Sonic Hedgehog Paracrine Signaling Activates Stromal Cells to Promote Perineural Invasion in Pancreatic Cancer

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Introduction

Perineural invasion (PNI) in pancreatic cancer is a common pathologic characteristic in which tumors invade the perineural space of local peripheral nerves and intimately disseminate along nerve fascicles (1). PNI is thought to contribute to both pain (2, 3) and local disease recurrence (2, 4, 5), and is an important prognostic factor for pancreatic cancer (6). In addition, pancreatic tumors are surrounded by a dense desmoplastic reaction (7), which is involved in pancreatic stellate cells (PSC; refs. 8 and 9) or fibroblasts (10) in the stroma, and exhibit neural alterations (11), such as hypertrophy and degeneration of intrapancreatic nerve fibers (12, 13). A positive correlation between fibrosis in the pancreatic tumor microenvironment and intrapancreatic neuropathy or PNI has been reported (14, 15).

Recently, a wide range of studies (16, 17) have shown that paracrine Sonic Hedgehog (SHH) protein, which is a hedgehog (Hh) pathway ligand and derived from pancreatic cancer epithelial cells, is the pivotal factor in both the regulation of the pancreatic tumor microenvironment and the promotion of tumor development and metastasis. Tumor-derived SHH protein acts on PSCs (18), whose activation, in turn, promotes the malignant behavior of pancreatic cancer cells, including reduced patient survival rates (19), uncontrolled growth (20), invasion (21), and therapeutic resistance (22).

As active stromal PSCs in the desmoplasia reaction are potentially associated with PNI in pancreatic cancer tissues (14, 15), we sought to explore the contribution of PSCS activated by paracrine SHH in tumor PNI in pancreatic cancer. We show that SHH overexpression in tumor cells activates the hedgehog pathway in PSCs in the stroma instead of activating tumor cells. These activated PSCs are essential for the promotion of pancreatic cancer cell migration along nerve axons and nerve outgrowth to pancreatic cancer cell colonies in an in vitro three-dimensional model of nerve invasion in cancer. Furthermore, the coimplantation of PSCs activated by paracrine SHH induced tumor cell invasion of the trunk and nerve dysfunction along sciatic nerves and also promoted orthotropic xenograft tumor growth, metastasis, and PNI in vivo models.

Conclusions: These results establish that stromal PSCs activated by SHH paracrine signaling in pancreatic cancer cells secrete high levels of PNI-associated molecules to promote PNI in pancreatic cancer. Clin Cancer Res; 20(16); 4326–38. ©2014 AACR.

References

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Sonic Hedgehog Paracrine Signaling Activates Stromal Cells to Promote Perineural Invasion in Pancreatic Cancer

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Abstract

Purpose: Pancreatic cancer is characterized by stromal desmoplasia and perineural invasion (PNI). We sought to explore the contribution of pancreatic stellate cells (PSC) activated by paracrine Sonic Hedgehog (SHH) in pancreatic cancer PNI and progression.

Experimental Design: In this study, the expression dynamics of SHH were examined via immunohistochemistry, real-time PCR, and Western blot analysis in a cohort of carcinomatous and nonneoplastic pancreatic tissues and cells. A series of in vivo and in vitro assays was performed to elucidate the contribution of PSCs activated by paracrine SHH signaling in pancreatic cancer PNI and progression.

Results: We show that SHH overexpression in tumor cells is involved in PNI in pancreatic cancer and is an important marker of biologic activity of pancreatic cancer. Moreover, the overexpression of SHH in tumor cells activates the hedgehog pathway in PSCs in the stroma instead of activating tumor cells. These activated PSCs are essential for the promotion of pancreatic cancer cell migration along nerve axons and nerve outgrowth to pancreatic cancer cell colonies in an in vitro three-dimensional model of nerve invasion in cancer. Furthermore, the coimplantation of PSCs activated by paracrine SHH induced tumor cell invasion of the trunk and nerve dysfunction along sciatic nerves and also promoted orthotropic xenograft tumor growth, metastasis, and PNI in vivo models.

