The slides were counterstained for nuclei with hematoxylin.

Statistical analysis

All statistical analyses were performed using the SPSS17.0 software (SPSS Inc., Chicago, USA). All data are expressed as the means ± standard deviation (SD). Differences between the groups were determined by analysis of variance (ANOVA) and Mann–Whitney test (2-tailed). $P<0.05$ was considered statistically significant.

Results

NE promotes neural invasion of pancreatic cancer cells in vitro

To investigate the effect of NE on neural invasion of pancreatic cancer cells, an in vitro neural invasion model was constructed as previously described by implanting a DRG adjacent to a colony of pancreatic cancer cells in growth factor-depleted Matrigel (28, 29) (Fig. 1A). We observed the neurite outgrowth from the DRG at 48 h (Fig. 1B). Pancreatic cancer cells made contact and invaded the DRG neurites approximately 5 days after implantation (Fig. 1C, D). To further determine the influence of NE on neural invasion of pancreatic cancer cells in vitro, we treated MIA PaCa-2 and BxPC-3 cells in the neural invasion model with different doses of NE for 7 days (Fig. 1 E, F). We found that NE produced dose-dependent enhancement in both the MIA PaCa-2 and the BxPC-3 neural invasion models (Fig. 1 G). These findings strongly indicate that NE plays a critical role in pancreatic cancer cell neural invasion development.

Activation of the STAT3 pathway by NE through β-adrenoceptor and PKA

To understand the molecular mechanisms underlying NE-induced pancreatic cancer cell neural invasion, we performed a Western blot analysis using total protein lysates
extracted from the MIA PaCa-2 and BxPC-3 cells treated with different doses of NE. As shown in Fig. 2A, pSTAT3 levels increased markedly after exposure to increasing concentrations of NE. There was no effect on total STAT3 levels. pCREB and pAKT levels were also increased after treatment with NE (Fig. 2E). Because NE interacts with adrenergic receptors (AR), we next examined the effect of β- and α-adrenergic receptor blockers on NE-induced pSTAT3 expression. We found that propranolol, a β-ARs blocker, prevented pSTAT3 induction by NE in both cell lines (Fig. 2B), but inhibition of α1 and α2, either individually or in combination, could not prevent pSTAT3 induction (Fig. 2C). Because PKA is the downstream signal of β-ARs, we further examined the effect of PKA on the NE-induced pSTAT3 activation. We found that 10 μM of KT5720, a PKA inhibitor, could prevent pSTAT3 activation with 10 μM of NE (Fig. 2D). These findings strongly indicate that the effects of NE on the activation of STAT3 are transmitted through the β-ARs/PKA signaling pathway.

**Effects of STAT3 blockade on NE-induced NGF, MMP2 and MMP9 expression in pancreatic cancer cells**

To further determine whether STAT3 blockade impacts NE-induced gene expression in pancreatic cancer cells, MIA PaCa-2 and BxPC-3 cells were pretreated with the STAT3 phosphorylation inhibitor AG490 (50 μM) for 1 h and then stimulated with 10 μM NE diluted in serum-free medium. Total protein lysates were harvested from cell cultures, and the levels of pSTAT3, NGF, MMP9 and MMP2 protein expression were determined using Western blot analysis. We found that AG490 significantly decreased the pSTAT3 activation induced by NE in both cell lines (Fig. 3A). Additionally, as shown in Fig. 3B, the protein expression levels of NGF, MMP9 and MMP2 increased upon treatment with 10 μM NE compared to the control, and pretreatment with AG490 significantly attenuated NE-induced protein expression in MIA PaCa-2 (Fig. 3C) and BxPC-3 (Fig. 3D) cells.

