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Systemic Depletion of Nerve Growth Factor Inhibits Disease Progression in a Genetically Engineered Model of Pancreatic Ductal Adenocarcinoma

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Abstract

Objectives—In pancreatic ductal adenocarcinoma (PDAC) patients, increased expression of proinflammatory neurotrophic growth factors (e.g. nerve growth factor (NGF)) correlates with a poorer prognosis, perineural invasion (PNI) and, with regard to NGF, pain severity. We hypothesized that NGF sequestration would reduce inflammation and disease in the KPC mouse model of PDAC.

Methods—Following biweekly injections of NGF antibody or control IgG, beginning at 4 or 8 wk of age, inflammation and disease stage were assessed using histological, protein expression, and qPCR analyses.

Results—In the 8 wk anti-NGF group, indicators of neurogenic inflammation in the dorsal root ganglia ([DRG], substance P and CGRP) and spinal cord (GFAP) were significantly reduced. In the 4 wk anti-NGF group, TRPA1 mRNA in DRG and spinal p-ERK protein were elevated, but GFAP expression was unaffected. In the 8 wk anti-NGF group, there was a 40% reduction in the proportion of mice with microscopic PNI and no macrometastases were observed.

Conclusions—Anti-NGF treatment beginning at 4 wk may increase inflammation and negatively impact disease. Treatment starting at 8 wk (after disease onset), however, reduces neural inflammation, neural invasion, and metastasis. These data indicate that NGF impacts PDAC progression and metastasis in a temporally dependent manner.

Keywords

nerve growth factor; pancreatic ductal adenocarcinoma; inflammation; perineural invasion; metastasis

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INTRODUCTION

Nearly all patients (80–100%) with pancreatic ductal adenocarcinoma (PDAC) exhibit neural inflammation (neuritis), pain and tumor cell invasion into the perineurium and/or endoneurium of nerve fibers (PNI).[1–5] Increased expression of members of the transforming, hepatocyte and endothelial growth factor families has been postulated as a driver of PNI, promoting “acinar to ductal metaplasia” and inflammatory desmoplasia, features seen in early stage neoplasia.[6–11] More recently however, ligands in the neurotrophin and glial cell line-derived neurotrophic factor (GDNF) families and their cognate receptors have been implicated in proliferation and invasiveness of PDAC. Many of these growth factors and their receptors exhibit increased immunoreactivity in intra-pancreatic nerves and pancreatic tissues.[12–21] Up regulation of these factors and/or receptors in patients has been correlated with a poor prognosis, extensive neural invasion, and, in the case of nerve growth factor (NGF), severe pain.[22–25]

The increased production/concentration of neurotrophic factors by tumor cells and intra-pancreatic nerve fibers raises the question as to whether suppression of growth factor signaling might inhibit the development and progression of pancreatic disease. Nerve growth factor (NGF) is a prime candidate for this role because of its strong neurotropic effects on peripheral neurons [reviewed in[26,27]]. NGF not only promotes neuronal survival but is also a key modulator of neurogenic inflammation and pain through its up regulation of inflammatory peptides (e.g. calcitonin gene related peptide (CGRP) and substance P (SP)) and its sensitizing effects on sensory neuron firing properties. Several completed and ongoing clinical trials have utilized NGF sequestration as a strategy to block pain signaling.[28–34] Preclinically, NGF sequestration has ameliorated pain behaviors in a variety of animal models with the most relevant being pancreatitis post-surgical pain, and metastatic cancer pain.[35–37]

Although the role of NGF in cancer pain has been documented, there is limited information available regarding how NGF signaling impacts cancer progression. Studies of human and murine PDAC cell lines indicate that NGF can promote proliferation, migration, and invasiveness of tumor cells.[20,38–40] Other *in vitro* and xenograft experiments show that NGF antibody (anti-NGF) treatment or siRNA-mediated knockdown of NGF reduces cell proliferation and inhibits growth of breast, prostate, and oral squamous carcinomas.[25,41,42] However, there are no studies that directly examine how suppression of NGF signaling affects PDAC in an *in vivo* transgenic model.

Genetically engineered mouse models (GEMMs) of PDAC that express the most common human mutation associated with the disease ($Kras^{G12D}$) provide an important physiologically relevant tool to investigate the role of growth factor signaling. These GEMMs share many of the pathological features of human PDAC including temporal progression of precursor lesions (pancreatic intraepithelial neoplasias, PanINs) to primary and metastatic tumors. With disease progression, intra-pancreatic nerve fibers exhibit hypertrophy, and mice exhibit pain-related behaviors that correlate with a significant up regulation of NGF and its receptor TrkA.[43] Interestingly, during initial acinar to ductal metaplasia and early PanIN development, the peripheral nervous system exhibits signs of

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injury that may be linked to an influx of pancreatic lineage cells and up-regulation of neural inflammatory markers.[44] These data are in line with other studies reporting that dissemination of pancreas lineage cells precedes tumor formation.[45,46] Because increased NGF/TrkA expression is correlated with greater inflammation, cell proliferation, invasion and poorer prognosis in both humans and xenograft models, we explored the hypothesis that NGF sequestration could reduce neural inflammation and impede PDAC development in a physiologically relevant GEMM.

