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PanIN neuroendocrine cells promote tumorigenesis via neuronal crosstalk

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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 crosstalk as a potential novel target in PDAC treatment.

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Keywords

Pancreatic Ductal Adenocarcinoma (PDAC); Pancreatic Intraepithelial Neoplasia (PanIN); Tumor Microenvironment; Sensory Nerves and Axons; Substance P (SP); Neurokinin 1 Receptor (NK1-R); Chromogranin A (CgA); Endocrine and Neuroendocrine Differentiation; Organoid; Sensory Ablation and Denervation

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 drive perineural invasion (4,5). In turn, human PDAC 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 targeting) functions (for review see Ref. (7)). Unmyelinated afferents, C and Aδ fibers, express the transient receptor potential vanilloid type 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) (12) signaling pathway, which is also an important driver of Kras–mediated oncogenesis in PDAC (13).

We hypothesized 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 pro-tumorigenic role of sensory nerves.

Materials and Methods

Mouse strains

Pdx1:Cre, *LSL-Kras*^{G12D} and *LSL-Trp53*^{R172H} mice (Jackson Laboratories) were bred to generate KC ^{Pdx1} and KPC^{Pdx1} mice. *Mist1:CreER*^{T2}; *LSL-Kras*^{G12D} and *LSL-tdTomato* mice were bred to generate KC^{Mist1}tdT mice. KC^{Mist1}tdT 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 lab) and 3T3 and 293T (ATCC) cells were maintained in RPMI 1640 or DMEM (Gibco) supplemented with 10% FBS (Sigma-Aldrich), GlutaMAX (Gibco) and 1x penicillin-streptomycin (Gibco). Human pancreatic ductal epithelial (HPDE) cells were cultured according to established protocols (15). Murine PanIN organoids (derived from KC^{Pdx1} and KC^{Mist1}tdT mice) were cultured according to published 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-Kras*^{G12D} allele genotyping (2016).

Neuronal co-culture

Dorsal root ganglia (DRGs) from 4 to 8 week C57BL/6J mice were harvested and cultured in supplemented Neurobasal medium (NBM; Gibco) as previously described (18). Growth factor-free NBM was used for all co-culture experiments. DRG neurons were plated with PDAC cell lines (5×10^3 cells/well) in the microfluidic device for 48 hours (Figure S1A) or with organoids (1×10^3 cells/well) in transwells (Corning) for 96 hours (Figure S1B). Organoid proliferation was measured with Cell Titer Glo assay for ATP (Promega) or MTT (Promega). Inhibitors L-733,060, RP 67580 and Stattic were used in some experiments.

Sensory denervation of KPC^{Pdx1} mice

7 day old KPC^{Pdx1} mice underwent a single subcutaneous injection of resiniferatoxin (RTX) or vehicle solutions. Early prophylactic denervation prior to 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.

Immunohistochemistry and immunofluorescence

Fixed cells and deparrafinized murine and human sections were subject to immunofluorescence (IF) and 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 ($\mu m/\mu m^3$) were calculated using Avizo 7.1 software (Burlington, MA, USA).

Histopathologic analysis

Histopathological analyses were performed on de-identified slides from 8, 12 and16-week old KPC^{Pdx1} pancreas. For each pancreas, 3–5 sections were sampled 150–200 μm apart and 5–15 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 percentage of total analyzed surface area occupied per lesion as previously described (Figure

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 (Figure 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) based on established protocols (14). tdTomato$^+$ or NK1-R$^+$ and NK1-R$^-$ PanIN cells (using the conjugated anti-NK1-R antibody) from KC^{Mist1}tdT mice were FACS sorted on a FACSAria III cell sorter (BD) based on established protocols (14). In clonogenic assays, sorted cells were cultured in 50% Matrigel (MG; Corning) with neurons (5000 sorted cells/well) or in organoid media (500 sorted cells/well) for 7 days (Figure S3A–D).

Reassociation assays

FACS sorted NK1-R$^-$ and NK1-R$^+$ cells were used to derive NK1-R^{low} and NK1-R^{hi}, organoids, respectively (Figure S3A–D). NK1-R^{low} and NK1-R^{hi} 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 manufacturer's protocol. NK1-R^{low} and NK1-R$^{hi/GFP}$ 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-R^{low} and NK1-R$^{hi/GFP}$ were plated at a 1:1 ratio (2×10^4 total cells/well) and subject to flow cytometry analysis at several time points.

Quantitative real-time PCR (qPCR)

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 *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 ΔC_t 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 (SP) Elisa

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., San Diego, CA) and were expressed as mean ± SEM. Comparisons between groups where data were normally distributed were made with Student t test, and comparisons with categorical independent variables were performed using Fisher's 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 to densities in pancreas from disease-free subjects. We aimed to characterize the nerve density in the *immediate* PanIN microenvironment compared to respective PanIN-free pancreas in isogenic mice to control for neural remodelling changes that may occur with genotype and disease state variables. We used 3D whole mount staining to reconstruct the fine intra-visceral axons (<5μm) that are not easily visualized on 2D histology (Figure S4A–B; ref (19)). In 12 week-old KPC^{Pdx1} mice, axons were observed in close proximity to PanIN epithelial cells in the immediate microenvironment (Figure 1A–B and Video V1). We quantified the nerve density surrounding PanINs versus their respective PanIN-free pancreas tissue in 12 week KC^{Pdx1} mice, which have only early PanINs, and age-matched KPC^{Pdx1} mice which have mostly late PanINs (Figure 1C–D and Figure S4C). KC^{Pdx1} and KPC^{Pdx1} mice had a 3.2± 0.84 and 10.1±2.4 fold increase in nerves densities compared to adjacent tissues, respectively. PanIN-associated fibers had a grossly aberrant branching pattern compared to 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 co-cultured DRG neurons with human PDAC or non-neoplastic human pancreatic ductal epithelial (HPDE) cells in a microfluidic system that allows axonal interactions and models intrapancreatic sensory innervation (Figure S1A). We used PDAC cell lines because of technical limitations of 3D culture of organoids in the device. The co-cultured PDAC cell lines, A6L and MIAPaCa-2, recruited 3.30 ± 0.31 and 3.43 ± 0.46 times more sensory axons, respectively, than the HPDE cells (Figure S4D–E). Co-cultured axons also expressed synapsin proteins, indicating active vesicular transport and neurotransmitter release (Figure S4F–G). 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