**Attenuation of NE-induced neural invasion and migratory, invasive ability of pancreatic cancer cells by STAT3 blockade**
To determine the effect of STAT3 blockade on NE-induced neural invasion of pancreatic cancer cells, a neural invasion model was co-cultured in 10 μM NE without (Fig. 3E) or with (Fig. 3F) AG490 treatment. We observed that AG490 significantly attenuated NE-induced neural invasion of MIA PaCa-2 and BxPC-3 cells (Fig. 3G). In addition, the nerve invasion index decreased significantly in the cells exposed to β2-adrenergic antagonists ICI118,551 and propranolol compared with metoprolol and control treatment groups (Supplementary Fig. 1). We further examined the effect of STAT3 blockade on NE-induced cancer cell migration and invasion. The MIA PaCa-2 and BxPC-3 cells were wounded by scratching and maintained at 37°C for an additional 12 hours. The treatment with 10 μM NE strongly promoted the flattening and spreading of both cell lines, whereas treatment with AG490 attenuated the NE-induced flattening and spreading of both cell lines (Fig. 4A, D). The results of the cell migration assay also indicate that treatment with 10 μM NE promoted the migration ability of both cell lines, whereas treatment with AG490 attenuated the migration ability of both cell lines (Fig. 4B, E). Similarly, treatment with 10 μM NE promoted the invasiveness of both cell lines, whereas treatment with AG490 attenuated the invasiveness of both cell lines (Fig. 4C, F, G, H). Thus, our data clearly establish that STAT3 blockade could attenuate NE-induced pancreatic cancer cell migration, invasion and neural invasion.

Neural invasion in vivo after systemic treatment with STAT3 blockade

The in vitro data demonstrated that the STAT3 phosphorylation inhibitor AG490 might inhibit NE-induced pancreatic cancer cell neural invasion, and a prior study showed that the NE level was higher in mice with sciatic nerve invasion than normal mice (31). Therefore, we further investigated the ability of STAT3 blockade to inhibit nerve invasion in vivo. The left sciatic nerve of mice was exposed (Fig. 5A), and MIA PaCa2 cells were implanted in a distal part of the left sciatic nerve (Fig. 5B). The right sciatic nerve was injected with saline and served as a sham operation control (Fig. 5C). Seven days after implantation of MiaPaCa2 cells, mice were randomized into two groups: intraperitoneal injection of AG490 (10 mg/kg) or vehicle. Mice were injected
twice a week for 7 weeks, and their sciatic nerve function was assessed weekly for 7 weeks. In the control group, mice began to develop left hind limb paralysis 4 weeks after tumor implantation. Of the 10 mice in this group, 7 were fully paralyzed by week 7. In contrast, 8 of the 10 mice that were treated with AG490 had normal hind limb function at week 7 (Fig. 5D). We used the sciatic nerve index (the hind limb paw span) as an additional measure of sciatic nerve function. In the control group (Fig. 5E, F), there was a statistically significant decrease in the left hind limb sciatic nerve index, whereas in the treatment group (Fig. 5G, H), no significant change in the sciatic nerve index was observed. All mice were killed at week 7, and their sciatic nerves were excised for histopathological analysis. Treatment with AG490 reduced pSTAT3 levels compared with the controls (Fig. 6A, B). The tumor diameter and proximal nerve diameter in the control group was larger compared with that in the treatment group (Fig. 6 C-H). These data indicate that AG490 inhibited tumor growth and invasion of the sciatic nerve proximally toward the spinal cord.

Discussion

In this study, we identified a critical role for the sympathetic neurotransmitter NE in pancreatic cancer PNI pathogenesis and its underlying mechanisms. We found that NE promotes PNI of pancreatic cancer cells in a concentration-dependent manner by activating STAT3 through the β-AR/PKA signaling pathway. Blocking STAT3 could inhibit NE-induced NGF, MMP2 and MMP9 expression and attenuate the migratory, invasive ability and PNI of pancreatic cancer cells. Furthermore, PNI of pancreatic cancer cells in vivo could be effectively treated by STAT3 blockade. Collectively, our in vitro and in vivo evidence strongly suggests that reciprocal signaling interactions between the sympathetic nerves and pancreatic cancer cells critically contribute to pancreatic cancer PNI pathogenesis and that inhibition of the activity of sympathetic nerves or STAT3 may be potential strategies for pancreatic cancer PNI therapy.

