Ablation of sensory neurons in a genetic model of pancreatic ductal adenocarcinoma slows initiation and progression of cancer

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Pancreatic ductal adenocarcinoma (PDAC) is characterized by an exuberant inflammatory desmoplastic response. The PDAC microenvironment is complex, containing both pro- and antitumorigenic elements, and remains to be fully characterized. Here, we show that sensory neurons, an under-studied cohort of the pancreas tumor stroma, play a significant role in the initiation and progression of the early stages of PDAC. Using a well-established autochthonous model of PDAC (PKC), we show that inflammation and neuronal damage in the peripheral and central nervous system (CNS) occurs as early as the pancreatic intraepithelial neoplasia (PanIN) 2 stage. Also at the PanIN2 stage, pancreas acinar-derived cells frequently invade along sensory neurons into the spinal cord and migrate caudally to the lower thoracic and upper lumbar regions. Sensory neuron ablation by neonatal capsaicin injection prevented perineural invasion (PNI), astrocyte activation, and neuronal damage, suggesting that sensory neurons convey inflammatory signals from Kras-induced pancreatic neoplasia to the CNS. Neuron ablation in PKC mice also significantly delayed PanIN formation and ultimately prolonged survival compared with vehicle-treated controls (median survival, 7.8 vs. 4.5 mo; $P = 0.001$). These data establish a reciprocal signaling loop between the pancreas and nervous system, including the CNS, that supports inflammation associated with oncogenic Kras-induced neoplasia. Thus, pancreatic sensory neurons comprise an important stromal cell population that supports the initiation and progression of PDAC and may represent a potential target for prevention in high-risk populations.

sensory neuron | pancreatic ductal adenocarcinoma | tumorigenesis | inflammation | PanIN

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers with a median survival of ~6 mo from diagnosis (National Cancer Institute). A number of unique features distinguish PDAC from other carcinomas, but the most striking is the exuberant desmoplastic infiltrate within tumors. This compartment exhibits an array of cell types, including activated myofibroblasts and myeloid-derived cells. Indeed, this inflammatory infiltrate is present at the inception of neoplasia and accumulates at a near exponential rate during progression to carcinoma and tumor formation. It provides a complex balance of pro- and antitumorigenic signals to neoplastic cells (and also to each other) that is a focus of intense investigation. The pancreas tumor microenvironment has been studied previously, but new tools [genetically engineered mouse models (GEMs) that faithfully recapitulate the salient features of human PDAC] now allow for a careful dissection of the stroma. Using these models, we showed that generalized inflammation is required for the development of precancerous pancreatic intraepithelial neoplasias (1) and that Hedgehog-dependent stromal elements, including activated myofibroblasts, serve to constrain tumor growth and spread (2). Other cellular components of the pancreatic inflammatory stroma have not been examined, and it is possible they also contribute to the complex balance of inputs, supportive and inhibitory, which underlie tumorigenesis and progression.

Previous reports indicate sensory neurons have a central role in benign inflammatory disease of the pancreas. Like most abdominal organs, the pancreas is innervated by sensory fibers from both the nodose (via the vagal nerve) and spinal ganglia (via splanchnic nerves) (3–7). In rodent models of acute or chronic pancreatitis, blockade of primary afferents from both nodose and spinal ganglia can moderate or prevent inflammation (8, 9) as well as associated pathology, even if done after the inciting injury (10, 11). In addition, autonomic neurons (sympathetic, parasympathetic, and enteric) (12) innervate the pancreas and interact with sensory fibers.

Because of the documented role of sensory neurons in the pathogenesis of pancreatitis (8–11, 13–15), a known contributor to the pathogenesis of PDAC, we hypothesized that sensory neurons innervating the pancreas provide key proinflammatory inputs that support the early stages of tumorigenesis. Here, using GEMs of PDAC, we provide evidence that bidirectional communication between the pancreas and sensory neurons is active well before the establishment of tumors. We detected inflammation in the spinal cord when histologically only precancerous lesions (pancreatic intraepithelial neoplasia, PanIN) were present. This was accompanied by perineural invasion (PNI) of sensory ganglia and spinal cord

Significance

In humans and genetically engineered mouse models (GEMs), the development of pancreatic ductal adenocarcinoma (PDAC) is accompanied by intimate neural–tumor interactions. Using a PDAC GEM that phenocopies the human disease, we found that many changes in peripheral and central nervous systems, indicative of injury and inflammation, arise at time points prior to overt tumor formation. Ablation of sensory neurons that innervate the pancreas, via neonatal capsaicin treatment, prevented neurogenic inflammation and delayed tumor formation. The slowing of PDAC in capsaicin-treated mice suggests the nervous system is not a bystander with respect to disease progression. Further studies are warranted to examine nervous system–tumor interactions and to identify potential targets for early detection, prevention, and treatment.

