Carcinogenesis vol.35 no.1 pp.103–113, 2014
doi:10.1093/carcin/bgt312
Advance Access publication September 25, 2013

The neurotrophic factor neurturin contributes toward an aggressive cancer cell phenotype, neuropathic pain and neuronal plasticity in pancreatic cancer

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Neurotrophic factors possess an emerging role in the pathophysiology of several gastrointestinal disorders, regulating innervation, pain sensation and disease-associated neuroplasticity. Here, we aimed at characterizing the role of the neurotrophic factor neurturin (NRTN) and its receptor glial-cell-line-derived neurotrophic factor receptor alpha-2 (GFRα-2) in pancreatic cancer (PCa) and pancreatic neuropathy. For this purpose, NRTN and GFRα-2 were studied in normal human pancreas and PCa tissues via immunohistochemistry, quantitative reverse transcription–polymerase chain reaction, immunoblotting and correlated to abdominal pain. The impact of NRTN/GFRα-2 on PCa cell (PCC) biology was investigated via exposure to hypoxia, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide viability and matrigel invasion assays in native and specific small interfering RNA-silenced PCCs. To assess the influence of NRTN on pancreatic neuroplasticity and neural invasion (NI), its impact was explored via an *in vitro* 'neuroplasticity assay' and a 3D neural migration assay. NRTN and GFRα-2 demonstrated a site-specific upregulation in PCa, predominantly in nerves, PCCs and extracellular matrix. Patients with severe pain demonstrated higher intraneural GFRα-2 immunoreactivity than patients with no pain. PCa tissue and PCCs contained increased amounts of NRTN, which was suppressed under hypoxia. NRTN promoted PCC invasiveness, and silencing of NRTN limited both PCC proliferation and invasion. Depletion of NRTN from PCa tissue extracts and PCC supernatants decreased axonal sprouting in neuronal cultures but did not influence glial density. Silencing of NRTN in PCCs boosted NI. We conclude that increased NRTN/GFRα-2 in PCa seems to promote an aggressive PCC phenotype and neuroplasticity in PCa. Accelerated NI following NRTN suppression constitutes a novel explanation for the attraction of PCC to nerves in the hypoxic PCa tumor microenvironment.

Introduction

The pancreas is a densely innervated organ, which is surrounded by numerous neural networks and receives input over splanchnic/vagal nerves and from intrapancreatic cholinergic ganglia (1,2). Despite this naturally rich innervation pattern, one can easily recognize that nerves in pancreatic ductal adenocarcinoma (PCa) undergo a prominent hypertrophy and a major increase in their density (3–5). Interestingly, the vast majority of these nerves are simultaneously infiltrated either by inflammatory cells ('pancreatic neuritis') or by PCa cells (PCC), resulting in neural invasion (NI) (3,6–9). The peculiarity about all these cardinal features of 'pancreatic neuropathy' is that their extent is closely correlated to the degree of 'neuropathic pain' sensation and survival of PCa patients (3).

Due to these remarkable neuropathic alterations and the associated agonizing pain syndrome in PCa, researchers recently turned their scope to the potential involvement of neurotrophic factors, especially that of the glial cell line-derived neurotrophic factor (GDNF) family. The GDNF family comprises the neurotrophic factors GDNF, artemin, neurturin (NRTN) and persephin (10). It was previously demonstrated that in addition to nerve growth factor (NGF), both artemin and GDNF are upregulated in PCa (3–5,11,12). A key discovery toward understanding the exact role of these factors in PCa was that they can actually exert a direct impact upon the biology of PCC. Indeed, NGF, artemin and GDNF can actively enhance the invasiveness and in part also the proliferation of PCC (4,13–17). Moreover, what distinguishes artemin and NGF from the rest is that they seem to be key players in the generation of neuroplastic alterations in PCa (4,5).

What has so far been neglected is the potential role of the GDNF family member NRTN in this visceral neuropathy. Especially, a deciding role for NRTN becomes very likely if one considers the physiological significance of NRTN for pancreatic innervation. Mice lacking the NRTN receptor glial-cell-line-derived neurotrophic factor receptor alpha-2 (GFRα-2) cannot develop normal pancreatic parasympathetic innervation and demonstrate no endocrine function at all (18). Therefore, we aimed at unfolding the role of NRTN in PCa and determined the expression of NRTN and GFRα-2 in normal human pancreas (NP) and PCa, and investigated the impact of NRTN upon PCC biology. Additionally, we evaluated the potential influence of NRTN on the generation of pancreatic neuroplasticity and NI in PCa via recently developed 3D *in-vitro* migration and neuroplasticity assays.