PNI causes frequent local recurrence even after radical resection and a poor
prognosis for pancreatic cancer (3, 32). Better understanding of PNI pathogenesis could bring about effective control of PNI-related morbidities, such as local recurrence and extrapancreatic spread. Initially, PNI was believed to be the lymphatic spread of tumors into nerves (9); however, later studies confirmed there were no lymphatic vessels in the nerve (8). The next predominant theory was that the nerve sheath served as a low-resistance path for tumor spread. However, using electron microscopy, pathologists observed that the nerve sheath is composed of three connective tissue layers, which is not a low-resistance path (10). Because certain carcinomas exhibit a predilection for PNI and others do not, researchers who focused on the effect of neurotrophins on cancer cells proposed the neurotropism theory, which suggested that tumor cells could be attracted by neurotrophins to invade the nerves (33-37). More recently, studies have demonstrated that PNI may involve reciprocal signaling interactions between tumor cells and nerves and that these invading tumor cells may have acquired the ability to respond to proinvasive signals within the peripheral nerve milieu (7). Therefore, this theory emphasized the signaling interactions between nerves and invading tumor cells. Current studies about PNI mostly focus on the interaction between cancer cells and nerves but do not consider the differences in nerve fiber quality in the PNI process. Ceyhan et al. (11) used tyrosine hydroxylase immunostaining to study the changes in sympathetic nerves in human pancreatic cancer tissues. They reported that sympathetic innervation was significantly reduced in pancreatic cancer, and noticeably decreased in patients with PNI. In the present study, we showed that the sympathetic neurotransmitter NE could promote PNI in a co-culture model using DRG and pancreatic cancer cells. This finding means that reciprocal signaling interactions between tumor cells and sympathetic nerves could be one cause of the pathogenesis in PNI of pancreatic cancer.

NE belongs to the group of classical neurotransmitters (38), and it is expressed from peripheral sympathetic nerve endings. It is also one of the most potent known stimulators of tumor cell migration (39). Previous studies have shown that NE
promotes pancreatic cancer cell invasiveness by modulating the expression of MMPs and VEGF. Similar results have also been found in ovary (19, 20), breast (21), colon (22), and prostate (23) cancers. Additionally, Schuller et al. (12) reported that NE was overexpressed in human pancreatic cancer tissues. Our current study shows that NE upregulates NGF expression in pancreatic cancer cells, so that NGF, as a neurotrophin, can attract neurite outgrowth toward the cancer colony. Our results also show that NE promotes MMP9 and MMP2 expression in pancreatic cancer cells, which would enhance the invasive ability of tumor cells toward neurites mediated by NE. These promising results suggest that the sympathetic neurotransmitter NE is a bridge connecting the signaling interactions between sympathetic nerves and tumor cells by the molecules NGF and MMPs. It has been shown that pancreatic cancer cells produce their own norepinephrine and epinephrine and that both agents stimulate cell proliferation and invasion by β-adrenergic signaling in an autocrine fashion (40). Given the crosstalk between sympathetic nerves and tumor cells, it is likely that NE produced by tumor cells may involve in PNI progression and inhibition of the activity of sympathetic nerves may reduce the PNI in pancreatic cancer, which awaits further experimental validation.

STAT3 is a member of the JAK/STAT signaling pathway and regulates a number of pathways important in tumorigenesis, including cell cycle progression, apoptosis, tumor angiogenesis, and tumor cell evasion of the immune system (25, 41-44). In this study, we demonstrated that STAT3 could be activated by NE through the β-AR/PKA signaling pathway and upregulation of the downstream gene expression of MMPs and NGF. Our findings were consistent with recent studies, which have suggested that NE-dependent effects on the growth of ovarian tumor cells in nude mice are mediated through the tumor cell cAMP/PKA signaling pathway (27). Furthermore, STAT3 is a downstream transcription factor of JAK, and JAK inhibition could also inhibit the STAT3 signaling pathway (45). In the present study, we found that AG490, a JAK-specific inhibitor, strongly suppressed NE-induced STAT3 activity in vitro and in vivo. Additionally, blocking STAT3 inhibited NE-induced migration, invasion and PNI of
pancreatic cancer cells by downregulating MMP9, MMP2 and NGF expression. Moreover, we observed that PNI of pancreatic cancer cells was blocked by treatment with a STAT3 phosphorylation inhibitor in vivo. However, further studies are required to examine the clinical relevance of this finding. Taken together, these results suggest that the STAT3 signaling pathway may contribute to the interaction between sympathetic nerves and pancreatic cancer cells. Therefore, STAT3 may be a potential therapeutic target for designing novel effective therapies for PNI in pancreatic cancer. Finally, our recent study has shown the existence and different effects of β1-AR and β2-AR on pancreatic cancer cells. It warrants further investigation to clarify the nature of β-adrenergic receptors involved.