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and evidence of injury to pancreatic sensory and sympathetic neurons. Ablation of sensory neurons at postnatal days 1–2 prevented injury to peripheral neurons and spinal cord inflammation. Surprisingly, sensory neuron ablation was associated with a dose-dependent and dramatic prolongation of survival in PDAC mice. The animals with the greatest degree of capsaicin-induced neuronal ablation (>80%) did not develop cancer (up to 18 mo when they were euthanized for analysis), whereas more than 90% of untreated mice succumbed to PDAC within 6 mo. These studies indicate that sensory neurons contribute significantly to the initiation and progression of PDAC and may be required for the development of this disease.

Results

Benign and Oncogene-Induced Pancreas Inflammation Elicits Activation of CNS Glia and Injury-Related Genes. In humans and the two mouse models used here (PKCT and PKCY mice, Fig. S1), pancreatitis is required for the initiation and development of PDAC (1, 16). Pancreatitis has also been shown to induce injury-like responses in sensory neurons (11). To further understand the relationship between tissue inflammation, tumor formation, disease progression, and the nervous system, we compared how benign acute pancreatitis, induced by repeated injections of cerulein, and Kras-driven precancerous inflammatory neoplastic disease (PanIN) affected the peripheral and central nervous systems. We first examined activation of spinal glial cells using neurochemical markers associated with CNS inflammation. Four regions of interest (ROIs), encompassing the dorsal columns, the canonical pain-transmitting anterolateral spinothalamic tract, and a medioventral nonpain-related region, were analyzed to determine the percent area covered by GFAP immunoreactivity, an indicator of astrocyte activation. Measures were made at the level of the thoracic spinal cord, which receives input from primary afferents that innervate the pancreas. Acute pancreatitis induced by cerulein (Fig. 1*A*) caused a significant increase in GFAP immunoreactivity in ROIs overlying the dorsal columns, anterolateral, and medioventral white matter tracts ($F = 73.06$, $P < 0.0001$, Fig. 1*B*).

Markers of spinal cord inflammation were also elevated in PKCT and PKCY mice at the PanIN and PDAC stages. The pattern of GFAP immunoreactivity was almost identical in PKCT (tomato) and PKCY (YFP) mice (Fig. 2*A*) and increased as mice aged and developed PDAC ($F = 21.45$, $P < 0.0001$, Fig. 2*B*). Phospho-ERK (p-ERK), another marker of inflammation (17–21), was also elevated in the thoracic spinal cord of PKCT mice (Fig. 2*C*). Thus, similar to cerulein-induced acute pancreatitis, Kras-induced neoplasia leads to increased inflammation of the spinal cord.

Chronic tissue inflammation, proinflammatory cytokine release, and physiological stress all change primary afferent gene expression. A particularly sensitive marker is activating transcription factor 3 (ATF3), a host defense transcription factor (22). We used ATF3 immunolabeling to determine if damage in the pancreas of either cerulein-treated or PKC animals caused an injury response in afferents of celiac, nodose, and T9–T12 dorsal root ganglia (DRG) (22–25). Whereas vehicle-treated mice lacked ATF3 expression in all ganglia, cerulein-treated mice showed a few positive cells in all ganglia examined. In contrast, PKC mice at the PanIN stage exhibited a significant increase in ATF3 immunoreactivity in nodose and DRG that was maintained throughout cancer progression (Kruskal–Wallis, $P = 0.002$, Fig. 3 *A* and *C*). ATF3 labeling was also increased in sympathetic celiac ganglia at the PanIN and PDAC stages (Kruskal–Wallis, $P = 0.003$, Fig. 3 *B* and *C*). Thus, both sensory and sympathetic postganglionic neuron injury occurs at precancerous PanIN stages and increases with progression to PDAC (Fig. 3*D*).