Conclusions: These results establish that stromal PSCs activated by SHH paracrine signaling in pancreatic cancer cells secrete high levels of PNI-associated molecules to promote PNI in pancreatic cancer. Clin Cancer Res; 20(16); 4326–38. ©2014 AACR.
Activated Stellate Cells Promote Perineural Invasion

Translational Relevance
Perineural invasion (PNI), in which tumor cells invade the perineural space of local peripheral nerves, is an important prognostic factor in pancreatic cancer. However, the mechanism of PNI remains unclear. Activated pancreatic stellate cells (PSC) are considered an important substance in the tumor microenvironment. This is the first study describing evidence that paracrine Sonic Hedgehog (SHH), which mediates the interaction between cancer cells and the stroma, may activate PSCs in the tumor microenvironment, and these PSCs with mutant alternative molecule expression contribute to pancreatic cancer PNI and ultimately tumor progression. These results suggest that PSCs affected by paracrine SHH signaling promote PNI within pancreatic cancer, thereby emphasizing the critical role of PSCs in the tumor microenvironment. This paracrine loop has important clinical implications as it involves novel pharmacologic targets that may be targeted to reduce tumor PNI and to improve the survival of patients with pancreatic cancer.

Materials and Methods
Patients and tissue samples and IHC
We obtained 70 pancreatic cancer samples from the surgical pathology bank at the Department of Pathology of the First Affiliated Hospital of the Medical College of Xi’an Jiaotong University after receiving the approval of the Ethical Committee of Xi’an Jiaotong University. The pathologic tumor–node–metastasis (TNM) status was assessed according to the criteria of the 6th edition of the TNM classification of the American Joint Commission on Cancer (2002). Informed consent was obtained from each patient. The 3-year follow-up for all of the cases was completed in December 2012. The other clinicopathologic data were presented in Supplementary Table S2. Standard IHC were carried out using SHH (Santa Cruz), Gli-1 (Santa Cruz), nerve growth factor (NGF; Santa Cruz), and S-100 (Boshide). The staining results were scored by 2 pathologists blinded to the clinical data as described by Sinicrope and colleagues (23).

Cell culture
The human pancreatic cancer cell lines AsPc-1, BxPc-3, CFPAC-1, MiaPaCa-2, Panc-1, and SW1990 were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured in DMEM (HyClone) supplemented with 10% FBS, 100 μg/mL ampicillin, and 100 μg/mL streptomycin. The cultures were incubated at 37°C in a humidified atmosphere with 95% O2 and 5% CO2. The cell lines were maintained directly from ATCC that conducts cell line characterizations or authentication by the short tandem repeat profiling and passaged in our laboratory for less than 6 months after receipt.

Western blot analysis
As described previously (24), standard Western blot analysis was carried out using whole-cell protein lysates and primary antibodies against SHH, Gli-1, NGF, MMP-2, and MMP-9 (Santa Cruz), and SMO (Bioworld); and a secondary antibody (anti-rabbit IgG or anti-mouse IgG; Santa Cruz). Equal protein sample loading was monitored using an anti-β-actin antibody (Santa Cruz).

Quantification of SHH in conditioned medium
SHH concentrations in the conditioned medium (CM) were quantified using an SHH Enzyme-Linked Immunosorbent Assay (ELISA) Kit (R&D) according to the manufacturer’s instructions.

Separation of dorsal root ganglions and coculture
Separation of the mouse dorsal root ganglions (DRG) has been described previously (25). Newborn mouse DRGs were stored on ice in DMEM and then seeded in 24-well Petri dishes containing 20 μl of Matrigel (Supplementary Fig. S1A). Next, coculture of tumor cells and DRGs were performed according to the protocol described by Ceyhan and colleagues (ref. 26; Supplementary Fig. S1B and S1C). See the Supplementary Materials and Methods for details.

Subcutaneous injections in athymic nude mice
Subcutaneous injections of tumor cells alone or tumor cells plus PSCs in athymic nude mice have been described previously (21). See the Supplementary Materials and Methods for details.

In vivo model of tumor sciatic neural invasion
As described previously (27), 6-week-old athymic nude mice were anesthetized for all procedures, and 3 μL of cell suspension (concentration of 1 × 10^5/μL) was slowly injected into the perineurium of the left sciatic nerve using a 10-μl microsyringe. Sciatic nerve function was measured weekly, and a small animal ultrasound (13-MHz) was used to measure the proximal sciatic nerve diameter and the primary sciatic nerve tumor diameter once per week. See the Supplementary Materials and Methods for details.

Orthotopic implantation in athymic nude mice
The cells were prepared as described for the subcutaneous injection model. Orthotopic implantation of pancreatic cancer cell lines was performed as described previously (21). Briefly, 30 μL of cell suspension (concentration of 1 × 10^6/μL) was injected into the pancreas of 8 mice to analyze...
tumor growth, PNI, and metastases in response to the overexpression of SHH.