Since 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 (21,25), may communicate with and regulate the PanIN epithelium. The KPC^{Pdx1} 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 (4) (data not shown). 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^{Pdx1} 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 (Figure 2A and Figure S5A–C). In areas of PanIN-free pancreas, NK1-R was expressed only in islets (Figure 2A, Figure 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 from16 patients containing synchronous PanIN lesions. NK1-R^+ and CgA^+ neuroendocrine cells were identified in one or more PanINs of all patients (Figure 2B and Figure S5D–E). 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 KPC^{Pdx1} mouse but not in human PanINs (Figure 2D). Even early KPC^{Pdx1} PanINs composed of a few neoplastic cells also expressed the NK1-R, suggesting a potential early and persistent role in PanIN evolution (Figure S5A).

Since 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 KC^{Mist1}tdT mouse 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 co-expressed tdTomato, confirming that these were acinar cell-derived neoplastic PanIN epithelial cells (Figure 2C and Figure S5F).

We incidentally saw expression of NK1-R on a subpopulation of CgA^+ enteroendocrine cells (EECs) in human duodenal villi (Figure 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 crosstalk, we utilized KC^{Pdx1} PanIN organoids in co-culture experiments. The organoid culture is a powerful tool to study non-cancerous 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 IF staining and immunoblotting (Figure 3A–B). In contrast to the low abundance NK1-R^+ cells *in vivo*, PanIN organoid cells uniformly expressed 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 (Figure S7A). PanIN organoids also diffusely expressed CgA (Figure S7B).

To recapitulate *in vivo* conditions with the potential for reciprocal signaling between neurons and cancer cells, we co-cultured DRG neurons with PanIN organoids in a transwell system (Figure S1B). Neurons increased organoid proliferation to a greater degree than 3T3 murine fibroblast cells (38.7 ± 3.5 fold versus 9.3 ± 0.80 fold), an established tumorigenic stromal cell type in the microenvironment (Figure 3C) (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 (Figure 3D and Figure 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% (Figure S7D). Neuronal conditioned media contained SP (20–40 pg/mL) as measured by ELISA. The decreased degree of proliferation in conditioned media compared to co-culture suggested that reciprocal crosstalk between organoids and neurons may help mediate neoplastic proliferation. Unlike neuronal co-culture, SP incubation alone did not cause organoid proliferation likely due to known peptide instability in solution (31) (data not shown). Very limited studies have shown that SP alone mediates proliferation of PDAC cell lines and the data are inconsistent (32,33). Since NK1-R antagonism only partly blocked PanIN organoid proliferation, additional neuronal factors such as nerve growth factor (NGF) (4) and glial derived neurotrophic factor (GDNF) (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 KC^{Mist1}tdT pancreas formed 6.1 ± 1.2 fold more organoid colonies in the presence of neurons (Figure 3E) as additionally confirmed by the proliferation assay (Figure S7E). Thus, sensory neurons supported the growth of organoid colonies from single primary cells in a 3D matrix, a process that additionally requires multiple growth factors (16).

PanIN neuroendocrine cells exert trophic influences

Given their similarity to intestinal enteroendocrine cells that are known to function as nerve-responsive niche cells (28), we investigated whether the subpopulation of NK1-R^+ PanIN cells could influence global PanIN growth. In clonogenic assays, FACS sorted NK1-R^- and NK1-R^+ cells from KC^{Mist1}Tdt PanINs were plated at low density with neurons or in organoid media (Figure 4A and Figure S3A). Strengthening our studies of SP-NK1-R mediated neuroepithelial crosstalk, only NK1-R^+ cells responded to DRG co-culture and formed 1.9 ± 0.09 fold more organoids than monoculture controls. Even though NK1-R inhibitors partially inhibited organoid proliferation in our prior studies, NK1-R^+ cells exclusively responded to neuronal co-culture. Thus, NK1-R^+ cells may be the sole sensory nerve-responsive PanIN cells *in vivo*. Surprisingly, the combination of NK1-R^- and NK1-R^+ cells formed the most colonies (3.5±0.21 fold over control) compared to the individual cultures, suggesting that the neuron-responsive NK1-R^+ cells potentiated global organoid

growth. This effect was accentuated in organoid media where combined NK1-R^{-} and NK1-R^{+} cells formed ~20 fold more colonies than the individual cultures. These data resonated studies of Paneth cells' striking potentiation of intestinal organoid growth (34).

Interestingly, conditioned media from NK1-R^{-} and NK1-R^{+} cells did not influence the cells' clonogenic potential in reciprocal experiments (data not shown). This indicated likely contact-dependent signaling. To better study NK1-R^{+} contact-mediated influences, we derived and validated new NK1-R^{low} and NK1-R^{hi} organoids (Figure S3A). Freshly sorted NK1-R^{-} cells, although initially depleted for *Tacr1* relative to NK1-R^{+} cells, expressed the receptor after one week in organoid culture (Figure S3B). Since the NK1-R may support Wnt signaling (35), receptor upregulation and universal expression in our organoid progenitor culture (Figure 3A) was not surprising. NK1-R^{hi} organoids were enriched for NK1-R protein on immunoblotting (Figure S3C) and did not contain any non-neoplastic cells as determined by *lox-Kras^{G12D}* genotyping (Figure S3D). In the reassociation assay, NK1-R^{low} and NK1-R$^{hi/GFP}$ organoids formed composite organoids when plated as single cells at a roughly *in vivo* ratio of 20:1 (Figure 4B–C and Figure S8A). This plating ratio was important so as to not drive the reaction in a biased direction. Surprisingly, the NK1-R$^{hi/GFP}$ cells maintained restrictive expansion within isolated areas of the composite organoids and no homogenous NK1-R$^{hi/GFP}$ organoids were seen. This finding was reminiscent of Paneth cell reassociation with intestinal cells into distinct regions within heterogeneous organoids (34). Furthermore, NK1-R^{low} and NK1-R$^{hi/GFP}$ organoid reassociation was a dynamic process with highly mobile organoids that appeared to preferably merge heterogeneously (Video V2). NK1-R$^{hi/GFP}$ cells also maintained a constant low cell population in composite organoids over time when plated at equal starting ratios (Figure 4D and Figure S8B). Strikingly, the NK1-R$^{hi/GFP}$ cell frequencies (ranging from 6.7% ± 0.11 to 9.9% ± 2.9) closely reflected their observed ratios in PanIN epithelium *in vivo* (Figure 2D). Even though NK1-R$^{hi/GFP}$ organoids grew comparatively to NK1-R^{low} organoids in individual cultures, they showed restricted expansion in the composite cultures.