Pancreatic Cells Invade the Nervous System at PanIN Stages. PNI is a common clinical feature of PDAC that coincides with tumor and

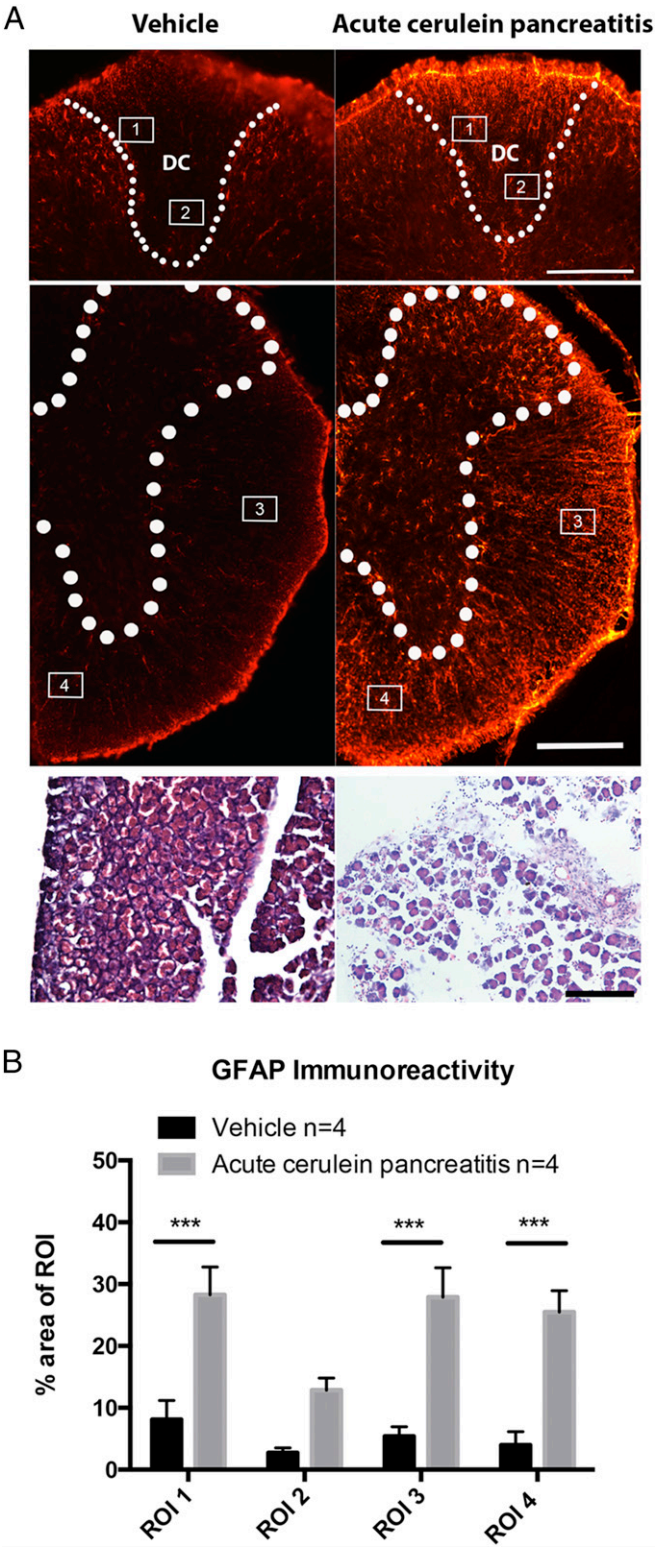

Fig. 1. Cerulein-induced acute pancreatitis causes spinal inflammation. (*A*) The thoracic spinal cord has increased GFAP immunoreactivity in cerulein-treated mice. H&E staining (*Lower*) shows cerulein-induced disruption in pancreatic histology. (*B*) Quantification of GFAP immunoreactivity across ROIs encompassing the dorsal columns, anterolateral, and medioventral white matter tracts of vehicle and cerulein-treated groups. ***$P < 0.001$, $n = 4$ per group. DC, dorsal columns. (Scale bar, 200 μm.)