Plasmid construction and transfections, cell proliferation assay, Matrigel invasion assay, apoptosis assessment, and real-time PCR assay

Details are described in the Supplementary Materials and Methods. The PCR primer sequences are listed in Supplementary Table S1. The comparative C(T) method (28) was used to quantify the expression of each target gene using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a normalization control.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package (version 13.0). The significance of the patient specimen data was determined using the Pearson correlation coefficient or Fisher exact test. The significance of the in vitro and in vivo data was determined using the Student t test (2-tailed), Mann–Whitney test (2-tailed), or one-way ANOVA. \( P < 0.05 \) was considered statistically significant.

Results

Overexpression of SHH in pancreatic cancer and its correlation with survival and patients’ clinicopathologic features

To determine whether SHH is overexpressed at the protein level in human pancreatic cancer tissues, we examined SHH expression in the surgical cases via Western blot analysis. The results show that the levels of SHH protein in primary pancreatic cancer tissues were significantly elevated compared with normal pancreatic tissues (Fig. 1A). To further confirm these results, we performed immunohistochemical staining of SHH on 70 cases of pancreatic cancer specimens and 8 cases of normal pancreatic specimens. Intensive IHC staining of SHH was detected in the cytoplasm of cancer cells and was undetected in stromal cells in tumor tissues, whereas rare staining events were observed in normal pancreatic tissues (Fig. 1B). The overexpression of SHH was observed in 55 of 70 (78.6%) pancreatic cancer samples compared with only 1 of 8 (12.5%) normal tissues \( (P = 0.0004, \text{Fisher exact test}; \text{Fig. 1C}) \). In addition, SHH overexpression was found in 4 pancreatic cancer cell lines

![Figure 1](image_url). Expression of SHH in pancreatic carcinoma and normal pancreatic (NP) tissues. A, representative Western blot analyses showing SHH expression levels in pancreatic carcinoma tissues and NP tissues. B, representative IHC images showing the overexpression of SHH in pancreatic carcinoma tissue (left) and normal expression levels of SHH in NP tissue (right; \( \times 100, \text{bar,}100 \mu \text{m}; \text{insets} \times 200 \)). C, compared with normal pancreas tissues, pancreatic carcinoma tissues displayed increased levels of SHH. Fisher exact test was used for statistical analyses. D, Kaplan–Meier survival analysis based on SHH expression levels in 70 patients with pancreatic carcinoma (log-rank test). The probability of patient survival among the following groups: SHH−, normal expression of SHH; SHH+, overexpression of SHH \( (P < 0.001) \).
(i.e., MiaPaCa-2, AsPc-1, SW1990, and CFPAC-1), whereas the other 2 pancreatic cancer cell lines (i.e., BxPc-3 and Panc-1) showed low levels of SHH expression (Supplementary Fig. S3A–S3C). The expression of SHH in normal PSCs was undetectable.

Kaplan–Meier analysis showed that the mean survival time for patients with pancreatic cancer with overexpression of SHH was 9.24 months, compared with 24.09 months for patients with pancreatic cancer with low expression of SHH (P < 0.001, log-rank test; Fig. 1D). Multivariate Cox regression analysis showed that SHH expression is an independent prognostic factor for poor survival in patients with pancreatic cancer (RR, 3.444; 95% confidence interval [CI], 1.564–7.582; Supplementary Table S3). Furthermore, correlation analysis demonstrated that the overexpression of SHH positively correlated with pancreatic cancer distant metastasis, TNM stage, and PNI (P < 0.05; Supplementary Table S4).

**SHH overexpression in tumor cells plays a role in PNI in pancreatic cancer**

To determine whether SHH overexpression is involved in the PNI of human pancreatic cancer, we analyzed SHH expression levels in the surgical cases either with or without PNI. The Western blot results showed that the relative SHH protein expression levels of PNI pancreatic cancer tissues were significantly increased compared with those of non-PNI pancreatic cancer tissues (P = 0.02; Fig. 2A and B).

We next examined the relationship between SHH expression and PNI in histologic sections from pancreatic cancer samples. Of the total 70 pancreatic cancer samples prepared for histologic sections, 43 (61.4%) displayed PNI, and 27 (38.6%) did not display PNI. Pancreatic cancer samples with PNI showed strong SHH immunoreactivity, whereas pancreatic cancer samples that lacked PNI showed mostly negative to faint SHH staining (Fig. 2C and Supplementary Fig. S3D). Strong immunoreactivity was generally observed in the cytoplasm and cell membranes of cancer cells. PNI was significantly associated with SHH protein expression in tumor cells (Fig. 2D).