Further characterization of NK1-R^{hi} organoids with qPCR showed significantly decreased expression of proposed pancreatic, and potentially neoplastic, progenitor markers relative to NK1-R^{low} cells, including *Lgr5* (29) and *Doublecortin-like kinase-1* (*Dclk1*) (14,36) (Figure 4E). 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-R^{low} organoids, NK1-R^{hi} organoids had decreased activation of the downstream canonical versus non-canonical pathway (Figure 4E and Figure S8C) as well as decreased expression of several Wnt receptor *Fzd* family members (Figure S8D). Thus, the expression profile of NK1-R^{hi} cells was less "progenitor"-like than of NK1-R^{low} 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^{+} cells appeared to function as trophic "niche" cells with the

potential to provide growth signals to NK-1R^{-} cells. The NK1-R^{+} PanIN cells were also Ki67^{-} on IF analysis, further suggesting that they were not proliferating progenitor cells *in vivo* (Figure S8E).

Substance P stimulates Stat3 phosphorylation in PanIN organoids

Since sensory neurons robustly increased PanIN organoid proliferation and selectively signaled to NK1-R^{+} 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 (Figure 5A–B). Stattic, a small molecule inhibitor of Stat3, decreased the proliferation of co-cultured organoids by 50.8%, suggesting that the neuron-mediated organoid proliferation is significantly mediated through Stat3 activation (Figure 5C). Indeed, the NK1-R^{+} PanIN cells *in vivo* had a significantly increased p-Stat3 expression (by 35.4 ± 7.9%) compared to NK1-R^{-} cells within the same PanIN lesion, suggesting that NK1-R^{+} PanIN cells may be more responsive to neuropeptide signaling (Figure 5D).

Sensory denervation of KPC^{Pdx1} mice decreases PanIN progression

Since 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 systemically denervated KPC^{Pdx1} mice with resiniferatoxin (RTX), a super agonist of the TRPV1 receptor which causes highly selective and rapid degeneration of the TRPV^{+} 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^{Pdx1} mice had significantly decreased corneal sensitivity to capsaicin for up to 12 weeks compared to control mice, indicating significant loss of TRPV1^{+} somatic innervation (Figure S9A). The 16 week control mice were more withdrawn and had a significantly decreased eye wipe response compared to 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 (Figure 6E and Figure S9B; ref. (44)). RTX treated mice had a significant decrease in pancreas sensory nerve density compared to 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 (Figure 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 to the control group, suggesting an influence on PanIN progression but not PanIN initiation (Figure 6A–B). Supporting this interpretation, there was no difference in ADM lesions between the two groups (Figure 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 versus 50% of control mice) (Figure 6C). The decreased dysplasia of denervated PanINs was further confirmed by a significantly lower Ki67 expression (Figure 6D and Figure 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 (Figure 6D and Figure S10A–B) as well as in NK1-R^+ cells (Figure S11A). Interestingly, denervation also led to a decrease in NK1-R^+ PanIN cell abundance (Figure 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 ref. (25,45)). Here, we describe nerves as bona fide members of the PanIN microenvironment and demonstrate that they support PanIN tumorigenesis likely through crosstalk with a novel population of neoplastic neuroendocrine cells (Figure 7).

Other examples of neuronal influences on non-neuronal tumors via crosstalk 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 cells in PanIN epithelium 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 potentiation 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 which can depend on close cell contact for signal transduction (48). Interestingly, the NK1-R^+ organoids highly expressed *Wnt7b* (Figure 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 Ki67$^-$ *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 cells 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 recruitment 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 (for review see ref. ((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. While we did not explore the effects of sensory denervation in altering the inflammatory compartment, our studies established an avenue of direct crosstalk between sensory nerves and the PanIN epithelium.