2. MATERIALS AND METHODS

2. 1 Animals

The KPC mouse model of PDAC was used for all experiments.[44] In this model the Pft1a/p48 promoter drives expression of a mutant Kras allele (LSL-Kras^{G12D}) and one allele of the p53 tumor suppressor gene is deleted in a Cre-dependent manner. Some KPC mice also expressed the fluorescent reporter protein tdTomato in a Cre-dependent manner. Mice were group-housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Division of Laboratory Animal Resources at the University of Pittsburgh. They were maintained in a 12-h light/dark cycle and temperature-controlled environment with ad libitum access to water and food. Mice were cared for and used in these studies following guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Anti-NGF Treatment

Mice were randomly assigned to receive biweekly intraperitoneal injections of anti-NGF (200μg/kg, Catalog # AF-556-NA, R&D systems, Minneapolis, Minn) or immunoglobulin G (IgG, 200μg/kg; R&D Systems) beginning at either age 4 or 8 wk of age. Unless mice succumbed to disease prematurely (n = 3), animals were euthanized via an overdose of inhaled isoflurane, perfused transcardially with saline at ≥16 weeks of age and tissue collected for analyses.

2.3 Antibody Immunolabeling

Mice were euthanized with inhaled isoflurane and perfused with saline. Superior cervical ganglia (SCG) and dorsal root ganglia (DRG) were removed, post-fixed for 30 min in 4% paraformaldeyhyde (PFA) and cryoprotected in 25% (wt/vol) sucrose in 0.1 M PB at 4°C. Pancreata were post-fixed overnight in 4% PFA with 15% (vol/vol) picric acid prior to cryoprotection. SCG and pancreata were embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, Calif), sectioned at 14 and 30 μm respectively and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pa). Sections were incubated in 10mM citrate buffer, pH 6.0 for 3–8 min at 95°C followed by 20 min at room temperature, then washed, blocked with 5% (vol/vol) normal horse serum in 0.1 M PB containing 0.25% Triton X-100 and incubated overnight in primary antibody. Antibodies used were: anti-tyrosine hydroxylase (1:500, AB152, Millipore, Temecula, Calif) or anti-CGRP (1:500, C8198, Sigma-Aldrich, St. Louis, Mo). Floating thoracic spinal cord sections (30 μm) were incubated overnight in anti-GFAP (1:500, 3670S clone GA5, Cell Signaling Technology,

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Danvers, Mass.) or anti-NeuN (1:500, MAB377 clone A60, Millipore). Following incubation with primary antibody, sections were washed and immunoreactivity detected using dye-conjugated secondary antibodies (1:500, Jackson ImmunoResearch Laboratories, West Grove, Pa). Sections were photographed using LAS software version 4.7 (Leica Microsystems, Buffalo Grove, Ill) and a Leica DM 4000B microscope and fluorescent intensity measured using Image J version 1.49 (NIH, Bethesda, Md).

2.4 Western Immunoblot Analysis

Total protein was extracted on ice by homogenization in 50 mM Tris·HCl lysis buffer (pH 7.4) containing 0.5% SDS and protease inhibitors (Cell Signaling Technology). Protein concentration was determined via bicinchoninic acid assay (Fisher Scientific) and aliquots (40μg) separated on 12% SDS/PAGE gels were transferred to PDVF membranes using the Transblot system (Bio-Rad, Hercules, Calif). Nonspecific binding was blocked using 5% (wt/vol) BSA, membranes were incubated overnight in primary antibodies directed against p-ERK (p44/42) or GFAP (1:1,000; Cell Signaling Technology) and protein bands detected using HRP-conjugated secondary antibodies (1:5,000). Membranes were washed and then probed with GAPDH or total ERK antibodies as loading controls. Densitometry readings were performed using SuperSignal Chemilumescent Detection reagents (Fisher Scientific), a LAS3000 imager (Fujifilm, Stamford, Conn), and Image J software. Protein levels were normalized to the IgG treated groups.

2.5 Semi-quantitative Real-time PCR

RNA from thoracic level DRG (T9–12) was isolated using the RNeasy mini kit (Qiagen, Germantown, Md), treated with DNase (Invitrogen, Pittsburgh, Pa) and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). SYBR Green PCR amplification was performed using a BioRad CFX connect real time system. After amplification, a dissociation curve was plotted against melting temperature to ensure amplification of a single product. All samples were run in duplicate and control reactions run (e.g., RT carried out without template). The relative fluorescence of SYBR Green bound to double-stranded DNA was compared with a passive reference for each cycle. Threshold cycle (Ct) values were used as a measure of initial template concentration. Fold changes in RNA levels were calculated by the ΔΔCt method using GAPDH as a reference standard: Ct values from duplicate samples were averaged and then subtracted from the reference standard, yielding ΔCt. Primer sequences are available upon request.

2.6 Histopathologic Analysis

Animals were examined for the presence of gross metastatic tumors at the time of dissection. The pancreas was then dissected *en bloc* with stomach and spleen and the number of additional organs involved was noted (observed range: 0–3). Hematoxylin and eosin stained sections of de-identified pancreas were used to define the disease stage for each animal (independently by Drs. Singhi and Hartman). Histopathology of lesions and fibro-inflammatory stroma were quantified by measuring the percentage of total analyzed surface area occupied. For each case, 10–15 fields of view were analyzed. Lesions were classified as low-grade (PanIN-1/2), high-grade (PanIN-3) or PDAC (tumor) based on the 2015 classification guidelines.[47]

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2.7 Statistical Analysis

All numerical data were compared using two-way ANOVA followed by Sidak's test for multiple comparisons. Categorical data (disease stage) were compared using a cumulative link model. Ordinal data (number of metastatic sites) were analyzed using Poisson's regression.