Materials and methods

Patients and tissues

PCa tissue samples from the pancreatic head were collected from patients following tumor resection (patient characteristics: Supplementary Table 1, available at *Carcinogenesis* Online). Tissue samples were processed as described previously (3,19). From all patients, informed consent was obtained for tissue collection. The study was approved by the ethics committee of the Technische Universität München, Germany.

Abdominal pain

In all PCa patients, individual pain degree (no pain/group 0, mild pain/group I and severe pain/group II) was prospectively registered and calculated prior to the operation, as described previously (20).

Immunohistochemistry

Consecutive 3 µm sections from paraffin-embedded NP and PCa samples were immunostained for NRTN (1:500) and GFRα-2 (1:300) (Abcam, Cambridge,UK), and for cytokeratin 19 (CK19) (1:400; Santa Cruz Biotechnologies, Heidelberg, Germany) as described previously (21). For double immunofluorescence analysis, Alexa® Fluor 488 and 594 antibodies (Invitrogen, Karlsruhe, Germany) in combination with 4′,6-diamidino-2-phenylindole nuclear stain were utilized, as described previously (22).

Histopathological analysis of tissue immunoreactivity

Histopathological analysis was performed by two independent observers (K.W., I.E.D.) blinded to patient data, followed by resolution of any differences

Abbreviations: CK19, cytokeratin 19; CP, chronic pancreatitis; DRG, dorsal root ganglia; ECM, extracellular matrix; FMI, forward migration index; GDNF, glial cell line-derived neurotrophic factor; GFRα-2, glial-cell-line-derived neurotrophic factor receptor alpha-2; NC, negative control; NGF, nerve growth factor; NI, neural invasion; NP, normal human pancreas; NRTN, neurturin; PCa, pancreatic cancer; PCC, PCa cell; siRNA, small interfering RNA.

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by joint review and consultation with a third observer (G.O.C.), as performed previously (20). In particular, the degree of immunoreactivity on each section and each tissue substructure was scored and added using a numerical scale (0: no staining, 1: weak staining, 2: moderate staining, 3: strong staining) and averaged among all patients to obtain the 'mean tissue immunoreactivity score'.

Immunoblot analysis

Protein extraction and immunoblot analysis of NP, PCa tissues and of PCC culture monolayers were performed by using NRTN and GFRα-2 antibodies (1:500) and mouse glyceraldehyde 3-phosphate dehydrogenase antibody (1:5000; Santa Cruz Biotechnologies) for equal loading followed by densitometric analysis via the ImageJ Software (National Institutes of Health), as described previously (23).

Enzyme-linked immunosorbent assay

In order to measure the amounts of soluble NRTN in PCC whole cell lysates, the Human Neurturin 'Super-X' Pre-Coated ELISA Kit (Antigenix America, Melville, NY) was utilized according to the instructions of the manufacturer. The lysates were prediluted 1:3 to achieve reliably detectable NRTN concentrations, and the correlation coefficient (R) for each assay was equal to 0.998.

Real-time Light Cycler® quantitative reverse transcription–polymerase chain reaction

Extraction of messenger RNA from PCC was prepared by using TissueLyser II and the RNeasy plus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Subsequently, RNA quantity and purity was determined using Nanodrop ND1000 (Peqlab, Erlangen, Germany). First strand complementary DNA synthesis was performed with the Transcriptor-First-Strand cDNA Synthesis kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Expression of *NRTN* (GeneBank, GeneID:4902) isoforms and of the reference housekeeping gene *cyclophilin-B* (*CypB*, GeneBank, GeneID:5478) was measured with the Roche LightCycler-480 Real-Time PCR System and LightCycler-480 SYBR Green I Master kit. In accordance with the Pfaffl method (24), relative *NRTN* expression in three different cell lysate samples was based on the mean crossing point deviation between the three samples normalized to the mean crossing point deviation for the reference gene, after efficiency correction of the PCR reactions. The relative expression of NRTN in samples was then normalized to the immortalized human pancreatic ductal epithelial cell line. All primers were obtained from Sigma–Aldrich (Munich, Germany; Supplementary Table 2, available at *Carcinogenesis* Online).