In summary, sensory nerves promote PanIN tumorigenesis potentially through direct crosstalk 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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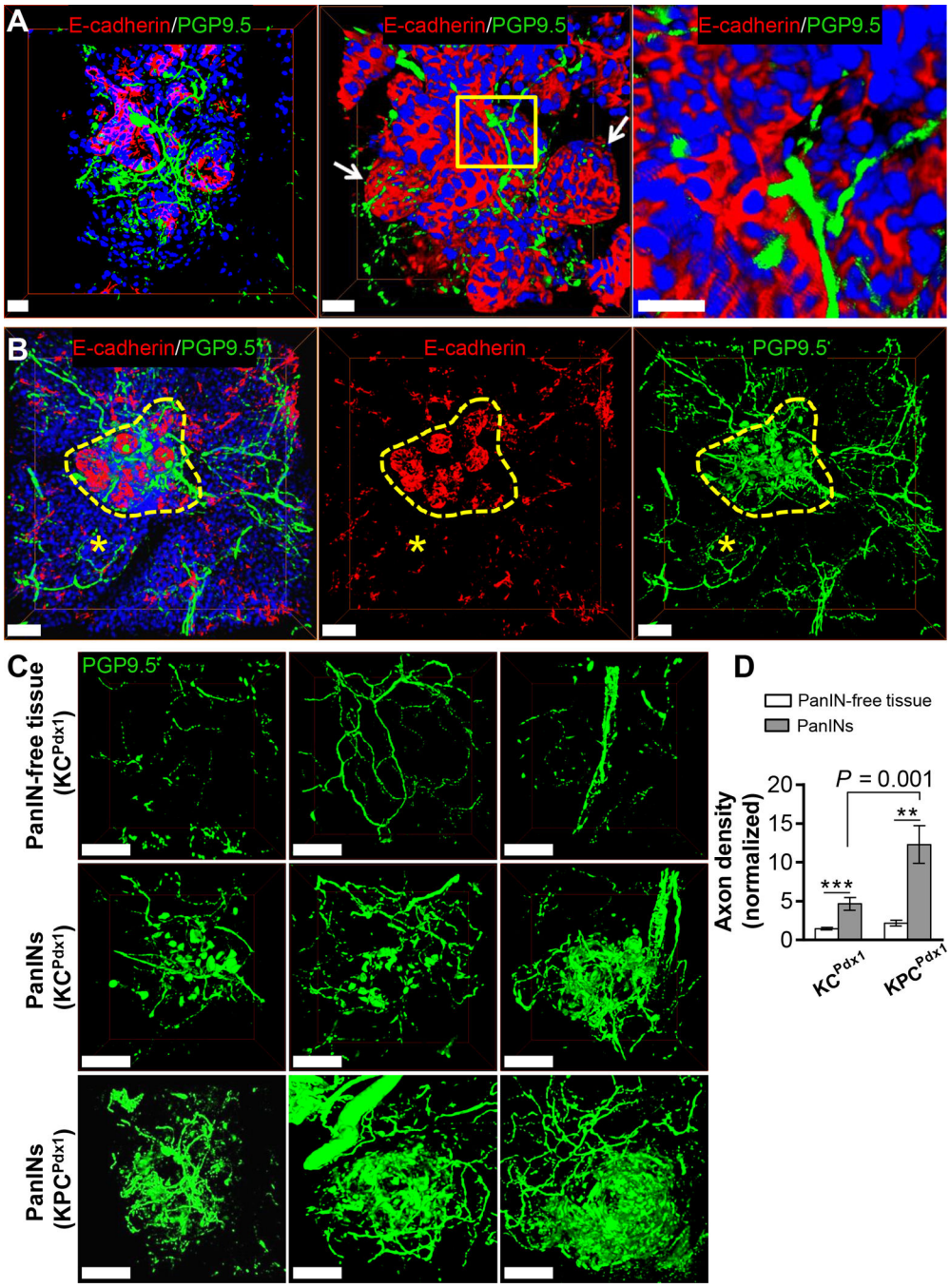

Figure 1. Innervation of PanINs

3D IF projections (scan depths = 150 μm) of PanIN epithelium stained with E-cadherin (red), axons with the pan-neuronal marker PGP9.5 (green) and nuclei with DAPI (blue) in mouse pancreas. (A) Late PanIN in a 12 week KPC^{Pdx1}mouse is a multilobar structure (arrows) with infiltrating axons (insert). Scale bars = 25 μm. (B) Increased innervation of an early PanIN lesion (yellow dashes) in a 12 week KC^{Pdx1} mouse pancreas compared to adjacent pancreas tissue (asterisks). Scale bars = 100 μm. (C) Sample projections of axons in PanIN-free tissue and PanIN lesions used for quantification of nerve densities (PanIN

epithelial staining not shown to highlight axonal network). Scale bars = 100 μm. (D) Axon density quantifications (n=6–13 scans for each group; n = 3–4 mice per group). The data represent mean ± SEM (two-tailed unpaired t test; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