**Overexpression of SHH in tumor cells activates the Hh pathway in PSCs in the stroma in vitro**

As the overexpression of SHH was positively associated with pancreatic cancer metastasis, TNM stage, and PNI, we investigated the impact of SHH at the cellular level on histologic activities in vitro. The data suggest that inhibition of the Hh pathway in pancreatic cancer cells by the blocking of SMO could influence cellular activities such as growth, invasion, and apoptosis (Supplementary Fig. S4). However, elevated autocrine SHH levels because of ectopically overexpressed SHH do not activate the Hh pathway in pancreatic cancer cell lines in vitro (Supplementary Fig. S5).

After confirmation of the high secretion of SHH on ectopic SHH-transfected cells (Fig. 3A), we investigated whether paracrine SHH expression in pancreatic cancer cells has an impact on PSC growth. Significant differences in PSC growth were observed after 48 hours of exposure with the various CMs (P < 0.05; Fig. 3B). The growth of PSCs cultured with CM derived from Panc-1 cells overexpressing SHH increased by 54.5% compared with vector cells (Fig. 3B). Similarly, the PSC migration toward CM derived from Panc-1 SHH cells was remarkably facilitated compared with CM derived from normal cells (P < 0.05; Fig. 3B). Notably, treatment with cyclopamine had a suppressive effect on the elevated migration associated with paracrine SHH or exogenous rSHH expression.

We further determined whether the Hh pathway in PSCs is activated via paracrine SHH in pancreatic cancer cells. Immunofluorescence analysis revealed that Gli-1, the activated Hh pathway transcriptional factor, was more stably and predominantly expressed in the cytosol and nucleus of PSCs cultured with CM derived from Panc-1 SHH cells, which could be abrogated by cyclopamine (Fig. 3C). Similar results were also verified by Western blot analysis and real-time PCR (Fig. 3D). Moreover, increased expression of Gli-1 was paralleled by elevated MMP-2, MMP-9, and NGF expression in PSCs cultured with SHH-rich CMs. Therefore, we found that paracrine SHH in pancreatic cancer cells activates the Hh pathway and promotes MMP-2, MMP-9, and NGF expression in PSCs in vitro.

**Paracrine SHH in pancreatic cancer cells promotes tumor growth and invasion**

To test the role of paracrine SHH to PSCs on tumor progression, we established the models of subcutaneous tumor xenograft in athymic nude mice by injection of tumor cells alone or tumor cells plus PSCs. Coinjection of PSCs resulted in a significant increase in tumor growth rates when comparing AsPc-1 cells alone with AsPc-1 cells coinjected with PSCs. Moreover, there was a notably reduction in tumor growth rates when AsPc-1 cells were coinjected with PSCs, in which the Hh receptor SMO was blocked with cyclopamine (Fig. 4A). Notably, the tumors in nude mice after cell injection showed an obvious increase in invasiveness to the surrounding tissue, including the sciatic nerve, skin, and muscles, in the AsPc-1 cells coinjected with PSCs compared with the AsPc-1 cells alone (Fig. 4B). However, pretreatment with cyclopamine in the PSCs attenuated the enhancement of tumor invasiveness resulting from coinjection.

At same time, we observed different histologic structures, especially in the stromal component in the tumor tissues. Only poorly differentiated gland-like structures assembled by tumor cells overexpressing SHH were observed in xenografts solely injected with AsPc-1, whereas abundant stroma composed of numerous fusiform PSCs surrounded the tumor epithelium (Supplementary Fig. S6A). Hedgehog pathway activation was confirmed via the analysis of SHH-stimulated PSCs, which showed increased cytoplasmic expression of the transcriptional factor Gli-1 (Supplementary Fig. S6B). Furthermore, real-time PCR showed that MMP-2, MMP-9, and NGF expression levels were increased in the AsPc-1 cells coinjected with PSC tumor, which was not observed with AsPc-1 cells injected alone or
with AsPc-1 cells coinjected with cyclopamine-pretreated PSCs (Supplementary Fig. S6B), thereby indicating that these PSCs are responsive to paracrine stimulation with SHH, resulting in the increased expression of MMP-2, MMP-9, and NGF. These data provide clues that the interaction between SHH protein secreted from the tumor cells and PSCs in the tumor microenvironment enhance tumor invasiveness and progression in pancreatic cancer.