PCC line cultures

Human PCC lines AsPC-1, BxPC, Capan1, Colo357, MiaPaCa-2, Panc1 and SU86.86 were purchased from ATCC (Rockville, MD), and human Schwann cells from ScienCell (Carlsbad, CA). T3M4 cells were a gift by Dr R.Metzgar (Durham, NC). The cell lines were routinely grown in complete medium, as shown previously (4). The cell lysates and supernatants were obtained at 100% cell confluence, and protein concentration was measured with the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Human pancreatic ductal epithelial cells were a gift from Prof. M.Tsao from Ontario Cancer Institute (Toronto, Ontario, Canada) and cultivated as published before (25,26).

Effect of hypoxia upon NRTN production by PCC

To investigate the effect of hypoxia upon NRTN production by PCC, sister clones of T3M4 cells were incubated under normoxic and hypoxic conditions (89.25% N_2 + 10% CO_2 + 0.75% O_2) after reaching 80% confluence for varying time periods, beginning with 15 min and gradually increasing to 30 min, 1, 2, 4, 6, 12 and 24 h at 37°C supplemented with 10% fetal bovine serm. Cells were then lysed with radioimmunoprecipitation buffer containing a protease inhibitor cocktail (Roche, Penzberg, Germany), and protein content was measured with the bicinchoninic acid assay.

Growth assay with NRTN-supplemented and NRTN-silenced PCC

To assess cell growth, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay was used as published before (27). Briefly, cells were seeded at a density of 5000 cells per well in 96-well plates, grown overnight and exposed to NRTN at concentrations of 10, 50, 100 and 500 ng/ml. The cell viability was measured at 0, 24, 48 and 72 h.

To evaluate cell growth after NRTN silencing, reverse small interfering RNA (siRNA) transfection of SU86.86 and T3M4 PCC was performed, where cell seeding and transfection were carried out simultaneously. Here, 13 ng of NRTN siRNA target sequence (CAA CUC CUA CGU UUA UUC AAG) or 13 ng of the negative control (NC) siRNA (Qiagen) was spotted to each well of a 96-well plate, followed by addition of HiPerFect (Qiagen) and OptiMEM medium (Gibco, Karlsruhe, Germany). Finally, cells were seeded at a density of 5000 cells per well in 96-well plates to yield a final siRNA concentration of 30 nM. The viability was measured at 0, 24, 48 and 72 h after seeding per transfection. All experiments were made in triplicates and repeated three times (4).

Matrigel-based invasion assay with NRTN-supplemented and NRTN-silenced PCC

Invasion assay was performed using BD Biocoat Matrigel 24-well invasion chambers with 8 µm pore size (BD Biosciences, Heidelberg, Germany), as described previously (4). To detect the influence on invasion, NRTN was added to the cells into the upper chamber (10 or 100 ng/ml) and incubated for 24 h. To assess the effect of NRTN blockade upon PCC invasiveness, PCC were siRNA-transfected (30 nM) against NRTN (28) and added to the invasion chambers. The assays were performed in triplicates and repeated five times.

In-vitro *neuroplasticity assay*

The potential of NRTN to induce neuroplastic alterations in PCa was evaluated in an *in-vitro* neuroplasticity assay (29). Here, cultures of isolated rat dorsal root ganglia (DRG) neurons were treated either with pancreatic tissue extracts derived from three PCa patients and three NP, or with supernatants of PCC lines. The neurons were seeded at 10 000 cells per well on poly-D-lysine-coated (40 mg/m^2; Sigma–Aldrich, Taufkirchen, Germany) 13 mm coverslips in 24-well plates (NUNC, Langenselbold, Germany) and supplemented with the amount of tissue extract or supernatant containing 50 µg of protein, thus equaling a final concentration of 100 µg protein/ml medium in each well. To elucidate the impact of NRTN on DRG neurite density, tissue extracts or PCC supernatants at a concentration of 3 µg/ml were treated with a NRTN-specific blocking antibody (mouse IgG_1; R&D Systems, Wiesbaden, Germany). Recombinant human NRTN (R&D Systems) was used as positive control (at 10 ng/ml based on previous dose titration analyses, ref. 29), and untreated DRG neurons cultivated in neurobasal medium as NC and non-immunized mouse IgG_1 isotype antibody (Sigma–Aldrich) as an additional control. NRTN-blocking antibody was added to the growth medium of DRG neurons as an additional control (+anti-NRTN). Each experiment was repeated three times in triplicates.