3. RESULTS

3.1 Anti-NGF Reduces Markers Associated With Neurogenic Inflammation and PDAC Progression

In the absence of intervention, PDAC progression and behavioral hypersensitivity in the KPC GEMM is associated with increased expression of growth factors, growth factor receptors, and neuropeptides associated with neurogenic inflammation (NI).[43,44] To determine if suppression of NGF signaling alters these PDAC-induced changes we used RT-PCR, protein immunoblotting and tissue immunolabeling to assess relative expression in anti-NGF and IgG treated KPC mice. We first examined mRNAs encoding the NGF receptors, TrkA and p75.[26,48] Both were significantly decreased in DRG of anti-NGF treated mice compared to IgG-treated mice, which was expected if sequestration of NGF ligand by antibody injection was effective. Specifically, there was a main effect of anti-NGF on TrKA mRNA regardless of age at onset of intervention [drug: $F(1,30) = 78.65$, $P < 0.0001$, Fig. 1A]. Anti-NGF also reduced p75 mRNA, with a greater reduction in the group that began treatment at 8 wk of age [time×treatment: $F(1,30) = 18.94$, $P = 0.0001$, Fig. 1B]. The decrease in NGF receptors was accompanied by a reduction in mRNAs that encode proinflammatory peptides. For example, *Tac1* (tachykinin 1), which encodes SP was decreased in DRG from KPC mice that began treatment at 8 wk of age as compared to IgG treated mice [time: $F(1,26) = 18.90$, $P = 0.0002$, Fig. 1C]. Compared to IgG treated mice, *CalcB* mRNA, which encodes CGRP, was decreased in mice that started anti-NGF at 4 wk of age but was down-regulated to a greater extent in mice that started treatment at 8 wk [time × treatment: $F(1,34) = 13.48$, $P = 0.0008$, Fig. 1D]. Anti-NGF treatment did not affect mRNA encoding transient receptor potential cation channel subfamily V member 1 (TRPV1, a channel shown to be important for inflammatory pain[49]) (Fig. 1E), but a significant increase in DRG mRNA encoding the related TRPA1 channel was seen in mice beginning anti-NGF treatment at 4 wk of age was measured [time × treatment: $F(1,30) = 4.536$, $P = 0.041$, Fig. 1F].

Perineural invasion in PDAC is associated with local nerve injury, neuritis and accompanied by increased spinal glial activation in both human and mouse.[5,44] To determine if anti-NGF impacts PDAC associated glial activation, levels of glial fibrillary acidic protein (GFAP) were assessed in the thoracic spinal cord, at the vertebral levels that innervate the pancreas.[50] Staining of thoracic spinal cord sections suggested a reduction in GFAP immunoreactivity and a lack of 'activated morphology' (as signified by the punctate nature of the staining) in the 8wk treatment group (Fig. 2A). The effects of anti-NGF of GFAP expression were quantified by immunoblotting of total protein from spinal cord, which showed a reduction of GFAP level in anti-NGF mice compared to IgG injected mice that was dependent on the age of treatment onset [time × treatment: $F(1,36) = 9.71$, $P = 0.004$]. KPC mice that began

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treatment at 4 wk exhibited no effect of anti-NGF on GFAP level whereas mice treated at 8 wk of age exhibited significantly lower GFAP immunoreactivity compared to all other groups (Fig. 2B).

Anti-NGF injections were also found to have an effect on spinal levels of phosphorylated ERK (p-ERK), an indicator of inflammation and pain[51] that is elevated at the PanIN stage of PDAC development.[5,44] Spinal p-ERK was increased in mice that began anti-NGF injections at 4 wk of age compared to all other groups, while there was no difference observed in the 8 wk anti-NGF group compared to either IgG treated group [time × treatment interaction: $F(1,36) = 8.51$, $P = 0.006$, Fig. 2C]. The differential increase in TRPA1 mRNA and p-ERK protein suggest that timing of anti-NGF intervention is a significant factor: at 4 wk, when animals are relatively healthy, anti-NGF may drive neural inflammation whereas intervention at 8 wk, when PanINs are present, inhibits neural inflammation.

3.2 Anti-NGF Does Not Directly Impact Primary Disease

To determine the effects of anti-NGF treatment on the initial occurrence of PDAC, sections of pancreas tissue were scored in a blinded manner to classify animals based on the most severe lesion present: normal, low grade PanIN, high grade PanIN, or PDAC (Fig. 3A). Specifically, the percent area occupied by low or high grade PanINs, surrounding fibro-inflammatory stroma, and tumors were measured in 10–15 fields of view from each animal. Using a cumulative link model to analyze the distributions of diagnoses, no significant differences were detected (Fig. 3B). Similar to human cancers, every case in the mouse was different and this variability prevented the detection of significant differences in any of the measured parameters (Fig. 3C–F). However, it is worth noting that 25% of the 8 wk anti-NGF exhibited no pancreatic disease whereas all mice in the other groups exhibited abnormal pancreata, suggesting that suppression of NGF signaling may slow the development of pancreatic disease.

3.3 Anti-NGF Therapy Reduces Tumor Cell Mobility and Metastases

To assess the effect of anti-NGF on PNI we crossed a Cre-dependent tdTomato allele into the KPC GEMM. TdTomato expression enabled tracking of pancreas-lineage cells that disseminated from the pancreas to the thoracic spinal cord (Fig. 4A,B). Pancreatic cell invasion of the thoracic spinal cord was present in 100% of mice that began treatment (anti-NGF or IgG) at 4 wk of age, whereas there was a significant reduction in the proportion of mice exhibiting PNI in the 8 wk anti-NGF group [Logistic Regression, $P = 0.045$, Fig. 4C]. In order to assess the extent of PNI, the average number of tdTomato+ cells in a $0.25mm^2$ region of interest (indicated in Fig. 4B) was determined in five random sections of spinal cord per mouse. There was a significant reduction in the extent of PNI in the 8 wk anti-NGF group relative to the 4 wk and IgG treatment groups [time × treatment: $F(1,18) = 16.57$, $P = 0.0007$, Fig. 4D].

