PanIN Neuroendocrine Cells Promote Tumorigenesis via Neuronal Cross-talk

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Abstract

Nerves are a notable feature of the tumor microenvironment in some epithelial tumors, but their role in the malignant progression of pancreatic ductal adenocarcinoma (PDAC) is uncertain. Here, we identify dense innervation in the microenvironment of precancerous pancreatic lesions, known as pancreatic intraepithelial neoplasms (PanIN), and describe a unique subpopulation of neuroendocrine PanIN cells that express the neuropeptide substance P (SP) receptor neurokinin 1-R (NK1-R). Using organoid culture, we demonstrated that sensory neurons promoted the proliferation of PanIN organoids via SP-NK1-R signaling and STAT3 activation. Nerve-responsive neuroendocrine cells exerted trophic influences and potentiated global PanIN organoid growth. Sensory denervation of a genetically engineered mouse model of PDAC led to loss of STAT3 activation, a decrease in the neoplastic neuroendocrine cell population, and impaired PanIN progression to tumor. Overall, our data provide evidence that nerves of the PanIN microenvironment promote oncogenesis, likely via direct signaling to neoplastic neuroendocrine cells capable of trophic influences. These findings identify neuroepithelial cross-talk as a potential novel target in PDAC treatment.

Introduction

Neuronal influences on tumorigenesis have been described in prostate (1) and gastric cancers (2) but their role in pancreatic ductal adenocarcinoma (PDAC) remains unclear. PDAC has a tropism for nerves and is associated with high rates of perineural invasion that portends a worse prognosis (3). Reciprocal molecular signaling between PDAC and nerves appears to cause global neural remodeling as tumors show increased neural hypertrophy (3). Whether nerves are selectively present in the neoplastic microenvironment and can drive tumorigenesis of PDAC or its precursor lesion, pancreatic intraepithelial neoplasia (PanIN), is unknown.

The pancreas is richly innervated by intrinsic, autonomic, and sensory nerves. Visceral sensory afferent nerves project from dorsal root ganglia (DRG) and vagal nodose ganglia (NG) neurons (6), and have both afferent (sensory) and efferent (tissue modifying) functions (for review see ref. 7). Unmyelinated afferents, C and Aδ fibers, express the transient receptor potential cation channel subfamily V member 1 (TRPV1) channel that mediates the release of inflammatory neuropeptides such as substance P (SP; 8). SP binding to its target G protein-coupled receptor (GPCR), neurokinin 1 receptor (NK1-R), activates several oncogenic pathways (for review see ref. 9) in non-neuronal human cancers (10, 11). The SP–NK1-R axis, for example, can stimulate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT; ref. 12) signaling pathway, which is also an important driver of Kras-mediated oncogenesis in PDAC (13).

We hypothesized that sensory nerves in the PanIN microenvironment communicated directly with the PanIN epithelium to drive tumorigenesis. We identified a small population of unique neoplastic neuroendocrine cells expressing the neuropeptide receptor NK1-R in both murine and human PanINs. Using organoid culture techniques, we demonstrated that these neuron-responsive NK1-R+ cells exerted trophic influences and potentiated global PanIN organoid growth. Accordingly, sensory denervation in a PDAC mouse model decreased the NK1-R+ cell population and protected against PanIN progression, confirming a protumorigenic role of sensory nerves.

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Materials and Methods

Mouse strains

Pdx1-Cre, LSL-KrasG12D and LSL-Trap53R172H mice (The Jackson Laboratory) were bred to generate KCflox and KPCflox mice. Mist1: CneERT2, LSL-KrasG12D and LSL-tdTomato mice were bred to generate KCflox/tdTom mice. KCflox/tdTom mice were injected with Tamoxifen and treated with cerulein as described previously (14). Animal studies were approved by the Animal Care and Use Committees of Johns Hopkins University School of Medicine, Memorial Sloan Kettering Cancer Center and Cold Spring Harbor Laboratory.