**Activated PSCs are essential to promote PNI in an in vitro model of nerve invasion in cancer**

As SHH overexpression in pancreatic cancer cells was involved in PNI, we hypothesized that SHH-activated PSCs may act as the "signal transit station" for interactions between tumor cells and nerves both to promote tumor invasion toward the nerves and induce nerve growth toward the tumor cells. Therefore, we evaluated the effect of PSCs...
Figure 3. SHH derived from pancreatic cancer cells modulates PSC proliferation and migration. A, enzyme-linked immunosorbent assay analysis of SHH concentrations in various CMs (*, \(P < 0.05\)). The CM derived from the pancreatic cancer cells AsPC-1, which expresses high levels of SHH, was used as a positive control, whereas that derived from PSCs lacking SHH expression was used as a negative control. B, PSCs were treated with or without rSHH (recombinant SHH, 2 \(\mu\)g/mL) in various CMs. Cell proliferation was quantified by MTT assay (left). The migration of PSCs in response to various CMs with or without rSHH was observed. The number of migrated cells was quantified by performing cell counts of 10 random fields at \(> 200\) magnification (right). *\(, P < 0.05\), compared with controls; **\(, P < 0.05\), compared with cells treated with 10 \(\mu\)mol/L cyclopamine. C, the expression of Gli-1 protein in PSCs in response to various CMs with or without 10 \(\mu\)mol/L cyclopamine treatment. Cells were labeled with fluorescence-conjugated Gli-1–specific antibody (green), and nuclei were stained with DAPI (blue; \(> 200\)). D, Western blot analysis of the expression of the indicated genes in PSCs in response to various CMs with or without 10 \(\mu\)mol/L cyclopamine treatment. β-Actin was used as an internal control (top). CM derived from Panc-1.SHH cells obviously increased Gli-1, MMP-2, MMP-9, and NGF expression in PSCs in vitro, and a significant decrease in Gli-1, MMP-2, MMP-9, and NGF expression was observed when the receptor Smoothened (SMO) was inhibited with cyclopamine in PSCs. Otherwise, we found no difference between the PSCs cultured with CMs derived from Panc-1.N1 cells with or without cyclopamine treatment. The comparative C (T) method was used to quantify the expression of the indicated genes using real-time PCR in PSCs in response to various CMs with or without 10 \(\mu\)mol/L cyclopamine treatment (bottom). GAPDH was used as an internal control.
on cancer cell migration and nerve outgrowth using the in vitro coculture models of nerve invasion (Supplementary Fig. S2). The ability of pancreatic cancer cells to invade the DRG and for the DRG axons to grow toward the pancreatic cancer colonies was assessed by calculating the nerve invasion index and DRG outgrowth index, respectively (Fig. 4D). We found that Panc-1.SHH cells increased the nerve invasion index and the DRG outgrowth index in the presence of PSCs compared with the absence of PSCs (Fig. 4C and D). In contrast, no significant enhancement of these

Figure 4. Paracrine SHH expression in pancreatic cancer cells promotes subcutaneous xenograft tumor growth and invasion in vivo, and the migration of pancreatic cancer cells to the DRG and the neurite outgrowth from the DRGs extended to the pancreatic cancer cell colonies in a 3D coculture model in vitro. A, Matrigel mixtures containing $5.5 \times 10^6$ AsPc-1 cells or mixed $5 \times 10^6$ AsPc-1 cells with $0.5 \times 10^6$ PSCs were implanted subcutaneously in the flank site of BALB/c nude mice. Meanwhile, a third group with mouse subcutaneous xenograft was established using $5 \times 10^6$ AsPc-1 cells with $0.5 \times 10^6$ cyclopamine-pretreated PSCs. Tumor volumes were determined by measuring the width and length of the tumors every 2 days (low). Mean ($n = 8$); bars, SD. B, representative photograph of AsPc-1 cell implantation, AsPc-1 plus PSCs coimplantation and AsPc-1 plus cyclopamine-pretreated PSC coimplantation mice bearing xenograft tumor. White arrows show the sciatic nerve. Note that sciatic nerves were involved in and infiltrated by tumor in the AsPc-1 cells cojected with PSCs in mice. C, a representative microphotograph of the experiments shows neural invasion at day 8 in different groups. In the 3D coculture model, pancreatic cancer cells gradually migrated to the DRG, and the neurite outgrowth extended to the pancreatic cancer cell colony from the DRGs, which provided an invasive pathway for the colony. PSCs promote neurotropism in Panc-1.SHH cells and DRG neurite outgrowth. The red lines indicate the initial edge of pancreatic cancer cell colonies. The black lines show the edge of outgrowing DRG neurite, which was attracted by pancreatic cancer cells. The green lines indicate the total distance measured between the pancreatic cancer cells colonies and the DRGs. D, nerve invasion index (left) and DRG outgrowth index (right) in different coculture groups at day 8. An illustration (top) showing the calculation of the nerve invasion index ($a/g$) and the DRG outgrowth index ($b/g$). *, $P < 0.05$. 
indexes between Panc-1.N1 cells with or without PSCs was observed. Furthermore, we observed a greater number of neurites growing toward the pancreatic cancer colonies in the group of Panc-1.SHH cells with PSCs (Fig. 4C and Supplementary Fig. 5C). In addition, in the group of Panc-1.SHH cells with PSCs, the numbers of axons from the DRG ventral to the colony side of the pancreatic cancer were increased compared with the DRG dorsal to the colony side of the pancreatic cancer. Collectively, these data suggest that PSCs activated by paracrine SHH in pancreatic cancer cells promote the interaction between pancreatic cancer cells and nerves in vitro, which is most likely the underlying cause of PNI.