In vitro and xenograft studies suggest growth factor signaling stimulates the invasive and migratory capabilities of cancer cells.[20,38–40] A prediction from these observations is that suppression of growth factor signaling in a GEMM should reduce metastases. We found that similar to other studies,[52,53] grossly detectable metastases were present in 30.2% (13/43) of

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untreated KPC mice. Importantly, anti-NGF treatment affected this critical disease feature in a manner dependent on the age of treatment onset. In the 4 wk group, the same number of mice exhibited metastasis regardless of treatment (Fig. 5). However, mice that began anti-NGF treatment at 8 wk of age exhibited a complete absence of grossly detectable metastatic tumors [Poisson regression, $P = 0.007$]. That later intervention reduces metastases suggests that anti-NGF treatment could be used as a potential neoadjuvant to restrict tumor cells to the pancreas, providing a greater chance for successful surgical intervention.

4. DISCUSSION

Inflammation and tissue injury are key features of PDAC that extend beyond the pancreas to the nervous system. Perineural invasion and increased levels of neurotrophic factors correlate with poorer prognosis.[22–25] In mice, neural inflammation and PNI occur early in the disease process and are accompanied by an up regulation of NGF.[43,44] In the current study, we present evidence that suppressing the rise in NGF can inhibit neural inflammation and restrict the dissemination of pancreas cells. If the extent of dissemination predicts the incidence of recurrence following pancreatic resection, the ability to inhibit cell migration from the diseased pancreas could significantly enhance the potential for successful treatment.

Our data show the effects of anti-NGF therapy depend on the time at which treatment begins. Intervention initiated at 4 wk (when mice are healthy) had no influence on PNI or spinal GFAP expression when assayed at 16 wk of age. However, the percent of mice with involvement of multiple (>1) additional organs was higher if anti-NGF treatment was begun at 4 wk, suggesting that sequestration of NGF prior to the appearance of disease may worsen the outcome. Mechanistically, this may relate to an increase in neurogenic inflammation, as suggested by the increased levels of TRPA1 and p-ERK in this group. This change in inflammation could be due to the long-term suppression of NGF signaling (i.e., starting at 4 wk), that may generate an imbalance in growth factor signaling that promotes the development and aggressiveness of the disease.[21,54,55] NGF sequestration may also impact other cell types in the tumor microenvironment. NGF is essential for the survival and differentiation of sympathetic neurons,[56] and anti-NGF treatment significantly reduced transcription of tyrosine hydroxylase (TH) in the postganglionic sympathetic neurons of the superior cervical ganglia (SCG) [$F(1,25) = 23.81$; $P < 0.001$, Supplementary Fig. 1]. Interestingly, both p75 and trkA are also expressed by immune cells.[57] Reduction of NGF could therefore impact neural-immune communication and in so doing impede immune surveillance of tumor progression.[12,20,21,54,55,58–67] Significant differences in the cellular microenvironment on which anti-NGF is acting (healthy at 4wk vs. pathological at 8wk) could also contribute to the time-dependent effects we observed.

In contrast to mice that began treatment at 4 wk, mice treated with anti-NGF beginning at 8 wk, when PanINs are typically present, exhibited significant differences in disease parameters. In 8 wk mice we observed a significant decrease in SP and CGRP in primary afferents as well as a reduction in GFAP, a marker of spinal astrocyte activation and inflammation. Secondly, anti-NGF starting at 8 wk correlated with a significant reduction in the migration of tdTomato-expressing cells to the spinal cord. The ability of pancreas-

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derived cells to migrate from the pancreas is proposed to reflect cells undergoing an epithelial to mesenchymal transition (EMT), during which invasive and stem-like properties are acquired.[68,69] Early EMT and dissemination of pancreas-derived cells (preceding tumor formation) have previously been documented in PDAC GEMMs.[44,45] Anti-NGF mediated inhibition of pancreatic cell invasion of the cord therefore suggests a reduction in metastatic potential, spinal inflammation and cancer-related pain. Finally, animals treated with anti-NGF starting at 8 wk exhibited a significant decrease in gross metastasis, with none of the treated mice exhibiting tumors outside of the pancreas. In humans, the absence of pancreatic cell dissemination, and ultimately metastasis, is the difference between a prognosis in which resection can produce long-term survival versus one in which surgery is unlikely to prevent recurrence.

Whether the primary effect of anti-NGF treatment is on sensory afferents, sympathetic efferents or both, remains to be determined. Early sensory denervation is sufficient to slow or block tumorigenesis in an animal model of PDAC,[44,70] but sympathetic denervation was sufficient to reduce tumor burden in a model of prostate cancer.[71–74] If sympathetic neurons have a prominent role in PDAC progression, a prediction is that anti-NGF would have a more robust effect than that seen in prior studies where only sensory neurons were depleted using neonatal capsaicin treatment[44,70] Our results show this does not occur, at least in the treatment paradigm employed (starting treatment at either 4 or 8 wk). Although not killing sensory or sympathetic neurons,[33] anti-NGF did decrease TH immunoreactivity in the SCG as well as reduced mRNAs encoding TrKA and p75, in DRG, which confirms anti-NGF bioactivity. Even so, this treatment was not sufficient for total denervation of the pancreas since TH and CGRP immunoreactivity were still present in nerve fibers within the pancreata of treated animals (Supplementary Fig. 2).