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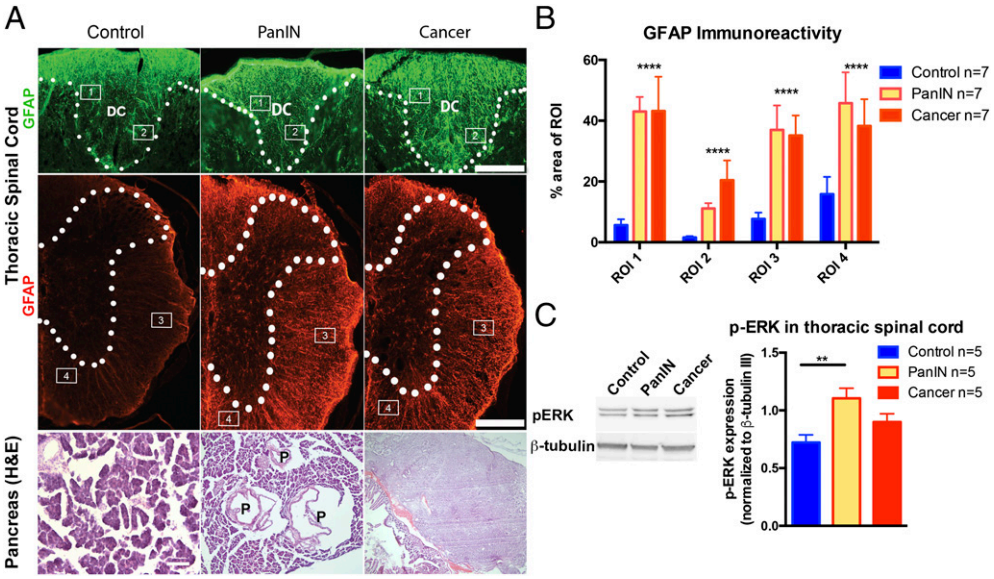

Fig. 2. Spinal inflammation is detected in early phases of PDAC. (*A*) Disease-stage–specific changes occur in GFAP staining in the thoracic spinal cord and pancreatic histology in PKCT and PKCY mice. (*B*) Astrocyte reactivity increases in regions of the spinal cord at the PanIN and cancer stage. (*C*) p-ERK is up-regulated in thoracic spinal cord of PKCT mice at the PanIN stage. ****$P < 0.0001$, $n = 5$–7 per group. DC, dorsal columns; P, PanIN lesion. (Scale bar, 200 μm.)

metastasis formation (26). Interestingly, a similar correlation occurs in PKCT mice where cells of pancreatic origin migrate to the DRG during PanIN and early cancer stages (Fig. 4). Tdtomato (Tdt)-positive pancreatic cells were also present in thoracic and lumbar (Fig. 4) spinal cord. Invasion into the lumbar spinal cord was observed only in mice that exhibited cell migration into the thoracic segment, suggesting Tdt cells enter at thoracic levels and migrate along the rostrocaudal axis to lumbar regions. Tdt cells were present in five of six mice examined at the PanIN stage and in seven of eight mice with primary tumors without metastases. The presence of pancreas-derived cells, which may activate glial defense mechanisms, could explain why regions of the spinal cord unrelated to pancreatic sensory innervation (i.e., ventral white matter) exhibit increased GFAP immunoreactivity. This migration may also underlie the increased ATF3 expression in PKC mice relative to mice with cerulein-induced inflammation.

Neonatal Ablation of Sensory Fibers Prevents CNS Inflammation in PKC Mice and Slows PanIN Progression. Because primary afferents contribute to inflammation in mouse models of pancreatitis, we examined whether sensory neuron ablation inhibited activation of spinal glial cells in PKCY mice. Postnatal days 1–2 (P2) mice treated with capsaicin had no loss of nodose neurons (Fig. 5*A*) but did have a reduction in neurons in thoracic DRG (Fig. 5*B*). A shift in the percentage of neurons with larger soma size also occurred (Fig. 5*C*), suggesting successful ablation of small diameter C fibers. Importantly, PKCY mice treated with capsaicin exhibited normal levels of GFAP immunoreactivity in the spinal cord (Fig. 5*D*), suggesting that activation of spinal astrocytes requires intact pancreatic sensory neurons.