Cell and organoid lines

A6L, MIA PaCa-2, and Capan-2 cells (Iacobuzio-Donahue laboratory) and 3T3 and 293T (ATCC) cells were maintained in RPMI-1640 or DMEM (Gibco) supplemented with 10% FBS (Sigma-Aldrich), GlutaMAX (Gibco), and 1× penicillin-streptomycin (Gibco). Human pancreatic ductal epithelial (HPDE) cells were cultured according to published protocols (15). Murine PanIN organoids (derived from KCflox and KPCflox mice) were cultured according to established protocols (16). A6L cells (2008) were authenticated with whole exome sequencing and confirmed to have mutant Kras (17). HPDE (2013), MIA PaCa-2 (2016), Capan-2 (2016), 3T3 (2015) and 293T (2016) cells were authenticated using short tandem repeat profiling. Organoids underwent lox-KrasG12D allele genotyping (2016).

Neuronal coculture

Dorsal root ganglia (DRG) from 4- to 8-week-old C57BL/6 mice were harvested and cultured in supplemented Neurobasal Medium (NB; Gibco) as previously described (18). Growth factor-free NB was used for all coculture experiments. DRG neurons were plated with PDAC cell lines (5 × 10^4 cells/well) in the microwell device for 48 hours (Supplementary Fig. S1A) or with organoids (1 × 10^5 cells/well) in Transwells (Corning) for 96 hours (Supplementary Fig. S1B). Organoid proliferation was measured with CellTiter-Glo assay for ATP (Promega) or MTT (Promega). Inhibitors L-733,060, RP 67580 and Statitic were used in some experiments.

Sensory denervation of KPCflox mice

7-day old KPCflox mice underwent a single subcutaneous injection of resiniferatoxin (RTX) or vehicle solutions. Early prophyllactic denervation before the development of PanINs obviated the concern of possible interactions of RTX with PanIN cells. Physiologic testing for somatic denervation was performed using the capsaicin-induced eye wipe response.

IHC and immunofluorescence

Fixed cells and deparaffinized murine and human sections were subject to immunofluorescence and hematoxylin and eosin (H&E) analyses as per standard protocols. Organoids were stained in whole mount in chamber slides (Lab-Tek). Pancreas tissues were optically cleared and stained as per established protocols for 3D analysis (19) and each view was scanned to a depth of 150 μm. Axon length densities (μm/μm^3) were calculated using Avizo 7.1 software.

Histopathologic analysis

Histopathologic analyses were performed on de-identified slides from 8-, 12-, and 16-week-old KPCflox mice. For each pancreas, 3 to 5 sections were sampled 150 to 200 μm apart and 5 to 15 random views were taken for each section. Lesions were classified as acinar to ductal metaplasia (ADM), PanIN1 (1A and 1B; “early”), PanIN2/3 (“late”) and PDAC (tumor) based on the classification consensus (20). PanIN and stroma burden were expressed as the percentage of total analyzed surface area occupied per lesion as previously described (Supplementary Fig. S2A; ref. 21). Proliferative activity, which correlates with degree of PanIN dysplasia (22), was assessed with Ki67 labeling. Cytokeratin 19 (CK19) staining of tumors further confirmed the presence of invasive cells in the stroma (Supplementary Fig. S2B). The fraction of mice with tumors at all ages was calculated.

Flow cytometry and clonogenic assays

Flow cytometry on live PanIN organoid and PDAC cells were performed using a FITC-conjugated anti-NK1-R antibody (Alomone) in a LSRFortessa 3 cell analyzer (BD Biosciences) based on established protocols (14). tdTomato+ or NK1-R– and NK1-R+ PanIN cells (using the conjugated anti-NK1-R antibody) from KPCflox/tdTom mice were FACS sorted on a FACSAria III cell sorter (BD Biosciences) based on established protocols (14). In clonogenic assays, sorted cells were cultured in 50% Matrigel (MG; Corning) with neurons (5,000 sorted cells/well) or in organoid media (500 sorted cells/well) for 7 days (Supplementary Fig. S3A–S3D).

Reassay reactions

FACS sorted NK1-R– and NK1-R+ cells were used to derive NK1-Rlow and NK1-Rhi organoids, respectively (Supplementary Fig. S3A–S3D). NK1-Rlow and NK1-Rhi organoids were dissociated into single cells and transduced with lentiviral constructs containing pLKO.1-puro-CMV empty vector and pLKO.1-puro-CMV-TurboGFP (Sigma), respectively, as per the manufacturer’s protocol. NK1-Rlow and NK1-Rhi/GFP organoids were plated as single cells at a 20:1 ratio (5 × 10^3 total cells/well) and imaged every 4 hours for 4 days in a microscope incubation chamber. NK1-Rlow and NK1-Rhi/GFP were plated at a 1:1 ratio (2 × 10^3 total cells/well) and subject to flow cytometry analysis at several time points.