That ablation (surgical or chemical) of adult sensory and/or sympathetic innervation of the pancreas is a useful strategy is suggested by studies demonstrating the efficacy of celiac plexus block or ganglion neurolysis for palliative pain management in PDAC patients with unresectable PDAC.[75–78] In one of the first double-blind placebo controlled study, patients with unresectable PDAC received either placebo or 50% ethanol-induced celiac plexus block.[79,80] Patients that received an ethanol-induced splanchnicectomy survived significantly longer than saline treated patients (median 9.15 vs. 6.75 months). An initial interpretation of this study was that patients lived longer because sensory depletion and resulting reduction in pain led to an improved quality of life. Unfortunately, subsequent studies failed to detect similar effects on survival, although all concluded that celiac block/neurolysis significantly improved quality of life.[76,78] In the context of the present study, results indicate that the time at which an anti-neural intervention is carried out is critical. Thus, neuron ablation or anti-NGF treatment must be performed within a window where inhibition of aberrant tumor-nerve interactions is effective. The challenge is to identify when this window of opportunity appears in each individual human patient.

Supplementary Material

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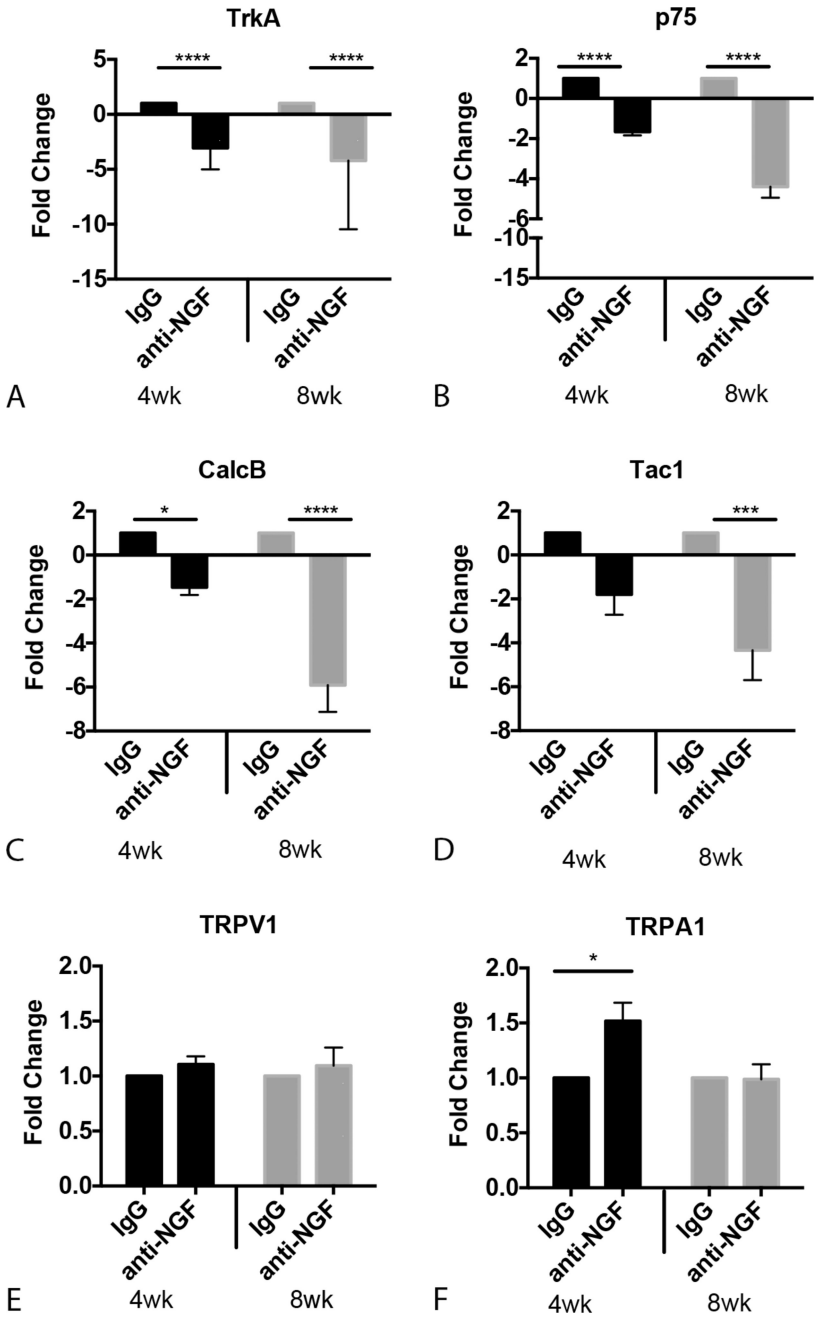

FIGURE 1.

Anti-NGF treatment alters levels of mRNAs encoding genes related to nociception and neurogenic inflammation in DRG T9–T12. A, TrkA and (B) p75 mRNA levels are significantly reduced following anti-NGF treatment regardless of age at onset of intervention. C, Substance P (Tac1) mRNA is significantly decreased in ganglia from mice treated with anti-NGF beginning at 8 wk of age. D, CGRP (CalcB) is significantly down regulated by anti-NGF. E) TRPV1 mRNA levels are unchanged by anti-NGF treatment. F, TRPA1 mRNA is significantly increased in thoracic DRG in the 4 wk anti-NGF group. Fold

change data were compared by two-way ANOVA followed by Sidak post-hoc test. *$P <$ 0.05, ***$P < 0.001$, ****$P < 0.0001$ n = 6–10/group.