**Activated PSCs promote pancreatic cancer cell invasion of peripheral nerves in vivo**

To mimic the natural tumor microenvironment, the ability of pancreatic cancer cell lines to invade nerves in vivo was investigated. Cancer cells were implanted in a distal part of the sciatic nerve as reported by Gil and colleagues (29). Mice were randomized into the following 4 injection groups: Panc-1.N1, Panc-1.N1 plus PSC, Panc-1.SHH, or Panc-1.SHH plus PSC (n = 8). One week after the implantation of cells into a distal site of the sciatic nerve in nude mice, sciatic nerve function was assessed weekly for 7 weeks.

In the Panc-1.SHH plus PSC group, mice began to develop right hind-limb dysfunction 1 week after cell implantation (Fig. 5A). Of the 8 mice in this group, all were fully paralyzed by week 7 (Fig. 5B). In the other groups, right hind-limb dysfunction began 3 to 4 weeks after cell injection. The sciatic nerve score among the 4 groups of mice was significantly different (Fig. 5B). As an additional measure of sciatic nerve function, we examined hind-limb paw span (sciatic nerve index) in all mice. In the Panc-1.SHH plus PSC group, a statistically significant decrease in the right hind-limb sciatic nerve index from weeks 1 to 4 was observed, whereas in the other groups, no significant change in the sciatic nerve index was found from weeks 1 to 4 (Fig. 5C). In addition, we used a high-frequency ultrasound weekly to measure sciatic nerve diameter at a site 5-mm proximal to the cancer cell injection site. In the Panc-1.SHH plus PSC group, we found that increasing proximal nerve invasion was associated with decreasing sciatic nerve function (Fig. 5D). In contrast, both parameters remained normal in the other groups.

All mice were euthanized at week 7, and the sciatic nerves were excised (Fig. 5D). Histologic evaluation of these nerves revealed that either the tumor diameter at the point of implantation (reflecting tumor growth) or the proximal nerve diameter (reflecting neural invasion) in the Panc-1.SHH plus PSC group were significantly larger than those in the other groups (Fig. 5D). These in vivo data confirm the propensity of Panc-1-derived tumors to invade along nerves, as predicted by the in vitro model.

**PSCs activated by paracrine SHH significantly promote tumor growth, metastasis, and PNI in vivo**

The observation of increased sciatic nerve invasion in the Panc-1.SHH and PSC coimplantation tumors led us to further investigate the effect of PSCs activated by paracrine SHH on in vivo pancreatic cancer growth. Panc-1.N1 and Panc-1.SHH, along with or without PSCs, were orthotopically implanted into the pancreas of nude mice, which were subsequently monitored for 6 weeks and euthanized when signs of cachexia were observed. As shown in Fig. 6A, Panc-1.SHH cells that were coinjected with PSCs resulted in a significant decrease in survival time compared with Panc-1.SHH cells alone. PSCs reduced the survival time in response to Panc-1 cells that expressed high levels of SHH compared with Panc-1.N1 and Panc-1.SHH cells that were both coinjected with PSCs. In addition, there was no difference in survival time when Panc-1 cells not expressing high levels of SHH were coinjected with PSCs (Fig. 6A).