Quantitative real-time PCR

RNA extraction and cDNA synthesis was done as described previously (21). qPCR was performed on a QuantStudio 6 Flex System (Applied Biosystems). TaqMan Universal PCR Master Mix (Applied Biosystems) was used with chromogranin A (CgA) and Gapdh primers, Mm99999915_g1 and Mm00514341_m1 (Thermo Fisher), respectively. All other cDNAs were amplified with PowerUp SYBR Green Master Mix (Applied Biosystems) with the primers listed in Supplementary Table S1. Relative amounts of mRNA were calculated by the comparative ΔCt method with Gapdh and/or Hprt as house-keeping genes.

Western blotting

Organoids were starved for 24 hours, dissociated and incubated with media with or without SP. Cell extracts were prepared according to standard protocols using cell lysis buffer (RIPA; Pierce) with protease and phosphatase inhibitors (Roche). Membranes were incubated with antibodies and developed with Trident ECL (Genetex). Densitometric analysis was done in ImageJ.

Substance P ELISA

Substance P (SP) concentration was quantified using a Fluorescent Immunoassay Kit as per the manufacturer’s protocol (Phoenix Pharmaceuticals, Inc.).
**Statistical analysis**

Data were analyzed using GraphPad Prism (GraphPad Software, Inc.) and were expressed as mean ± SEM. Comparisons between groups where data were normally distributed were made with the Student t test, and comparisons with categorical independent variables were performed using Fisher exact test. Detailed protocols and standard procedures are described in Supplementary Materials and Methods.

**Results**

**PanINs and PDAC cells demonstrate neurotropism**

Increased global nerve density has been suggested in pancreas from human PDAC (3) and murine PanINs (23) compared with densities in pancreas from disease-free subjects. We aimed to characterize the nerve density in the immediate PanIN microenvironment compared with respective PanIN-free pancreas in isogenic mice to control for neural remodeling changes that may occur with genotype and disease state variables. We used 3D whole mount staining to reconstruct the fine intra-visceral axons (<5 μm) that are not easily visualized on 2D histology (Supplementary Fig. S4A and S4B; ref 19). In 12-week-old KPC<sup>Pdx1</sup> mice, axons were observed in close proximity to PanIN epithelial cells in the immediate microenvironment (Fig. 1A and B; Supplementary Video SV1). We quantified the nerve density surrounding PanINs versus their respective PanIN-free pancreas tissue in 12-week KC<sup>Pdx1</sup> mice, which have...
only early PanINs, and age-matched KPC<sup>Pdx1</sup> mice, which have mostly late PanINs (Fig. 1C and D; Supplementary Fig. S4C). KCPdx1 and KPCPdx1 mice had a 3.2 ± 0.84 and 10.1 ± 2.4-fold increase in nerves densities compared with adjacent tissues, respectively. PanIN-associated fibers had a grossly aberrant branching pattern compared with acinar axons. Similar to our findings, neoplasia-associated nerves in prostate cancer show evidence of new sprouting and axonogenesis (1).

Having observed markedly increased axon density around PanIN lesions in vivo, we investigated whether neoplastic pancreatic cells actively recruited more axons. We cocultured DRG neurons with human PDAC or non-neoplastic HPDE cells in a microfluidic system that allows axonal interactions and models intrapancreatic sensory innervation (Supplementary Fig. S1A). We used PDAC cell lines because of technical limitations of 3D culture of organoids in the device. The cocultured PDAC cell lines, A6L and MIA PaCa-2, recruited 3.30 ± 0.31 and 3.43 ± 0.46 times more sensory axons, respectively, than the HPDE cells (Supplementary Fig. S4D and S4E). Cocultured axons also expressed synapsin proteins, indicating active vesicular transport and neurotransmitter release (Supplementary Fig. S4F and S4G). Reflective of these in vitro findings, PanIN cells likely actively recruit axons to their microenvironments. Interestingly, mutations in axon guidance genes, such as semaphorins, in human PDAC are significant, associated with a worse prognosis and may explain increased axon recruitment (24).