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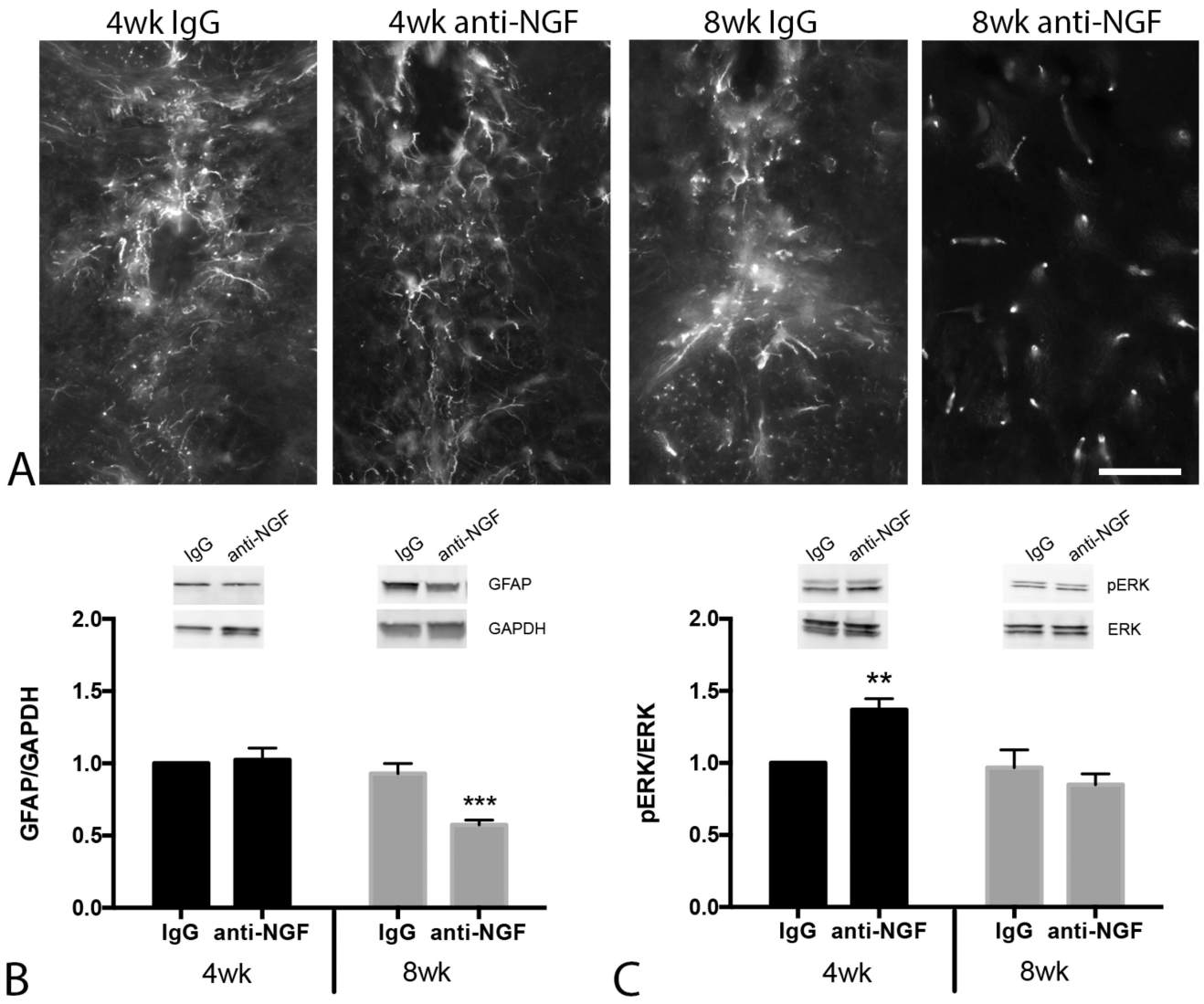

FIGURE 2.

Anti-NGF treatment reduces levels of the inflammatory marker GFAP in the thoracic spinal cord. A, High power micrographs show astrocyte morphology and GFAP immunoreactivity in the central canal region of the thoracic spinal cord across experimental groups. Note the lack of 'activated' astrocyte morphology in 8 wk treatment group. B, Western blots confirm reduced GFAP expression in 8 wk treatment group. Inset: Representative blot compares relative GFAP expression in IgG and anti-NGF treated mice. Band densities were normalized to GAPDH level. C, Mice that began anti-NGF at 4 wk of age exhibited significantly higher p-ERK expression. Inset: Representative western blot shows pERK expression (normalized to total ERK) in thoracic spinal cord. Immunoblot data were analyzed by two-way ANOVA followed by Sidak's test for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$ n = 7–11/group. Scale bar in A =50μm.