A significant increase in tumor volume in mice injected with Panc-1.SHH cells plus PSCs was observed (Fig. 6B). Tumors in the Panc-1.SHH cell plus PSC group displayed increased metastatic disease in the liver, spleen, and peritoneum (Fig. 6C). Next, we evaluated the PNI in orthotropic xenograft tumors via histopathologic examination. The results (Fig. 6D) showed a significant difference in the incidence rates of neural invasion between the 4 groups, thereby suggesting that PSCs activated by SHH paracrine signaling derived from tumor cells regulate neural invasion in the tumor microenvironment during tumor growth in vivo.

**Discussion**

A prominent neural phenomenon in pancreatic cancer has been characterized by PNI of cancer cells, which is closely associated with the severity of intrapancreatic neuropathic alterations, including increased neural density and hypertrophy (30). Those neuropathic changes, referred to as "neural remodeling," are associated with desmoplasia in pancreatic cancer and chronic pancreatitis (2). This observation has provided insight into the function of tumor stroma PSCs in the regulation of the tumor microenvironment; however, until now, the potential important function of PSCs in PNI was unclear. In this report, we hypothesized that PSCs may act as the "signal transit station" for tumor cell–nerve interactions both to promote tumor invasion toward nerves and to induce nerve growth toward tumor cells. Our results show that the abnormally high expression levels of SHH in pancreatic tumor cells is associated with nerve invasion and metastasis and represents an important independent prognostic factor. We further showed that PSCs activated by paracrine SHH displayed significantly increased MMP-2, MMP-9, and nerve growth-related factor NGF expression levels and promoted the interaction between tumor cells and nerves, resulting in pancreatic cancer PNI. In addition, we provide evidence that inhibition of the Hh pathway in PSCs abrogated pancreatic cancer development and sciatic nerve invasion in subcutaneous xenograft tumors.

As an essential developmental signal, SHH is critical for maintaining tissue polarity (31) and stem cell populations (32). A recent retrospective study has indicated that SHH is
Weeks after tumor injection

Sciatic nerve score

Sciatic nerve index (mm)

Week

0 1 2 3 4 5 6 7

Panc-1-N1

Panc-1-N1 + PSC

Panc-1-N1.SHH

Panc-1-N1.SHH + PSC

Ultrasound

In situ image

H&E staining

Panc-1-N1

Panc-1-N1.SHH

Panc-1-N1 + PSC

Panc-1-N1.SHH + PSC

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frequently amplified in pancreatic cancer, and the over-expression of SHH may be important in the acquisition of an invasive phenotype in pancreatic cancer (33, 34). However, the impact of SHH expression on pancreatic cancer patient survival and the mechanism by which SHH regulates pancreatic cancer cell metastasis remains unclear.

Our results were consistent with other previous findings (35) in which SHH was frequently overexpressed in pancreatic cancer tissues, and it significantly correlated with tumor metastasis, advanced clinical stage, and nerve invasion. Importantly, we also found that the overexpression of SHH in pancreatic cancer was a strong and independent predictor of cancer-specific poor survival rates. These findings underscore the potentially important function of SHH involved in an underlying biologic mechanism in the progression of pancreatic cancer, and the upregulation of SHH

Figure 6. PSCs activated by paracrine SHH significantly promote tumor growth, metastasis, and PNI in vivo. Two different SHH expression-level pancreatic cancer cell lines, Panc-1.N1 and Panc-1.SHH, either with or without PSCs, were orthotopically implanted into the pancreas of nude mice (n = 8 per group), which were subsequently monitored for 6 weeks and euthanized when signs of cachexia were observed. A, survival curves of mice with orthotopic tumors at different groups. B, representative images (left) and graphical representation (right) of primary tumor volume (mm$^3$) in mice with orthotopic tumors. *, P < 0.05. C, a graphical representation of the number of mice with metastasis in different organ sites. D, a graphical representation of PNI in mice with orthotopic tumors.