PanIN neuroendocrine cells express the substance P neuropeptide receptor

Because we observed axons in close proximity to PanIN epithelial cells and showed that PDAC cells robustly recruited sensory axons, we hypothesized that nerves, like other stromal components (21; for review see ref. 25), may communicate with and regulate the PanIN epithelium. The KPC<sup>Pdx1</sup> PanIN epithelia were negative for expression of several nerve-responsive elements such as the neurotrophic receptors p75, TRKA, TRKB, and TRKC that can be expressed in human PDAC (data not shown; ref. 4). The well-characterized sensory neuropeptide SP and its receptor, NK1-R, have been shown to mediate neurogenic inflammation in the pancreas (26) and promote mitogenesis in several cancers (9–11). We discovered that a subpopulation of PanIN epithelial cells in the KPC<sup>Pdx1</sup> mouse expressed NK1-R. These cells were present as a low-abundance population in the PanIN epithelium and expressed NK1-R in a cytoplasmic and membranous pattern (Fig. 2A and Supplementary Fig. S5A–S5C). In areas of PanIN-free pancreas, NK1-R was expressed only in islets (Fig. 2A; and Supplementary Fig. S5D–S5F).

Figure 2.
PanIN neuroendocrine cells express the neuropeptide receptor NK1-R. Confocal immunofluorescence analyses of murine and human PanINs stained with E-cadherin (gray) and nuclei with DAPI (blue). A, PanIN in a KPC<sup>Pdx1</sup> pancreas (white dashes) next to an islet (yellow dashes). Note PanIN NK1-R<sup>+</sup>/CgA<sup>+</sup> cells (arrowheads) and NK1-R<sup>+</sup>/CgA<sup>−</sup> cells (arrows). B, Human PanIN lesion with NK1-R<sup>−</sup>/CgA<sup>−</sup> cells (arrowheads) and NK1-R<sup>+</sup>/CgA<sup>−</sup> cells (arrows). C, tdTomato<sup>+</sup> PanINs in a KCP<sup>mutT</sup> pancreas. All PanIN NK1-R<sup>+</sup> cells are tdTomato<sup>+</sup> (arrowheads). All scale bars, 25 μm. D, Quantification of NK1-R<sup>+</sup> cells/PanIN. Seventy sections from mice of different ages (n = 10) and 33 sections from patients (n = 16) were quantified. The data represent mean ± SEM (two-tailed unpaired t test; *, P ≤ 0.05).
Supplementary Fig. S5A). NK1-R expression in islets raised the question of whether PanIN NK1-R⁺ cells also displayed features of endocrine differentiation. PanIN NK1-R⁺ cells expressed the pan-endocrine marker chromogranin A (CgA) but not insulin, confirming their identity as neuroendocrine cells.

We surveyed PDAC tumor sections from 16 patients containing synchronous PanIN lesions. NK1-R⁺ and CgA⁺ neuroendocrine cells were identified in one or more PanINs of all patients (Fig. 2B; Supplementary Fig. S5D and S5E). As in the mouse, NK1-R expression was limited to islets in disease-free areas. The frequency of NK1-R⁺ cells per lesion increased with PanIN grade in KPCPdx1 mouse but not in human PanINs (Fig. 2D). Even early KPCPdx1 PanINs composed of a few neoplastic cells also expressed the NK1-R, suggesting a potential early and persistent role in PanIN evolution (Supplementary Fig. S5A).

Because NK1-R and CgA are generally expressed by islet cells, we investigated whether the NK1-R⁺ cells were intrinsic neoplastic cells, or alternatively, of an endocrine lineage that had been incorporated into the PanIN epithelium. We stained for NK1-R in the lineage traced KCMist1tdTmouse that expresses tdTomato only in cells that have undergone Cre-mediated recombination as directed by the acinar-specific Mist1 promoter (27). All NK1-R⁺ cells in the PanIN epithelium coexpressed tdTomato, confirming that these were acinar cell-derived neoplastic PanIN epithelial cells (Fig. 2C; Supplementary Fig. S5F).