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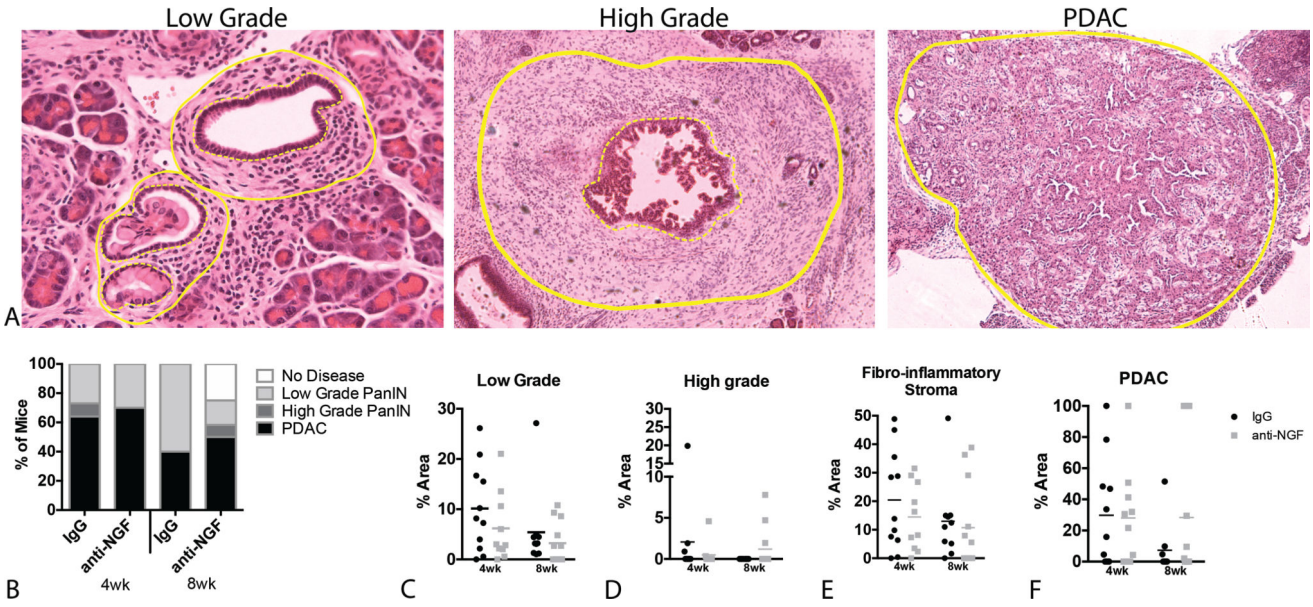

FIGURE 3.

Anti-NGF does not alter the development of primary pancreatic disease. A, Hemotoxylin and eosin staining of sections of pancreas from low- and high-grade PanIN lesions and PDAC tumor. Fibro-inflammatory stroma is demarcated between dotted and straight lines. B, The distribution of diagnoses was similar across treatment groups when compared using a cumulative link model. C–F, Charts indicate the percent area occupied by lesion, fibro-inflammatory stroma and PDAC tumor in pancreata of KPC mice. No significant difference in area is found in comparison of all groups using a two-way ANOVA. n = 10–12/group.

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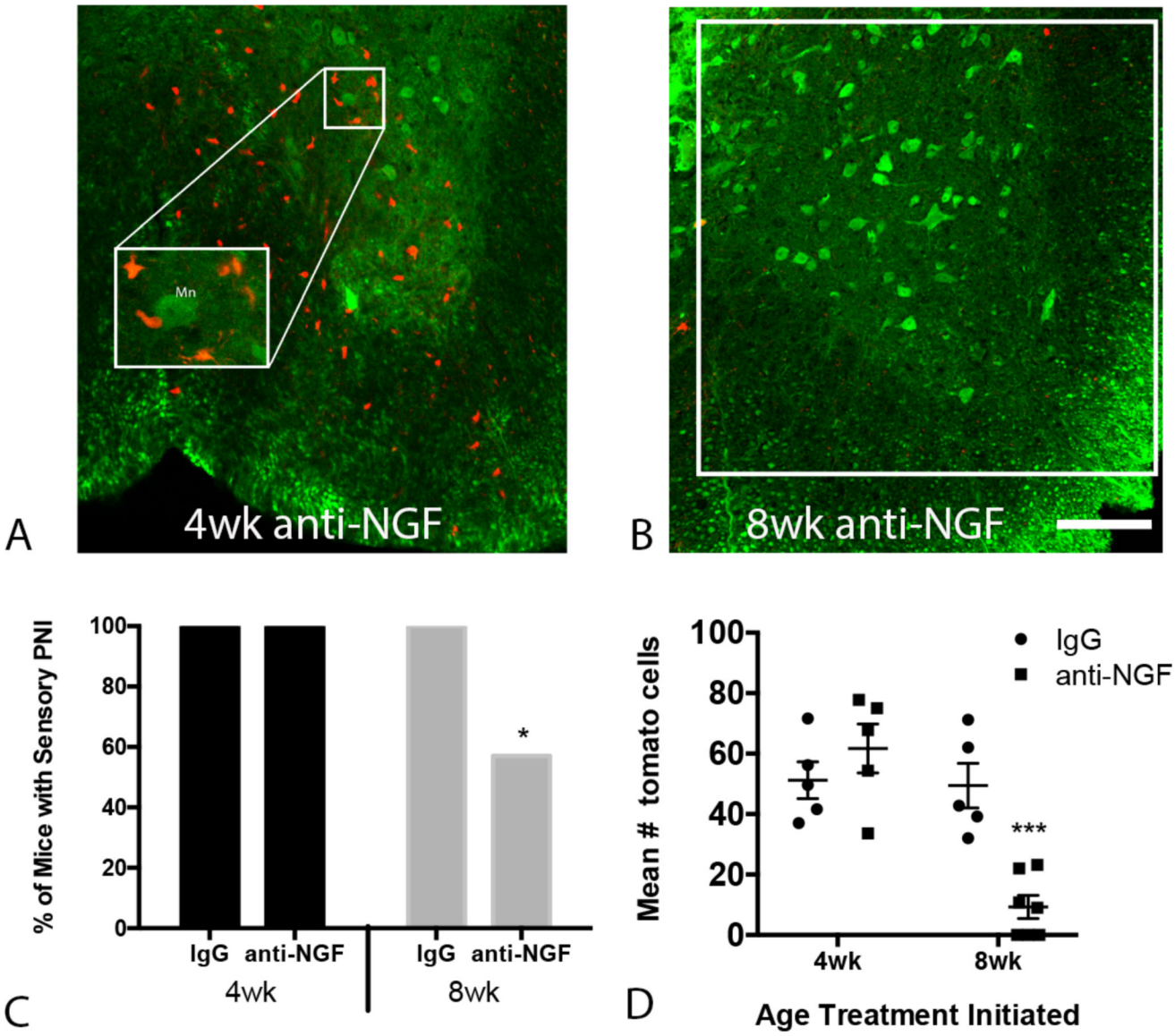

FIGURE 4.