Sensory neuron ablation attenuates inflammation in models of pancreatitis, suggesting these neurons provide proinflammatory signals (8, 10, 11, 13, 14, 27). To examine how sensory neurons impact PDAC initiation and progression, we first determined if neonatal capsaicin had direct effects on pancreatic cells because previous studies using cancer-derived cell lines showed it affected apoptosis and proliferation (28–34). The neurotoxic effects of neonatal capsaicin treatment are assumed to be via binding to TRPV1, the vanilloid receptor specific for capsaicin. At the dose used in this and the majority of studies, a single treatment ablates unmyelinated fibers and a subset of Aδ-myelinated afferents (35–39). This specificity of action correlates to what is known about the developmental expression of TRPV1, i.e., that it is expressed embryologically in virtually all C fibers and a subset of Aδ fibers and then becomes restricted to a subset of these fibers in adulthood (40). To determine whether the P2 pancreas is affected by capsaicin treatment via a receptor-mediated mechanism, we used semiquantitative real-time PCR to measure TRPV1 transcript level. Although highly expressed in the thoracic DRG ($\Delta CT = 6.61 \pm 0.23$, $n = 8$ normalized to GAPDH), where significant neuron loss is measured in adults, (Fig. 5*B*), TRPV1 was undetectable in the P2 pancreas ($n = 7$). The absence of TRPV1 in the P2 pancreas suggests that neonatal capsaicin could not affect pancreatic cells via a receptor-specific mechanism. However, as noted

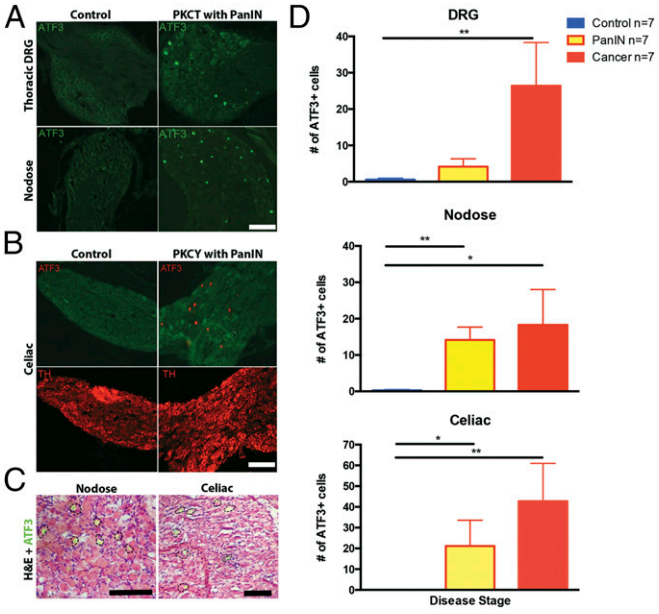

Fig. 3. Pancreatic disease increases ATF3 in sensory afferents. (*A*) Nuclear localization of ATF3 immunoreactivity in DRG (*Top*) and nodose ganglia (*Bottom*) of PKCT mice at the PanIN stage. (*B*) ATF3 is also elevated in celiac ganglia of PKCY mice. (*C*) H&E staining shows ATF3-IR in neurons of nodose and celiac ganglia. (*D*) Summary of increases in neuronal ATF3 expression in sensory and autonomic ganglia during PanIN and cancer stages. *$P < 0.05$, **$P < 0.01$, $n = 7$ per group. (Scale bar, 100 μm.)