We incidentally saw expression of NK1-R on a subpopulation of CgA⁺ enteroendocrine cells (EEC) in human duodenal villi (Supplementary Fig. S6A), suggesting similarities between PanIN and intestinal neuroendocrine cells. Even though PanIN neuroendocrine cells were a subpopulation in the epithelium, we hypothesized they may have the potential to affect global PanIN biology through paracrine niche influences, similar to the way EECs can indirectly regulate crypt stem cells (28).

**Sensory neurons promote PanIN organoid proliferation**

To investigate whether PanIN NK1-R expression could mediate neuroepithelial cross-talk, we used KC177 PanIN organoids in coculture experiments. The organoid culture is a powerful tool to study noncancerous pancreatic epithelial cells, such as PanIN cells, as it allows for propagation of these cells without the need of a mesenchymal niche while preserving the characteristics of the source epithelium (16, 29). All PanIN organoid lines studied expressed cytoplasmic and membranous NK1-R as detected on immuno-fluorescence staining and immunoblotting (Fig. 3A and B). In contrast with the low abundance NK1-R⁺ cells in vivo, PanIN organoid cells uniformly expressed NK1-R, supporting the potential for sensory innervation to affect PanIN biology in vitro (Fig. 3A and B).

![Figure 3](image_url)

**Figure 3.**

Sensory neurons increase PanIN organoid proliferation. **A,** Immunofluorescence confocal projection of a PanIN organoid stained with NK1-R (red), EpCAM (gray), and nuclei (blue); scale bars, 25 µm. **B,** Western blot analysis for NK1-R expression in mouse brain (positive control) and PanIN organoids (β-actin, loading control). **C,** Proliferation of cocultured PanIN organoids (RLU, random luminescence units; n = 3–4). Note break in y-axis. Representative bright field (BF) images of viable MTT-stained organoids (arrowheads) in this experiment; scale bars, 200 µm. **D,** Effects of NK-1R inhibitors on organoids cocultured with neurons (n = 4–6). Monoculture controls not shown. RLU values are normalized to monoculture controls in each experiment. **E,** Live confocal images of organoid colonies from FACS sorted and cocultured tdTomato⁺ single PanIN cells from a KC177tdT mouse pancreas; scale bars, 500 µm. All cells are tdTomato⁺ (inserts); scale bars, 50 µm. Organoid colony (>20-µm diameter) counts (n = 2). The data represent mean ± SEM (two-tailed unpaired t test; ***, P ≤ 0.001; ****, P ≤ 0.0001).
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NK1-R, possibly indicating enrichment of receptor expression in organoid culture. The universal expression of NK1-R in organoid cells and minimal expression in PDAC cell lines was confirmed by flow-cytometry studies (Supplementary Fig. S7A). PanIN organoids also diffusely expressed CgA (Supplementary Fig. S7B).

To recapitulate in vivo conditions with the potential for reciprocal signaling between neurons and cancer cells, we cocultured DRG neurons with PanIN organoids (Supplementary Fig. S1B). Neurons increased organoid proliferation to a greater degree than 3T3 murine fibroblasts (38.7 ± 3.5-fold vs. 9.3 ± 0.8-fold), an established tumorigenic stromal cell type in the microenvironment (Fig. 3C; ref. 30). Neuron-mediated PanIN organoid proliferation could be partially but significantly blocked by two NK1-R antagonists L-733,060 and RP 67580, by 43.2 and 32.3%, respectively (Fig. 3D; Supplementary Fig. S7C). To confirm that the neuronal effect on organoid proliferation was mediated by a soluble factor, we incubated organoids with neuronal conditioned media and found 3.1 ± 1.4-fold increased proliferation that was also blocked by L-733,060 by 31.5% (Supplementary Fig. S7D). Neuronal conditioned media contained SP (20–40 pg/ml) as measured by ELISA. The decreased degree of proliferation in conditioned media compared with coculture suggested that reciprocal cross-talk between organoids and neurons may help mediate neoplastic proliferation. Unlike neuronal coculture, SP incubation alone did not cause organoid proliferation likely due to known peptide instability in solution (data not shown). Conversely, limited studies have shown that SP alone mediates proliferation of PDAC cell lines and the data are inconsistent (32, 33). Because NK1-R antagonism only partly blocked PanIN organoid proliferation, additional neuronal factors such as nerve growth factor (4) and glial cell-derived neurotrophic factor (5) could drive PanIN oncogenesis but were not explored in this study.