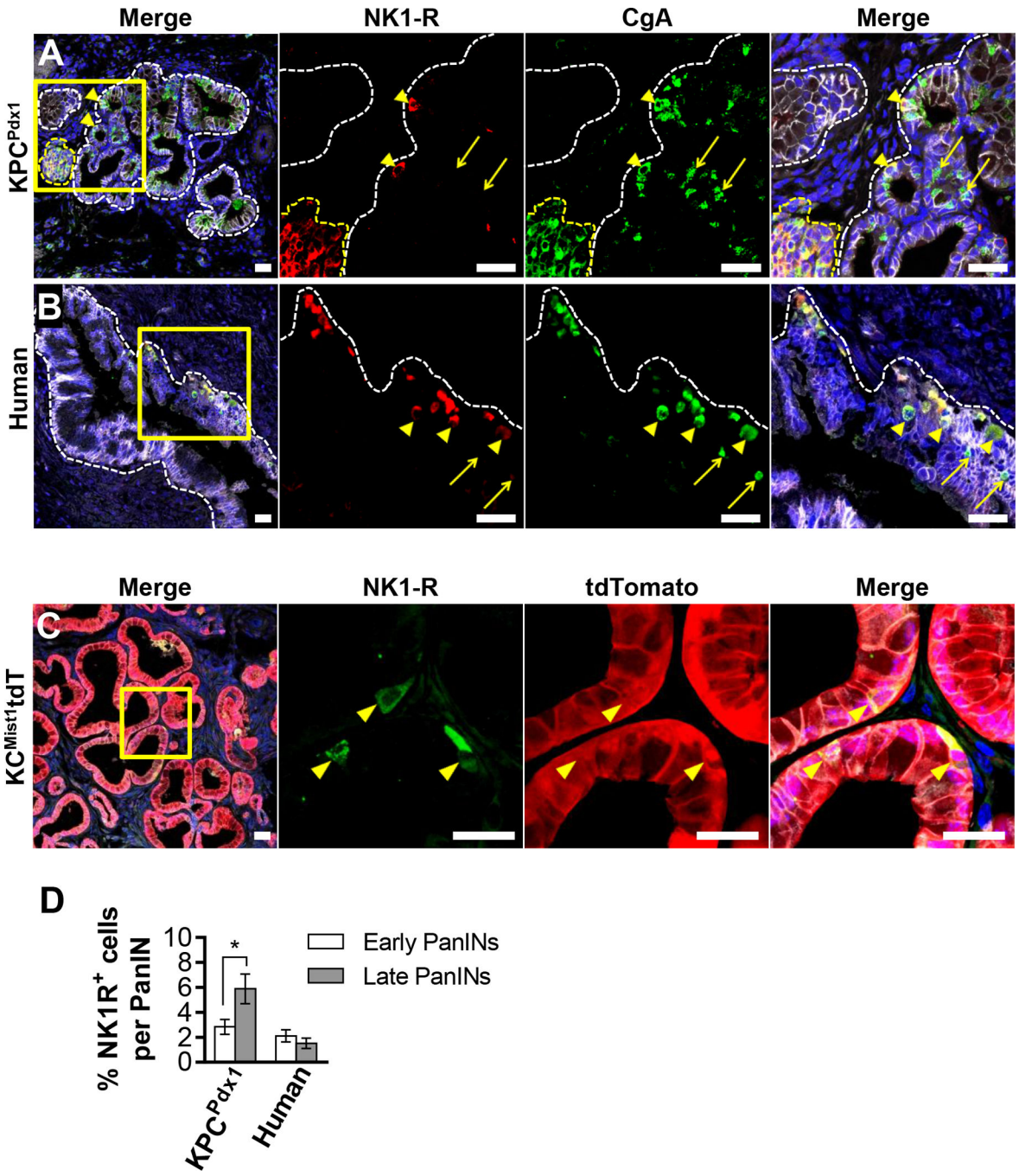

Figure 2. PanIN neuroendocrine cells express the neuropeptide receptor NK1-R

Confocal IF analyses of murine and human PanINs stained with E-cadherin (gray) and nuclei with DAPI (blue). (A) PanIN in a KPC^{Pdx1} pancreas (white dashes) next to an islet (yellow dashes). Note PanIN NK1-R^+/CgA^+ cells (arrowheads) and NK1-R^-/CgA^+ cells (arrows). (B) Human PanIN lesion with NK1-R^+ /CgA^+ cells (arrowheads) and NK1-R^-/CgA^+ cells (arrows). (C) $tdTomato^+$ PanINs in a $KC^{Mist}tdT$ pancreas. All PanIN NK1-R^+ cells are $tdTomato^+$ (arrowheads). All scale bars = 25 μm. (D) Quantification of NK1-R^+ cells/PanIN. 70 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; *$P \leq 0.05$).

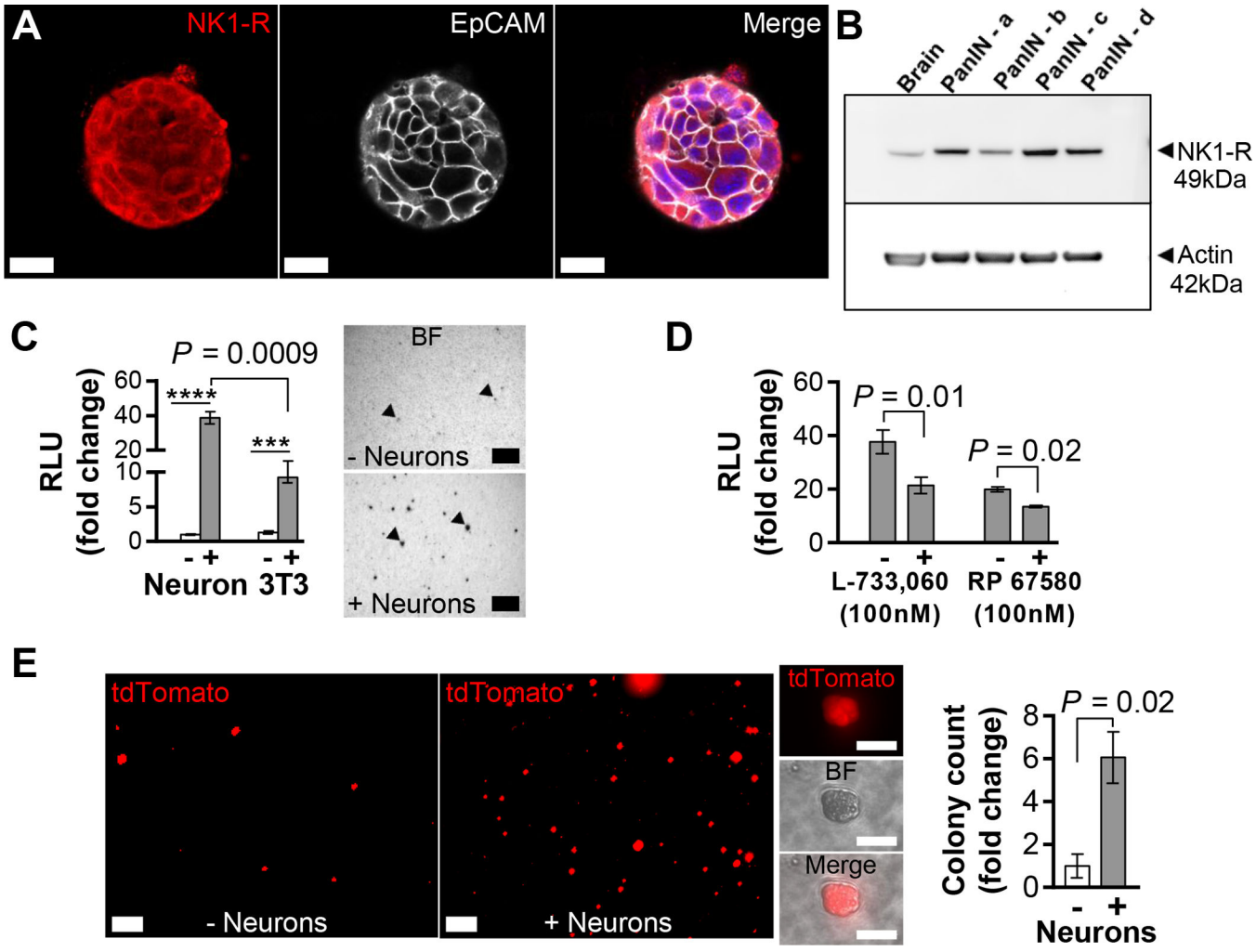

Figure 3. Sensory neurons increase PanIN organoid proliferation

(A) IF 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 co-cultured 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 co-cultured 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 co-cultured tdTomato^{+} single PanIN cells from a KC^{Mist}tdT 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; ***$P \leq 0.001$, ****$P \leq 0.0001$).