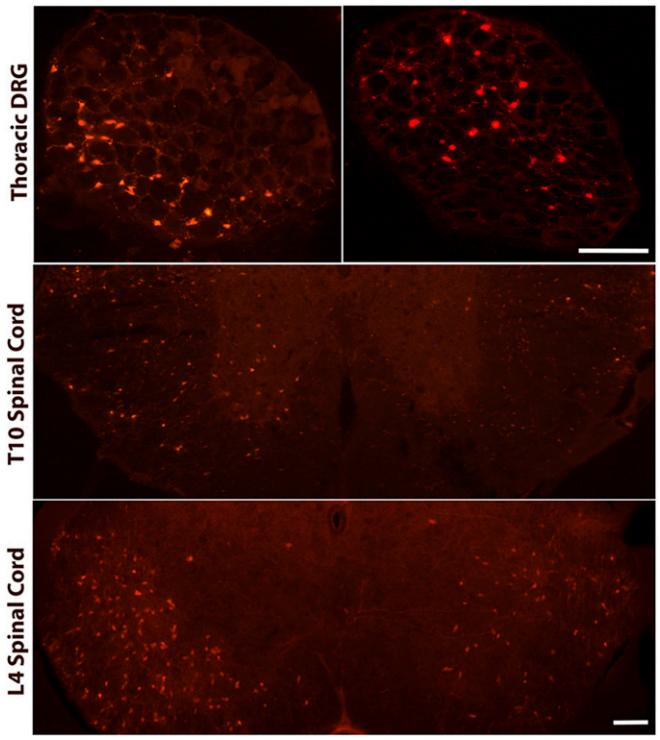

Fig. 4. Pancreatic cells invade the DRG and spinal cord before PDAC development. Individual Tdtomato positive cells are present in thoracic DRG and thoracic and lumbar regions of the spinal cord. (Scale bar, 200 μm for DRG and 500 μm for spinal cord sections.)

by Diaz-Laviada and Rodriguez-Henche (41), for many tumor cell lines, capsaicin effects, especially with respect to alterations in apoptosis or proliferation, are TRPV1 independent (33, 42, 43). We therefore examined pancreata from P2 vehicle- and capsaicin-treated mice stained for markers of apoptosis (anticaspase 3) and proliferation (anti-Ki67) 2 h posttreatment. There was no significant difference for either marker in pancreata from mice treated with capsaicin compared with vehicle-treated controls (Fig. S2).

To examine the long-term effect of neonatal sensory neuron ablation on cancer progression, we assessed the presence of precancerous PanIN lesions in pancreata from 10-wk-old PKC mice that were treated with vehicle or capsaicin at P2. At 10 wk, there were significantly fewer grade 1 and 2 PanINs in pancreata from mice treated with capsaicin (Fig. 6 *A*–*C*). There was a trend for a decrease in grade 3 PanINs. We next performed a survival study of PKCY mice treated with neonatal capsaicin and found that capsaicin treatment significantly prolonged survival of PKCY mice compared with vehicle-treated controls (median survival, 7.80 vs. 4.53 mo, $P = 0.0001$, Fig. 6*D*). Moreover, this increase in survival is likely an underestimate as a number of capsaicin-treated mice were euthanized to allow histological analysis of disease progression even though no tumors were detected by ultrasound (these mice are indicated by arrows in Fig. 6*D*). In some of the longest-lived cases (two of the mice euthanized at 18.9 mo) only benign metaplasia with low-grade inflammation was observed. These data suggest that sensory innervation of the pancreas is required for both the initiation and progression of PanINs and that ablation of innervation can significantly increase survival.

Discussion

PDAC is characterized by a complex and exuberant desmoplastic inflammatory stroma. Whereas some aspects of the tumor microenvironment support tumor growth, others can inhibit tumorigenesis. In humans, pancreatic inflammation is one of the most significant risk factors for the development of PDAC. The data presented here demonstrate that at the PanIN stage of PDAC in the PKC model, spinal cord inflammation and PNI of both the spinal cord and peripheral ganglia are already well under way. Stopczynski et al. (26), showed that in the PanIN stage, sprouting of sensory fibers and increases in neurotrophic factors accompany an increase in mRNAs encoding CGRP, TRPV1, and TRPA1 in DRG, all hallmarks of pancreatic inflammation driven by increased activity in pancreatic sensory neurons, i.e., neurogenic inflammation. Growth-factor–related changes, as well as chemokine release, support PNI (44–47), a common clinical feature of several cancers including up to 100% of PDAC cases (48–50).

In mouse models of both acute and chronic pancreatitis, silencing of pancreatic afferents can block neurogenic inflammation (10, 11). Thus, the present study was formulated to test the hypothesis that ablation of sensory neurons (to silence primary afferents throughout PDAC progression) might slow tumorigenesis. Results indicate that loss of sensory neurons slows the development of PanIN lesions and significantly increases overall survival, and both of these phenomena are accompanied by the absence of spinal cord inflammation.