Having observed that DRG sensory neurons exerted a robust proliferative influence on PanIN organoids, we hypothesized that neurons could support the survival of primary PanIN cells. FACS-sorted PanIN cells from the KCATminTdt pancreas formed 6.1 ± 1.2-fold more organoid colonies than single cells at a roughly in vivo ratio of 20:1 (Fig. 4B and C; Supplementary Fig. S8A). This plating ratio was important so as to not drive the reaction in a biased direction. Surprisingly, the NK1-Rhi/GFP cells formed 3.5-fold more colonies from 6.7% to 9.9% closely reflected their observed ratios in PanIN epithelium in vivo (Fig. 2D). Further characterization of NK1-Rhi/GFP organoid dissociation was a dynamic process with highly mobile NK1-Rhi/GFP organoids that appeared to preferably merge heterogeneously (Supplementary Video SV2). NK1-Rhi/GFP cells also maintained a constant low cell population in composite organoids over time when plated at equal starting ratios (Fig. 4D; Supplementary Fig. S8B). Strikingly, the NK1-Rhi/GFP cell frequencies (ranging from 6.7% ± 0.11% to 9.9% ± 2.9%) closely reflected their observed ratios in PanIN epithelium in vivo (Fig. 2D). Even though NK1-Rhi/GFP organoids were observed to grow comparably to NK1-Rlow organoids in individual cultures, they showed restricted expansion in the composite cultures.

Further characterization of NK1-Rhi organoids with qPCR showed significantly decreased expression of proposed pancreatic, and potentially neoplastic, progenitor markers relative to NK1-Rlow cells, including Lgr5 (29) and doublecortin-like kinase-1 (Dclk1; Fig. 4E; refs. 14, 36). Hedgehog ligands, Shh and Ihh, which cooperate with mutant Kras in early PanIN development and are potentially activated in neoplastic “progenitor” cells (37), were also decreased. The Wnt pathway is a critical mediator of progenitor biology for review see ref. 38 and is notably activated in pancreatic injury and in organoid cultures (29). Like Hedgehog signaling, the canonical Wnt pathway is also critical for early PanIN development (39). Despite expressing many Wnt ligands similar to NK1-Rlow organoids, NK1-Rhi organoids had decreased activation of the downstream canonical versus noncanonical pathway (Fig. 4E; Supplementary Fig. S8C) as well as decreased expression of several members of the Wnt receptor, frizzled (Fzd), family.
Thus, the expression profile of NK1-R<sup>hi</sup> cells was less "progenitor"-like than of NK1-R<sup>low</sup> cells.

Considering together their ability to greatly potentiate composite organoid growth despite being a restricted subpopulation and their lower expression of known pancreatic progenitor markers and pathways, NK1-R<sup>+</sup> cells appeared to function as trophic "niche" cells with the potential to provide growth signals to NK-1R<sup>−</sup>/C<sup>0</sup> cells. The NK1-R<sup>+</sup> PanIN cells were also Ki67<sup>−</sup> on immunofluorescence analysis, further suggesting that they were not proliferating progenitor cells in vivo (Supplementary Fig. S8E).

**SP stimulates STAT3 phosphorylation in PanIN organoids**

Because sensory neurons robustly increased PanIN organoid proliferation and selectively signaled to NK1-R<sup>+</sup> PanIN cells, we hypothesized that SP should activate oncogenic pathways. Organoids incubated with SP were screened for known SP-mediated signaling pathways (9, 12, 40). SP rapidly phosphorylated JAK2 and STAT3 in the PanIN organoids but did not affect MAPK or AKT phosphorylation (Fig. 5A and B). Stat3, a small-molecule inhibitor of STAT3, decreased the proliferation of cocultured organoids by 50.8%, suggesting that the neuron-mediated organoid proliferation is significantly mediated through STAT3 activation (Fig. 5C). Indeed, the NK1-R<sup>+</sup> PanIN cells in vivo had a significantly increased p-STAT3 expression (by 35.4% ± 7.9%) compared with NK1-R<sup>−</sup> cells within the same PanIN lesion, suggesting that NK1-R<sup>+</sup> PanIN cells may be more responsive to neuropeptide signaling (Fig. 5D).