Anti-NGF inhibits perineural migration of pancreatic lineage cells into the thoracic spinal cord. Representative micrographs of the ventral thoracic spinal cord of KPC mice treated with anti-NGF starting at 4 wk (A) or at 8wk of age (B). Pancreas-lineage cells are identified by tdTomato expression (red); neurons are immunolabeled with anti-NeuN (green). C, The proportion of mice exhibiting perineural invasion is reduced in the 8 wk anti-NGF group (logistic regression analysis, P t6 = 0.045). D, The number of tdTomato positive cells (sum of 5 tissue sections per mouse) is reduced in mice that received anti-NGF treatment beginning at 8 wk (two-way ANOVA followed by Sidak's post-hoc test). Cells within a 0.25mm^2 region of interest (indicated in panel B) were counted. *P < 0.05, ***P < 0.001, n = 5–8/group. Scale bar = 100μm. Mn = motor neuron

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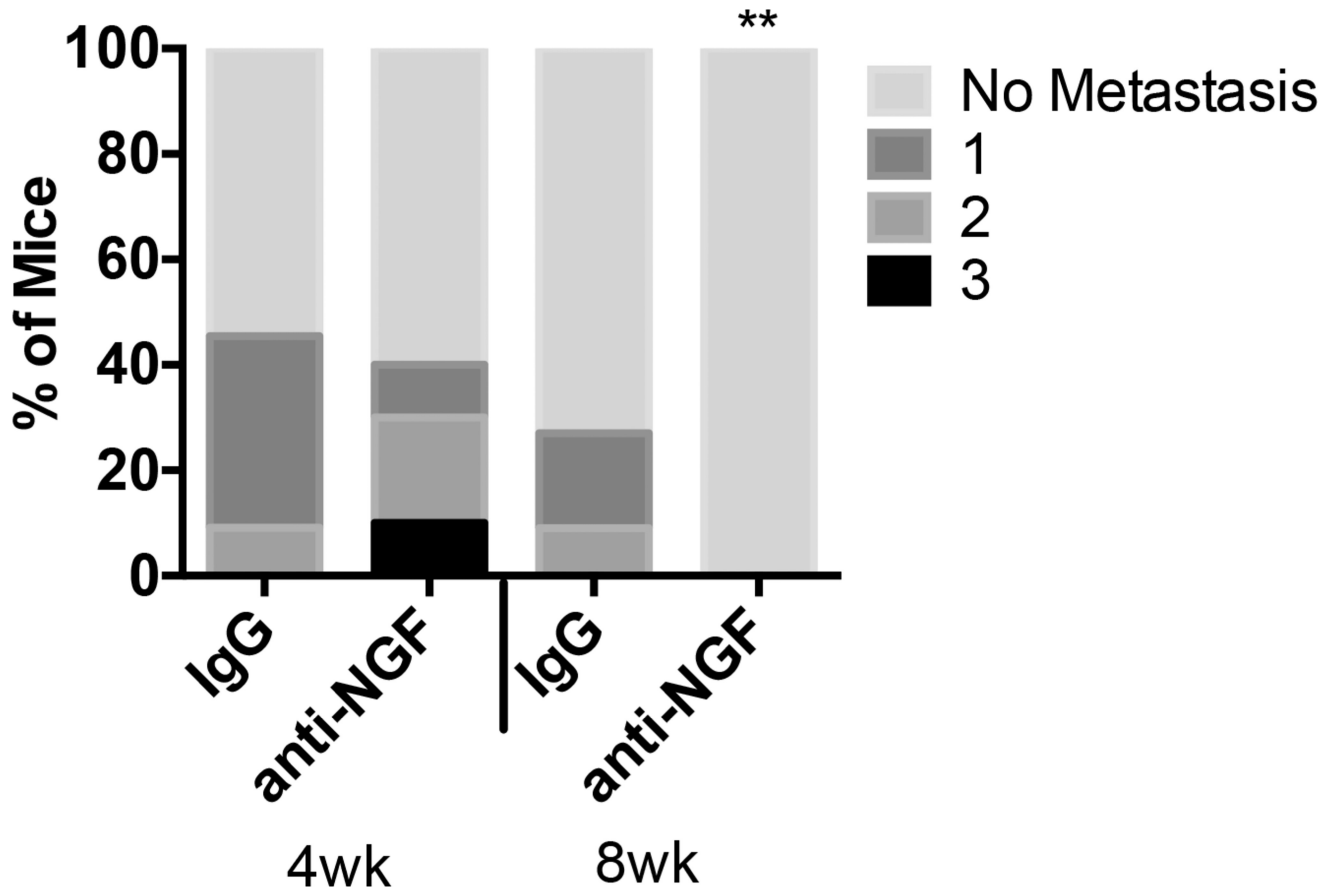

FIGURE 5.

Anti-NGF inhibits metastases. Mice were examined for the presence of gross metastatic tumors at the time of euthanasia and dissection. The number of extrapancreatic sites involved (0–3) was compared by a two-way Poisson regression analysis. Anti-NGF treatment beginning at 8 wk of age was associated with a complete absence of grossly detectable metastatic tumors, ** $P < 0.01$, n = 10–12/group.