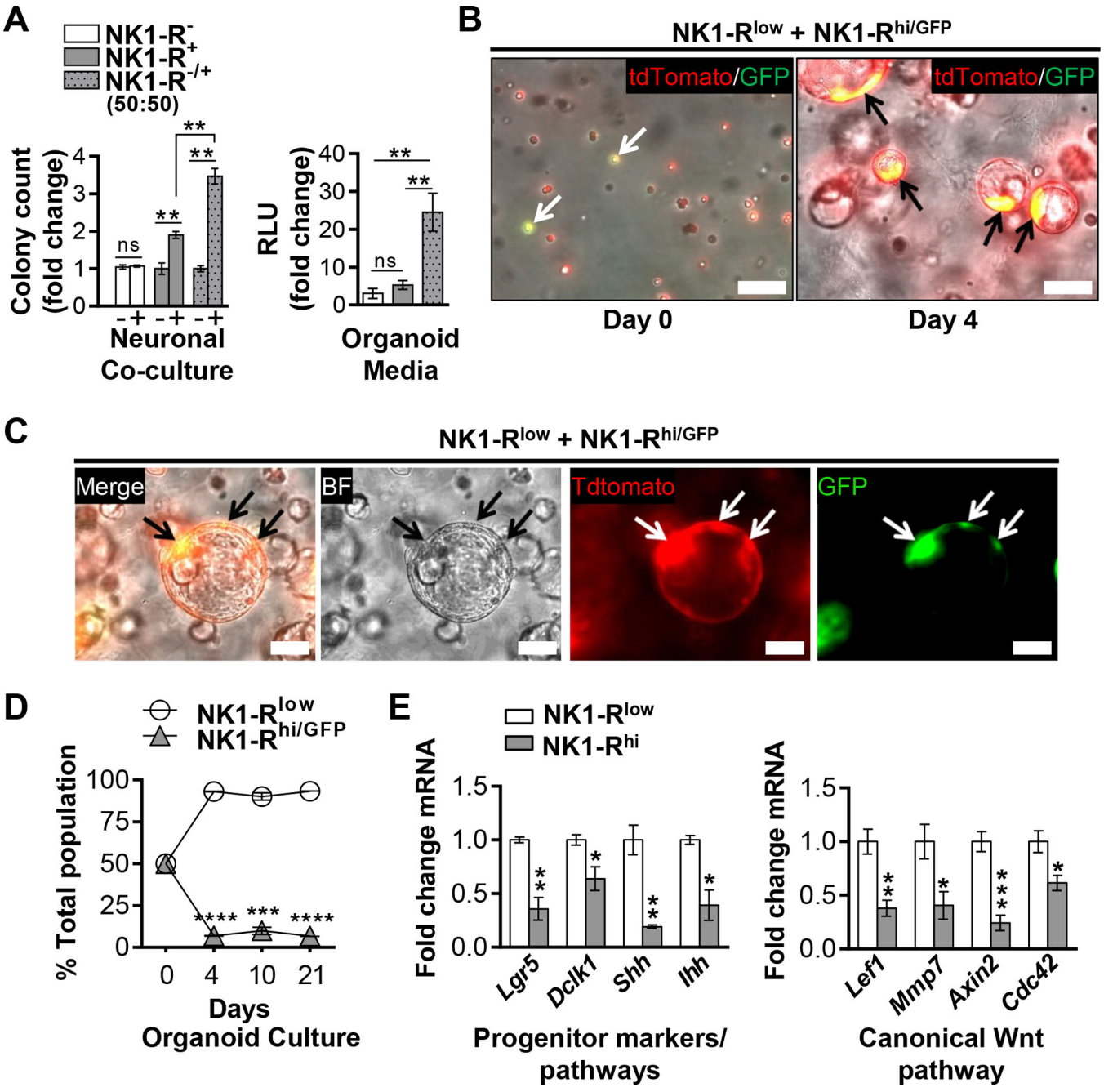

Figure 4. PanIN neuroendocrine cells exert trophic influences

(A) Clonogenic assays of FACS sorted NK1-R^{-} and NK1-R^{+} KC^{Mist}tdT single PanIN cells (n=3–4 for each experiment; RLU = random luminescence units). Data are normalized to the monoculture controls and the NK1-R^{-} group in the co-culture and organoid media experiments, respectively. (B–C) Z-stack live IF and bright field (BF) images of the reassociation of single NK1-R$^{hi/GFP}$ (yellow and green; arrows) and NK1-R^{low} (red) organoid cells into composite organoids. Scale bars = 100 μm. (D) Population fractions of NK1-R^{low} (GFP^{-}) and NK1-R$^{hi/GFP}$ (GFP^{+}) organoid cells cultured together as single cells at a 1:1 ratio (on day 0) as determined by FACS analysis over time. (E) qPCR analysis. The

data represent mean ± SEM (two-tailed unpaired t test; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$).