**Sensory denervation of KPC<sup>Pat1</sup> mice decreases PanIN progression**

Because nerves were abundant in the PanIN microenvironment and promoted PanIN organoid proliferation, we hypothesized that sensory axons may play an important role in PanIN growth in vivo. A possible role of sensory nerves on PanIN tumorigenesis has been suggested by studies that show capsaicin, a TRPV1 antagonist at high doses, decreases tumor growth in PDAC mouse models (41, 42). However, the mechanism for capsaicin’s chemoprotective effects and the specific role of decreased intrapancreatic innervation is unknown. We systematically denervated KPC<sup>Pat1</sup> mice with resiniferatoxin (RTX), a super agonist of the TRPV<sup>+</sup> sensory afferent nerve fibers (43).

In these studies, effective sensory denervation was confirmed by both the capsaicin-induced eye wipe response as well as sensory axon density counts in pancreas tissue. Denervated KPC<sup>Pat1</sup> mice had significantly decreased corneal sensitivity to capsaicin for up to 12 weeks compared with control mice, indicating significant loss of TRPV<sup>+</sup> somatic innervation.
Supplementary Fig. S9A). The 16-week control mice were more withdrawn and had a significantly decreased eye wipe response compared with earlier time points, likely reflecting tumor-associated morbidity. Although we had evidence for robust somatic sensory denervation, it was critical to confirm intrapancreatic loss of TRPV1⁺ fibers as we hypothesized local effects of nerves on PanINs. Pancreatic sensory nerve densities were quantified with SP staining, which overlaps exclusively with TRPV1 (Fig. 6E; Supplementary Fig. S9B; ref. 44). RTX-treated mice had a significant decrease in pancreas sensory nerve density compared with control mice at 8 and 12 weeks but not at 16 weeks, suggesting likely axon...
regeneration over time. Interestingly, at this later time point the regrowth of pancreatic visceral sensory fibers was not associated with an increase in the eye wipe response (Supplementary Fig. S9A), suggesting maintenance of peripheral somatic denervation.

The denervated mice had reduced late-stage, but not early-stage, PanIN burden at all time points compared with the control group, suggesting an influence on PanIN progression but not PanIN initiation (Fig. 6A and B). Supporting this interpretation, there was no difference in ADM lesions between the two groups (Supplementary Fig. S9C). In another study, STAT3 inactivation in the neoplastic epithelium of a different PDAC murine model also prevented PanIN progression but not initiation (13). Therefore, STAT3 may have minimal or no effects on PanIN initiation, which most likely depends on cell-autonomous mutation-driven mechanisms. Denervated mice also had a significant decrease in the PanIN-associated fibro-inflammatory stroma. Consistent with their impaired PanIN progression, overall fewer denervated mice developed PDAC (7.7% of denervated vs. 50% of control mice; Fig. 6C). The decreased dysplasia of denervated PanINs was further confirmed by a significantly lower Ki67 expression (Fig. 6D; Supplementary Fig. S9D). Furthermore, consistent with the observation that SP activated STAT3 in PanIN organoids, PanINs in axotomized pancreata had profoundly decreased STAT3 phosphorylation in PanIN epithelial cells (Fig. 6D; Supplementary Fig. S10A and S10B) as well as in NK1-R+ cells (Supplementary Fig. S11A). Interestingly, denervation also led to a decrease in NK1-R+ PanIN cell abundance (Fig. 6D). Taken together, these findings demonstrated that decreased sensory intrapancreatic innervation reduced PanIN progression to PDAC in vivo, potentially through both impaired epithelial STAT3 activation and neuroendocrine cell maintenance.