That the peripheral nervous system plays a role in tumor progression has been reported recently for two types of visceral tumors; however, the focus of these studies was on the autonomic portion of the peripheral nervous system. Magnon et al. (51), used a mouse model of prostate cancer and found that chemical or surgical ablation of hypogastric nerves was associated with decreased tumorigenesis. They proposed that sympathetic postganglionic neurons regulated the initiation of the disease, whereas postganglionic parasympathetic neurons were implicated in advanced disease. Zhao et al. (52), used three models of gastric cancer and found that surgical vagotomy slowed and/or decreased tumor progression and increased the effectiveness of chemotherapy. Parasympathetic postganglionic neurons were also implicated based on changes in signaling pathways downstream of the type 3 muscarinic receptor expressed by these neurons, and that botox was able to slow tumor growth. Unfortunately, the role of sensory neurons was not examined in this study despite the fact that 85% of

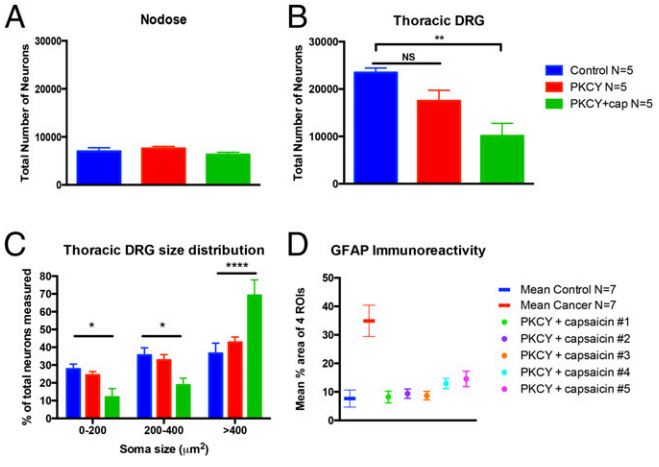

Fig. 5. Neonatal capsaicin ablates DRG neurons. (*A*) The total number of nodose ganglion neurons is unchanged in adult mice treated with capsaicin at P2. (*B*) In contrast, capsaicin causes a significant loss of thoracic DRG neurons. (*C*) A rightward shift in somal diameters of the remaining neurons indicates selective reduction of small diameter afferents. (*D*) Neonatal capsaicin prevents spinal inflammation. The mean percent area of the four ROIs (designated in Fig. 2*A*) covered by GFAP immunoreactivity is plotted as a mean ($n = 7$ per group) and compared with the mean of the four ROIs for individual animals treated with neonatal capsaicin. PKCY mice treated with capsaicin exhibit GFAP immunoreactivity levels similar to control. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

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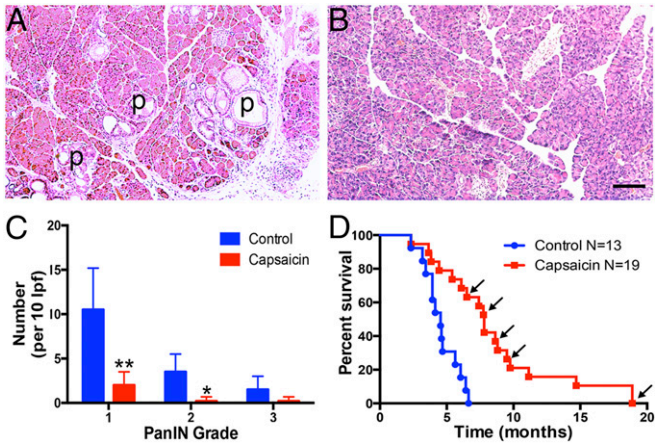

Fig. 6. Neonatal capsaicin slows development of PanIN lesions and prolongs survival of PKCY mice. Pancreata of 10-wk-old PKC mice were treated with vehicle (*A*) or capsaicin (*B*). Vehicle-treated mice exhibit numerous PanIN lesions (P), whereas pancreata from capsaicin-treated mice do not. (*C*) There were significantly fewer total PanIN lesions and virtually no lesions above grade 1 in capsaicin-treated mice; $*P < 0.05$, $**P < 0.001$. (*D*) Capsaicin-treated mice survive longer than vehicle-treated PKCY mice. Arrows indicate mice that were euthanized for histological analysis while otherwise healthy (no tumors upon ultrasound).

vagal fibers are from sensory neurons originating in the nodose ganglia (53).