In summary, this study provides critical insight into the reciprocal signaling interactions between sympathetic nerves and pancreatic cancer cells in pancreatic cancer PNI progression by upregulating the expression of NGF and MMPs and the migration and invasion of tumor cells through activating the β-AR/PKA/STAT3 signaling pathway. Our study not only provides a novel mechanism for pancreatic cancer PNI progression but also undercovers molecular signaling as a promising new molecular target for designing novel therapeutic modalities to reduce pancreatic cancer PNI.

References

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**Figure legends**

**Figure 1.** Effect of the neurotransmitter norepinephrine (NE) on neural invasion of pancreatic cancer cells in vitro. Mouse dorsal root ganglia (DRG) and pancreatic cancer cells were co-cultured to assess the interactions between nerve and cancer cells. A, DRG were implanted adjacent to a colony of pancreatic cancer cells in growth factor-depleted Matrigel matrix. B, Neurites grew out of the DRG at 48 h. C, The interaction of MIA PaCa-2 pancreatic cancer cells and neurites 7 days after implantation. D, A magnified image of tumor cells invading a nerve (white arrows). E and F, Tumor cells were grown in Matrigel adjacent to the DRG. Images show representative experiments of neural invasion by MIA PaCa-2 cells taken at day 7 in control conditions allowing for neural invasion (E) and with 10 μM NE promoting...
neural invasion (F). **G**, Dose-dependent effects of NE on the neural invasion index of human pancreatic cancer cells MIA PaCa-2 and BxPC-3 at day 7 (*, #, *P* <0.05 in comparison of the NE-treated group with the control group).

**Figure 2.** The influence of NE on pSTAT3 expression in pancreatic cancer cells. **A**, The pancreatic cancer cell lines MIA PaCa-2 and BxPC-3 were serum-starved for 12 h before the addition of increasing concentrations of NE. Cell lysates were harvested, and the level of protein expression was determined using Western blot analysis. Equal protein sample loading was monitored by probing the same membrane filter with an anti-β-actin antibody. **B**, Treatment with propranolol, a β1/β2-adrenergic receptor blocker, for 1 h prior to exposure to 10 μM of NE prevented an increase in pSTAT3 expression. **C**, Pretreatment of tumor cells with prazosin (an α1 blocker), yohimbine (an α2 blocker), or a combination of both did not prevent NE-induced increases in pSTAT3. **D**, The PKA-specific inhibitor KT5720 (10 μM) reduced the increase in pSTAT3 that was induced by 10 μM NE treatment. **E**, The influence of NE on phosphorylation of AKT and CREB in pancreatic cancer cells. The unphosphorylated proteins AKT and CREB were used as controls to ensure equal loading of proteins.

**Figure 3.** Effects of treatment with AG490, a STAT3 phosphorylation inhibitor, on the NE-induced neural invasion of and gene expression in human pancreatic cancer cells in vitro. Serum-starved pancreatic cancer cell lines MIA PaCa-2 and BxPC-3 were pretreated with the STAT3 phosphorylation inhibitor AG490 (50 μM) for 1 h and then stimulated with 10 μM NE diluted in serum free medium. Total protein lysates were harvested from the cell cultures, and the level of pSTAT3 (**A**), NGF, MMP9 and MMP2 (**B**) protein expression was determined using Western blot analysis. Equal protein sample loading was monitored by probing the same membrane filter with an anti-β-actin antibody. **C** and **D**, The levels of NGF, MMP9 and MMP2 expression were quantitated and expressed as fold changes. **E** and **F**, Tumor cells were grown adjacent to the DRG in Matrigel. Images show representative experiments of neural
invasion by MIA PaCa-2 cells taken at day 7 in 10 μM NE without (E) or with (F) AG490 treatment. G, Nerve invasion index in control, 10 μM NE and 10 μM NE with AG490 groups at day 7. *, P<0.05 compared with control group. #, P<0.05 compared with 10 μM NE group.