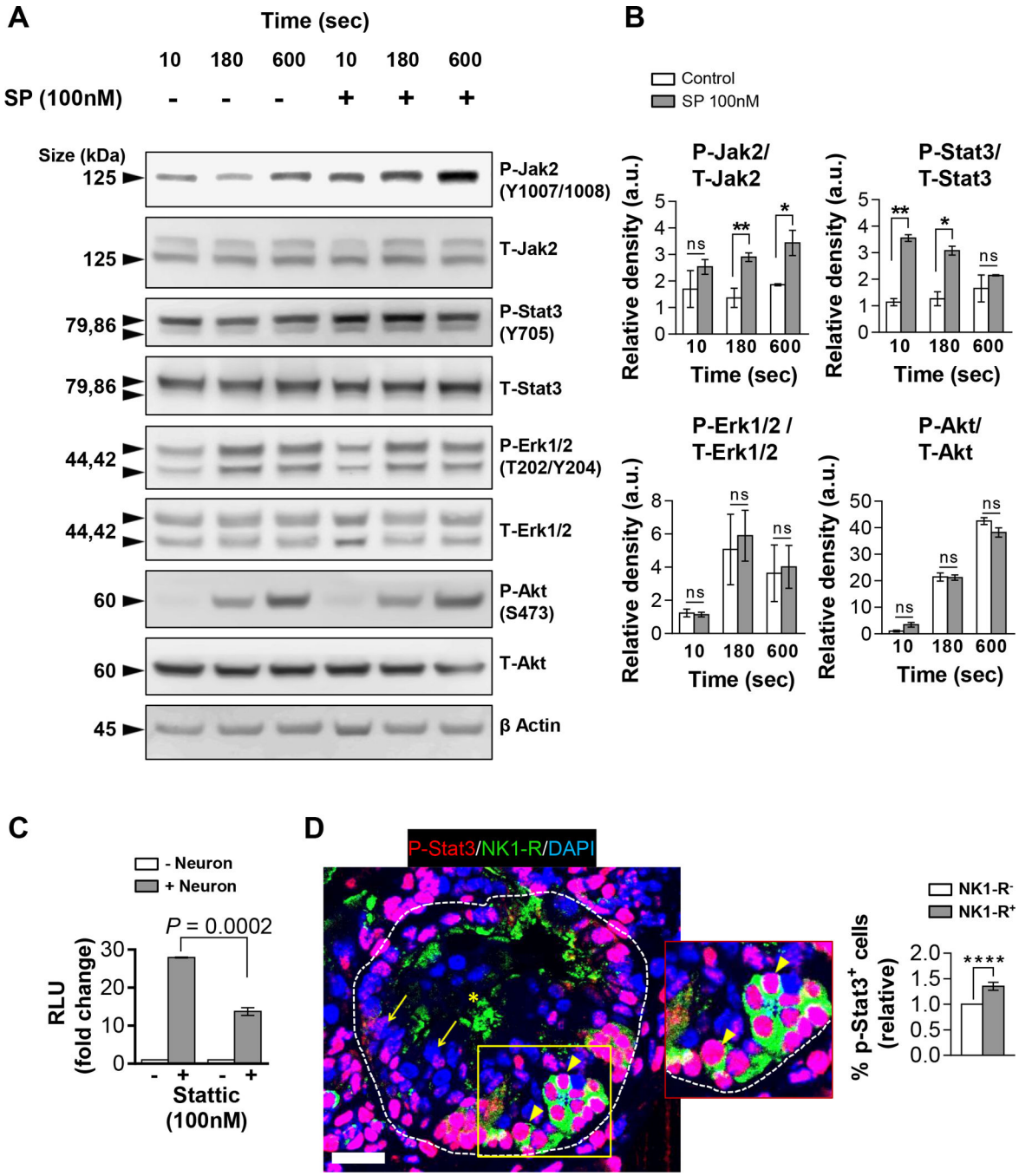

Figure 5. SP induces Jak2 and Stat3 phosphorylation in PanIN organoids

(A) Western blot analysis for the proteins shown from PanIN organoids incubated with media -/+ SP. (B) Quantification of relative band densities (a.u. = arbitrary units) of phospho-protein/total protein (β actin = loading control) (n=3). (C) Proliferation of co-cultured PanIN organoids with Stat3 inhibitor, Stattic (n=3). RLU (random luminescence units) values are normalized to monoculture controls. (D) Representative IF confocal projection of a PanIN lesion (dashes) in a 12 week KPC^{Pdx1} pancreas. p-Stat3 (red) expression in NK1-R^+ cells (green; short arrows) versus NK1-R^- cells (long arrows) within

the same lesion. Non-specific staining in PanIN lumen (asterisks). Scale bar = 25 μm. Quantification of p-Stat3^{+} nuclei within the same PanIN lesions of 12-week control KPC^{Pdx1} mice (n=5; n=17 scans). The data represent mean ± SEM (two-tailed unpaired t test; *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$).