Discussion

PanIN progression is not only accompanied by accumulating genetic mutations and cellular atypia but also the development of a complex tumor-associated stroma that promotes tumor progression through direct and indirect influences on the neoplastic epithelium (for review see refs. 25, 45). Here, we describe nerves as bona fide members of the PanIN microenvironment and demonstrate that they support PanIN tumorigenesis likely through cross-talk with a novel population of neoplastic neuroendocrine cells (Fig. 7).
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Figure 7.
Neuroepithelial cross-talk. Sensory innervation of a pancreatic PanIN lesion. The sensory neuron residing in the dorsal root ganglion (DRG) projects a visceral sensory afferent nerve into the pancreas. Vagal nodose ganglia (NG) projections are omitted. The terminal axon in the microenvironment is in close proximity to the PanIN epithelium. SP binding its membrane receptor, NK1-R expressed on NK1-R+ neuroendocrine cells (red), leads to STAT3 phosphorylation and maintenance of the NK1-R+ cells, which signal to potentiate PanIN growth. Treatment with resiniferatoxin (RTX) results in loss of the intrapancreatic sensory fibers regardless of the origin of the projections.

Other examples of neuronal influences on non-neuronal tumors via cross-talk with the neoplastic stem cell niche are emerging. For example, autonomic nerves signal directly to gastric stem cells to upregulate the oncogenic Wnt pathway and drive gastric tumorigenesis (2). Here, we propose neuromodulation of PanIN neuroendocrine cells that influences global PanIN progression. Even though human PDACs commonly express endocrine markers (46, 47), a functional role of endocrine differentiation in PanIN and PDAC has not been established. In our study, NK1-R+ neuroendocrine PanIN cells appeared in the earliest murine neoplastic lesions, exclusively responded to neuronal signaling and functioned as trophic "niche" cells in organoid culture. NK1-R+ cells' dramatic potentiating of composite organoid proliferation depended on contact mediated signaling, the exact mechanism of which needs to be elucidated. NK1-R+ cells may serve as a source of Wnt ligands that are critical for PanIN tumorigenesis (39) and that can depend on close cell contact for signal transduction (48). Interestingly, the NK1-R+ organoids highly expressed Wnt7b (Supplementary Fig. S8C), which mediates pancreatic progenitor cell growth during development (49). To formally establish whether NK1-R+ cells are required for PanIN progression in vivo, future studies should employ such strategies as toxin-dependent elimination of NK1-R+ cells in PanIN epithelium, as has been done for DCLK1+ PanIN progenitor cells (36).

The NK1-R+ cells were K167+ in vivo but expectedly proliferated under permissive organoid culture conditions (29). Intriguingly, the NK1-R+ cells recapitulated their in vivo role as a restricted subpopulation when associated with NK1-R+ cells. Thus, neuronal activation of STAT3 in NK1-R+ cells in vivo may promote neuroendocrine cell maintenance rather than proliferation, reflecting the complex role of STAT3 in cancer epithelium (for review see ref. 50). Furthermore, NK1-R+ cells may exclusively influence PanIN versus tumor biology as they were not expressed in tumors or PDAC cell lines. The long-term effects of intrapancreatic sensory denervation on tumor progression and survival require further investigation. Also, the SP–NK1-R axis is likely not involved in axon reorganization by PDAC cell lines that do not notably express the NK1-R. Such time- and context-specific roles of oncogenic factors are well described in PanIN and PDAC tumorigenesis (25).

SP-induced JAK2 and STAT3 phosphorylation in the PanIN organoids provided evidence for neuropeptide activation of a key transcription factor in PanIN cells. Epithelial STAT3 activation by stromal cell–derived IL-6 is critical to Kras-mediated PanIN tumorigenesis (13). Here, we show neuronal SP in the microenvironment can serve as an additional activator of STAT3, as axotomized mice had markedly decreased PanIN p-STAT3 expression. Notably, p-STAT3 was also reduced in NK1-R+ cells, suggesting the potential for a more complex role of nerves in mediating stromal-epithelial signaling. Although we did not explore the effects of sensory denervation in altering the inflammatory compartment, our studies established an avenue of direct cross-talk between sensory nerves and the PanIN epithelium.

In summary, sensory nerves promote PanIN tumorigenesis potentially through direct cross-talk to unique neoplastic neuroendocrine cells. Our studies define a unique subpopulation of neural-responsive PanIN neuroendocrine cells capable of exerting trophic influences to stimulate global PanIN growth. These results highlight the heterogeneous nature of the neoplastic epithelium and its associated microenvironment, and illustrate the potential for their dynamic interactions. Disruption of neuronal influences, perhaps even at the tumor stage, may have therapeutic implications in the chemoprevention and/or treatment of human pancreatic cancer.
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