**Figure 4.** Influence of AG490 on NE-induced pancreatic cancer cell migration and invasion. MIA PaCa-2 (A, B, C and G) and BxPC-3 cells (D, E, F and H) were treated with control, 10 μM NE and 10 μM NE with AG490, respectively. For cell scratch-wound assays, the cultures were wounded by scratching and were then maintained at 37°C for an additional 12 h. Cell cultures were photographed, and cell migration was assessed by measuring gap sizes (inserted number represents the percent area of the gap ± SD) (A and D). For cell migration assays, the cells were maintained at 37°C for an additional 24 h. Representative tumor cells that migrated through the membrane were photographed, while the numbers of cells that migrated through the membrane without Matrigel were counted in 5 random fields identified within the lower surface of the membrane and expressed as a % of the control (inserted numbers). Data represent the means ± SD of triplicates (B and E). For cell invasion assays, the cells were maintained at 37°C for an additional 48 h. Representative tumor cells that invaded through the Matrigel were photographed, while the number of invasive cells that penetrated through the Matrigel-coated filter were counted in 5 random fields, identified within the lower surface of the filters and expressed as a % of control (inserted numbers). Data represent the means ± SD of triplicates (C and F). To assay the effects of NE as chemoattractant on pancreatic cancer cell invasion, pancreatic cancer cells (3×10^5) in a 300 μl volume of serum-free medium were placed in the upper chambers, and 10 μM of NE-containing medium was placed in the lower chambers to act as a chemoattractant. The untreated cell cultures were given arbitrary invasiveness percentages of 100% (G and H). *, P<0.05 compared with control group. #, P<0.05 compared with 10 μM NE group.

**Figure 5.** Analysis of the therapeutic effect of AG490 on neural invasion modeled in
vivo. MIA PaCa2 cells were implanted in a distal part of the left sciatic nerve and treated starting at day 7 with vehicle or AG490 (10 mg/kg) twice a week for 7 weeks. 

**A**, The left sciatic nerve of mice was exposed. **B**, The pancreatic cancer cell line MIA PaCa2 was implanted in the left sciatic nerve of mice. **C**, Postoperative picture of the mouse, the right sciatic nerve served as a sham operation control (injected with saline). **D**, The mean left sciatic nerve scores of control and AG490-treated mice were measured weekly for 7 weeks. **E-H**, The sciatic nerve indexes (paw span in mm) of control mice (E and F) and AG490-treated mice (G and H) were measured 1 and 7 weeks after tumor implantation. The left (E, G) side sciatic nerve was injected with MIA PaCa2 cells (n=10), while the right (F, H) side was injected with saline (n=10).

**Figure 6.** Neural invasion in vivo after treatment with AG490. **A**, Representative images of immunohistochemical staining of tumors for phosphorylated STAT3 in control mice. **B**, Effect of treatment with AG490 on levels of phosphorylated STAT3. **C** and **D**, In situ images of the sciatic nerve in a control mouse (C) and an AG490-treated mouse. **E** and **F**, H&E staining. Control (E) and AG490-treated (F) MIA PaCa2 tumors. **G**, Histological analysis of nerve specimens to measure primary tumor sizes in control and AG490-treated mice (n = 10). **H**, The proximal diameter of the sciatic nerve (5 mm from implantation site toward the spinal cord) was measured in control and AG490-treated mice (n = 10). *P < 0.05 in a comparison between the AG490-treated and control groups.
Guo K, et al, Figure 3

(A) Western blot analysis showing the effect of AG490 and NE on pStat3, β-Actin, and AG490 in MIA PaCa-2 and BxPC-3 cells.

(B) Western blot analysis showing the effect of NGF, MMP9, MMP2, and β-Actin in MIA PaCa-2 and BxPC-3 cells.

(C) Bar graph showing the protein expression levels of NGF, MMP9, and MMP2 in MIA PaCa-2 cells treated with control, 10 μM NE, and NE + AG490.

(D) Bar graph showing the protein expression levels of NGF, MMP9, and MMP2 in BxPC-3 cells treated with control, 10 μM NE, and NE + AG490.

(E) Images of neural invasion in MIA PaCa-2 and BxPC-3 cells.

(F) Bar graph showing the neural invasion index in MIA PaCa-2 and BxPC-3 cells treated with control, 10 μM NE, and NE + AG490.
Guo K, et al, Figure 4

A

0 h

12 h

Control

NE

NE+AG490

B

Migration

C

Invasion

D

0 h

12 h

Control

NE

NE+AG490

E

Migration

F

Invasion

G

Control

10 μM NE

H

Control

10 μM NE

Graph showing the effects of different conditions on migration and invasion.
Guo K, et al, Figure 6
Molecular Cancer Therapeutics

Interaction of the Sympathetic Nerve with Pancreatic Cancer Cells Promotes Perineural Invasion through the Activation of STAT3 Signaling

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Mol Cancer Ther Published OnlineFirst January 3, 2013.

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