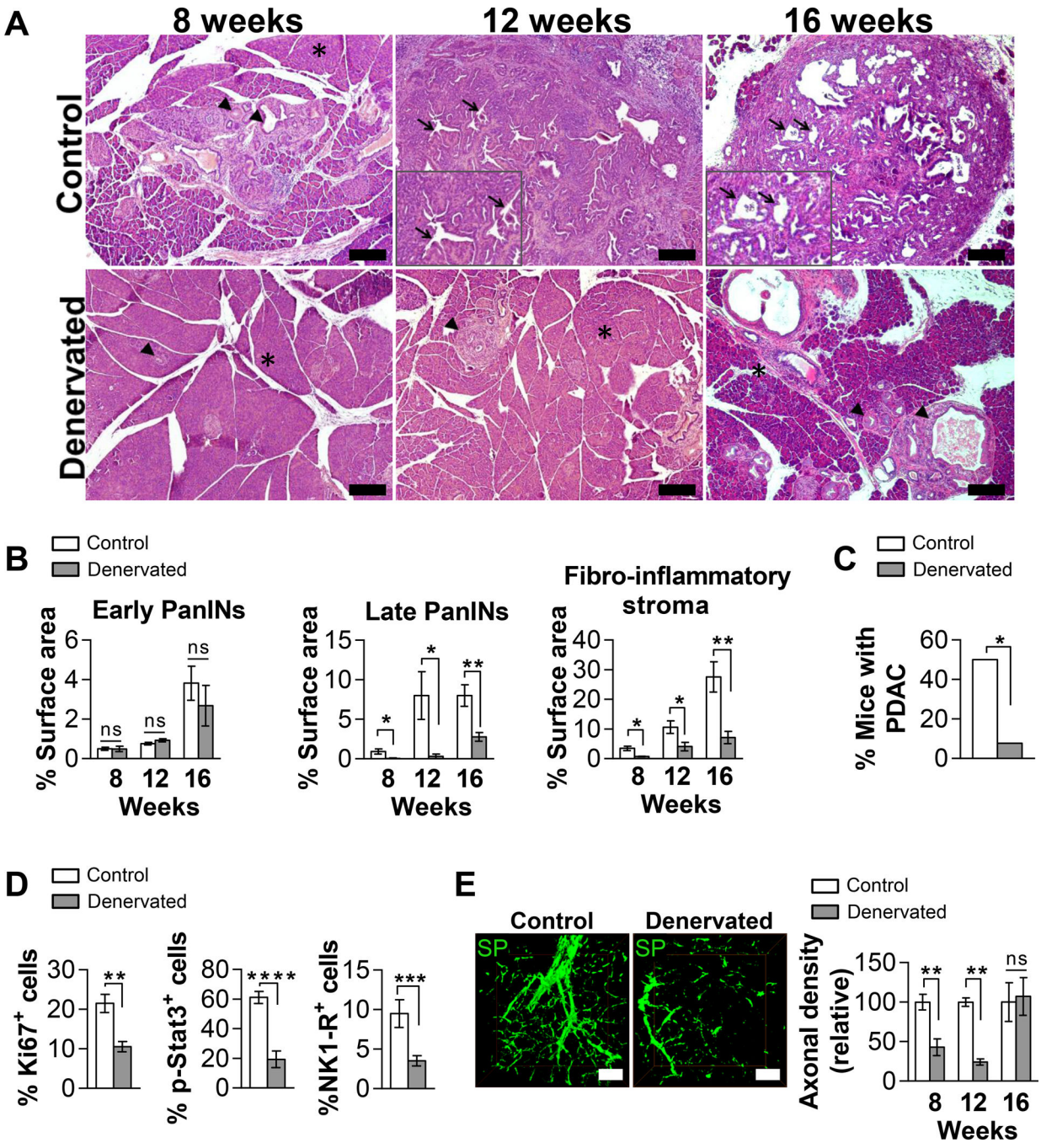

Figure 6. Sensory denervation decreases PanIN progression to PDAC

(A) Representative H&E pancreas sections. Normal pancreas (asterisks), PanINs (arrowheads) and highly dysplastic ducts of tumors (inserts; arrows) are shown. Scale bars = 200 μm. (B) Histopathologic quantifications of PanIN and stroma burden (n=4–5 mice per group per time point). (C) Percentage of 8, 12 and 16 week-old mice with tumors (n=12 control and n=13 denervated). (D) Quantifications of Ki-67^{+}, p-Stat3^{+} and NK1-R^{+} cells per PanIN (n=13–44 PanINs per analysis and 3–5 mice per group). (E) Representative 3D IF projections of substance P (SP) axons (green). Pancreatic axon density quantifications (n=3

views per mouse; n=3 mice per group per time point). Scan depth = 150 μm. Scale bars = 50 μm. The data represent mean ± SEM (two-tailed unpaired t test or Fisher's exact test; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$).

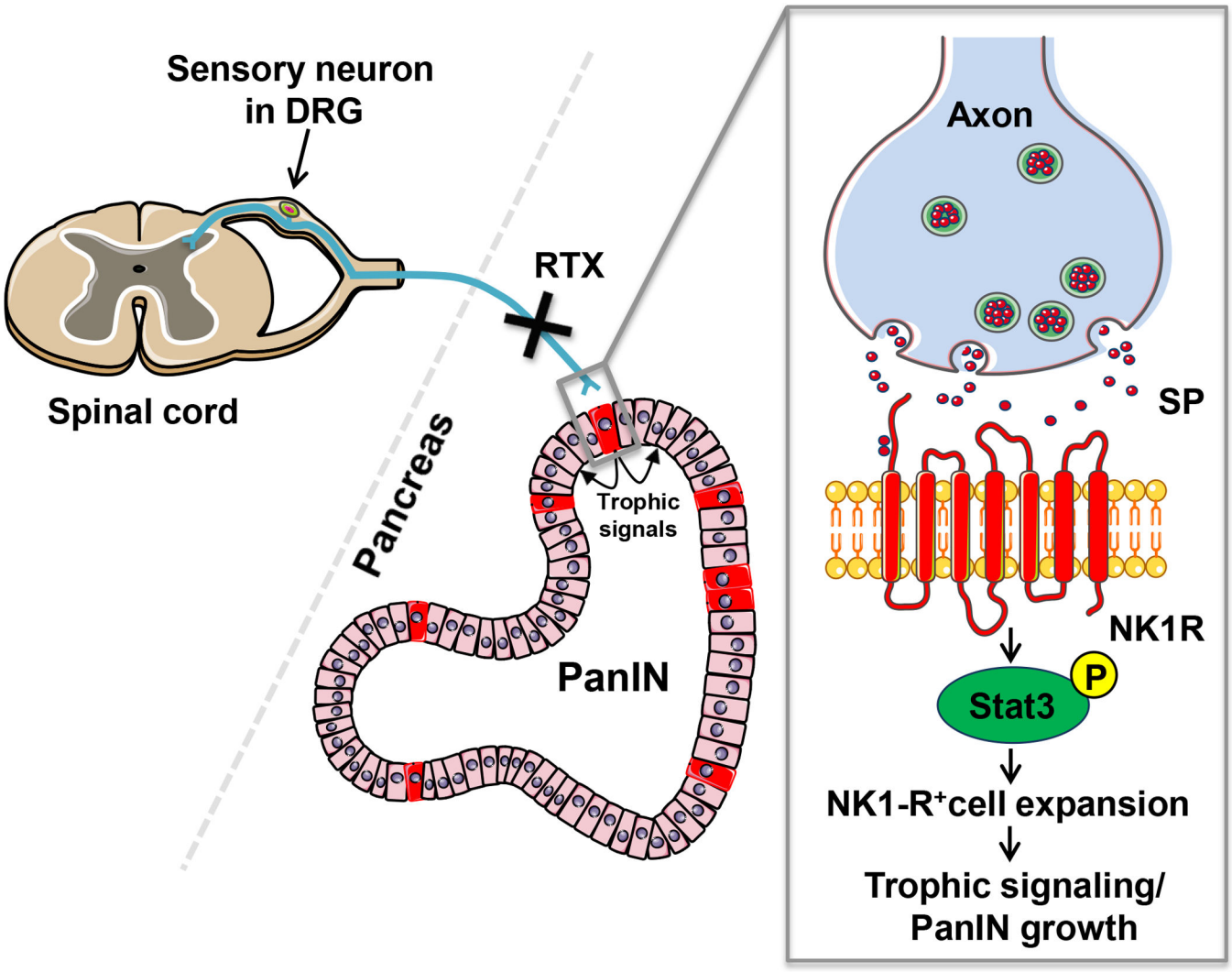

Figure 7. Neuroepithelial crosstalk

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, NK-1R 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.