Figure 5. Activated PSCs promote the pancreatic cancer cell invasion toward murine sciatic nerves in vivo. A, representative images of mice 4 weeks after tumor implantation showing right hind limb paralysis in the Panc-1-N1.SHH plus PSC group (top) compared with the normal function observed in the Panc-1-N1 group (bottom). B, the mean right sciatic nerve function scores of different groups. The nerve score of 4 indicates normal function, and 1 indicates total paralysis. C, sciatic nerve index (paw span in millimeters) of different groups at 1, 4, and 7 weeks after tumor implantation. D, using ultrasound, the degree of nerve invasion was determined by measuring the tumor diameter at the implantation site (“tumor growth”) and the nerve diameter 5-mm proximal along the sciatic nerve (“tumor nerve invasion”). Representative high-resolution ultrasound of sciatic neural invasion in mice of different groups at week 4. The red dotted lines delineate the sciatic nerves; white bar, 5 mm. In situ image of the sciatic nerve tumors in different groups showing that either the tumor diameter at the point of implantation or the proximal nerve diameter in the Panc-1.SHH plus PSC group was larger compared with those in the other groups. The arrowheads indicate the sciatic nerve; white bar, 5 mm. HE staining confirmed cancer cell invasion along the nerve in the murine model. The tumor diameter at the implantation site was increased in the Panc-1.SHH plus PSC group compared with the other groups; importantly, the proximal nerve diameter was significantly larger in the Panc-1.SHH plus PSC group than in the other groups. Black bar, 5 mm.
in pancreatic cancer may play a pivotal role in cancer–nerve interactions. In addition, the levels of SHH protein expressed in pancreatic cancer tissues with or without PNI, as determined by IHC and Western blot analysis, showed that SHH overexpression in tumor cells was involved in PNI in pancreatic cancer.

Multiple reports have described tumor cell autonomous (36, 37) and stromal cell-paracrine functions (17, 18, 38, 39) for SHH signaling in tumors. Our observations suggest that SHH signaling induces pancreatic cancer development via a paracrine manner to regulate the tumor microenvironment rather than an autocrine manner. Using a coculture system of transfective pancreatic cancer cells and primary human PSCs in vitro, we found that paracrine SHH protein expressed in cancer cells activates the Hh pathway in PSCs, thereby promoting PSC proliferation and migration and increasing MMP-2, MMP-9, and NGF expression levels. In our in vivo experiments, we found that paracrine PSCs activated by SHH significantly promote animal xenograft tumor growth and invasion; however, in PSCs pretreated with cyclopamine to block the Hh pathway, the effect described above was reversed. Taken together, our observations suggest that in the pancreatic tumor microenvironment, paracrine SHH protein derived from tumor epithelial cells activates the Hh signaling pathway in neighboring stromal PSCs and subsequently starts the transcription of the downstream genes to promote tumor progression.

Ceyhan and colleagues (30) have suggested that both fibrosis in chronic pancreatitis and desmoplastic reactions in pancreatic cancer seem to have a significant impact on increased pancreatic neuropathy and the severity of neural invasion. It has also been shown that stromal PSCs may play a regulatory role in the interaction between cancer cells and nerves (40, 41). It has been reported (42) that immortalized human PSC lines secrete NGF when stimulated by TGF-β, and the supernatants of hPSC strongly stimulated neurite outgrowth (11).

Our coculture in vitro study shows that activated PSCs in the tumor microenvironment enhance both DRG neurite outgrowth toward pancreatic cancer colonies and pancreatic cancer cell migration toward DRGs. In our in vivo study, we used a nude mouse sciatic nerve invasion model to expand on the mechanism of PNI and to especially examine tumor cell invasion and proliferation along nerves. PSCs in the presence of pancreatic cancer cells with high SHH expression levels resulted in enlarged tumor masses, accelerated nerve dysfunction, and enhanced PNI. These findings, along with those of studies conducted in 3D coculture assays, suggest the existence of interactions between pancreatic cancer cells and PSCs via paracrine SHH protein, and this mutual interaction potentially promotes PNI in pancreatic cancer. In addition, orthotopic tumor models were examined to confirm that SHH paracrine signaling induces molecular alterations, including increased NGF, MMP-2, and MMP-9 expression levels in the stromal PSCs that promote PNI and metastasis. It has been reported (43) that neurotrophins, such as NGF, can stimulate tumor cell proliferation and invasion, and contribute to promote neurite growth in pancreatic cancer. Other studies (44, 45) have reported increased expression of MMP-2 and MMP-9 in response to the stimulation of glial-derived neurotrophic factor (GDNF) and NGF in pancreatic cancer cells, which is thought to contribute to PNI. These results are consistent with the hypothesis (46) that PSCs may regulate the expression of molecules involved in PNI and induce neuronal plasticity, which renders nerves more vulnerable to invasion.

In conclusion, our results suggest that PSCs affected by paracrine SHH signaling promote PNI in pancreatic cancer, thereby emphasizing the critical role of PSCs in the tumor microenvironment. We believe that this paracrine loop has important clinical implications as it involves novel pharmacologic targets that may be targeted to reduce tumor neuroinvasion and to improve the survival of patients with pancreatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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