The potential role of sensory neurons and their association with sympathetic and parasympathetic neurons remains to be defined. Mantyh and colleagues have documented in detail that autonomic nerves, especially sympathetic postganglionic fibers, are affected in cancer progression (5, 54–56). Moreover, both sympathetic and parasympathetic neurons release molecules that can activate sensory neurons; sympathetic postganglionic neurons release three molecules of ATP for every molecule of norepinephrine. Sensory neurons express both ionotropic and metabotropic receptors for ATP and its breakdown products (e.g., ADP). Sensory neurons also express nicotinic receptors (e.g., α3, β4, α7) (57, 58) that can be activated by acetylcholine released from postganglionic parasympathetic neurons. That sensory neurons play a direct role in tumor formation has also been reported for basal cell carcinoma (59). In these studies, tamoxifen-inducible Cre drivers were used to delete the hedgehog suppressor Ptch1 in adult mouse skin. This strategy was particularly effective at producing tumors in touch domes, sensory structures containing specialized epithelium (Merkel cells) innervated by large, low-threshold, mechanically responsive sensory fibers. Once tumor formation was underway (timed by application of tamoxifen), denervation dramatically suppressed tumorigenesis, suggesting that sensory neurons directly contribute to an environment that supports tumor production.

The data reported here also indicate that sensory neurons and the spinal cord are extremely sensitive to pancreatic disease progression. Although at the early PanIN stage the pancreas does not exhibit major inflammation, astrocyte activation in the spinal cord was equivalent to that seen in response to acute pancreatitis. The activation of spinal cord glia affected all regions of the white matter although pancreatic sensory fibers run primarily in the dorsal columns (60–63) and anterolateral regions of the spinal cord (64). In patients with pancreatic cancer, peripheral nerve damage associated with PNI has been linked to spinal cord glial activation in T10–L1 (65). Thus, the widespread appearance of activated astrocytes is likely related to the migration of pancreatic cells from the pancreas to the peripheral nervous system where they track into the thoracic and lumbar spinal cord. It should be noted that the tumorigenic potential of these cells is unknown; however, spinal metastases are rare in patients with PDAC. At later disease stages, tumors can be found in both peripheral nerves and spinal cord in the PKC model (26), but at the time in which spinal inflammation is first observed, no obvious tumors were detected. Importantly, the migration of cells is not restricted to animal models. Circulating pancreatic cells have been identified even in patients with pancreatitis and precancerous lesions, but no cancer diagnosis (1).

A central role of primary afferents in pancreatic disease has been reported for models of pancreatitis and diabetes, where sensory neuron-released CGRP played a role in initiation and prevention of islet cell inflammation (10, 11, 66). In these cases, the link between sensory neurons and pancreatic cells relates to the ability of the sensory system to regulate neurogenic inflammation. But, could there be a more direct and fundamental interaction between these two cell types? It is well documented that sensory neurons have efferent function through the wide range of small molecules they release in the periphery such as glutamate, ATP, CGRP, and SP (27, 67–69). Moreover, sensory, sympathetic, and parasympathetic neurons express receptors for these molecules that when stimulated, release additional substances that modulate pancreatic cellular function. These interactions are present in virtually all tissues (e.g., prostate, stomach, skin) in which recent studies have invoked a role for the peripheral nervous system in tumorigenesis. These convergent observations suggest a common mechanism by which predisposing genetic mutations hijacks the normal homeostatic process regulated by the peripheral nervous system to produce an environment that supports tumorigenesis.

Methods

Animals were cared for and studies were performed in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh/University of Michigan and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Two mouse models of PDAC, PKCY, and PKCT were generated (1, 26) (Fig. S1). Disease stage was assessed using H&E staining. Pancreatitis was induced using repeated cerulein injections (1, 10, 70). Sensory neurons were ablated via neonatal capsaicin treatment (20 μL, 50 mg/kg, i.p.) at 1–2 d of age (P2). More detailed information including the protocols for assessing spinal inflammation can be found in *SI Methods* (23, 26, 71–81).

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