After 24 h of treatment, cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline, immunostained with the neuronal marker β-III Tubulin (1:200; Chemicon Int.) or the glial marker glial fibrillary acidic protein (1:400; DAKO, Hamburg,Germany). Neurite and glia density were measured as described previously (29).

3D extracellular matrix-based migration assay

For NI analysis, the standardized 3D extracellular matrix (ECM) gel-based *in-vitro* migration assay was utilized (7,30). Briefly, 100 000 native or NRTN siRNA-silenced T3M4 cells were suspended in ECM gel and placed at exact 1 mm distance next to DRG neurons isolated from newborn Wistar rats (29). To enable interaction of T3M4 with DRG, a 1 mm long ECM 'bridge' was placed between the suspensions (Figure 5A) (7). The migratory behavior of T3M4 cells toward DRG was recorded via digital time-lapse microscopy (Observer D1; Carl Zeiss Imaging, Munich, Germany), equipped with a CO_2 incubation chamber, an AxioCam camera and a plan-neoluar ×10/0.3 PH1-M27 objective over a total observation time of 12 h per movement front. Single pictures were taken at 5 min intervals, compiled as a video and subsequently used to quantify the migratory behavior of T3M4 cells. For this purpose, the movement of PCC was tracked with an ImageJ-based 'manual tracking' plug-in, and the collected data were subsequently imported to the 'chemotaxis/migration tool' provided by Ibidi (www.ibidi.com). This tool employs several morphometric parameters including *velocity* at which the PCC migrate toward neuronal structures, the accumulated and Euclidean (direct linear) distance that PCC cover when migrating toward DRG and the forward migration index (FMI) describing the neurite-targeted PCC migration, as demonstrated previously (30). At the start of each experiment, 30 cells at each front were randomly selected for morphometric analysis. Every experiment was repeated three times.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 (La Jolla, CA). The Mann–Whitney *U*-test was applied for two-group analysis. To compare more than two groups, the one-way analysis of variance followed by the Bonferroni's *post hoc* test was used. The growth assays were compared by first calculating the area under the curve of the growth curves and subsequent comparison of multiple groups. For the multivariate analysis on pain and NRTN/GFRα-2, the NRTN/GFRα-2 tissue/neural immunoreactivity, age,

gender and Union Internationale Contre Cancer stage of patients were included as independent variables and the presence of pain (i.e. pain versus no pain) as the dependent variable. The multivariate analysis was conducted as binary logistic regression in the IBM SPSS Statistics 21 software. Results are expressed as mean ± standard error of the mean. Two-sided *P* values were always computed, and an effect was considered statistically significant at a *P* value ≤0.05.

Results

NRTN and GFRα-2 are upregulated in PCa

We first investigated the distribution of NRTN and GFRα-2 in NP and PCa. In NP, NRTN and GFRα-2 were hardly detectable, where NRTN was only faintly present in acini and some nerves, and GFRα-2 in ducts and occasionally in nerves (Figure 1A). In sharp contrast, there was an overall upregulation of NRTN and GFRα-2 in PCa, predominantly in nerves, islets and ECM (Figure 1A, F and G; Supplementary Table 3, available at *Carcinogenesis* Online). PCC demonstrated a prominent immunoreactivity for NRTN and somewhat for GFRα-2 (Figure 1A; Supplementary Table 3, available at *Carcinogenesis* Online). Remarkably, the nerves, which were particularly immunoreactive for NRTN and GFRα-2, were the ones that concurrently revealed NI (Figure 1A). In order to prove the presence of NRTN and GFRα-2 in PCC, double immunofluorescence labeling of PCa tissues with the PCC marker CK19 together with either NRTN or GFRα-2 was performed (Figure 2A). Interestingly, PCa tissues contained several cancer cell foci with colocalization of CK19 with either NRTN or GFRα-2; however, of note, not all, but rather a subset of cancer cells was observed to exhibit such a colocalization (Figure 2A). Importantly, this analysis confirmed the specific labeling of intrapancreatic nerves by NRTN/GFRα-2 (Figure 2A).

Accordingly, quantitative histopathological analysis showed increased immunoreactivity scores of nerves and ECM for NRTN (Figure 1D and F) and GFRα-2 (Figure 1E and G) in PCa. There was no difference for the immunoreactivity of vessels, ducts and acini between PCa and NP (Supplementary Table 3, available at *Carcinogenesis* Online).

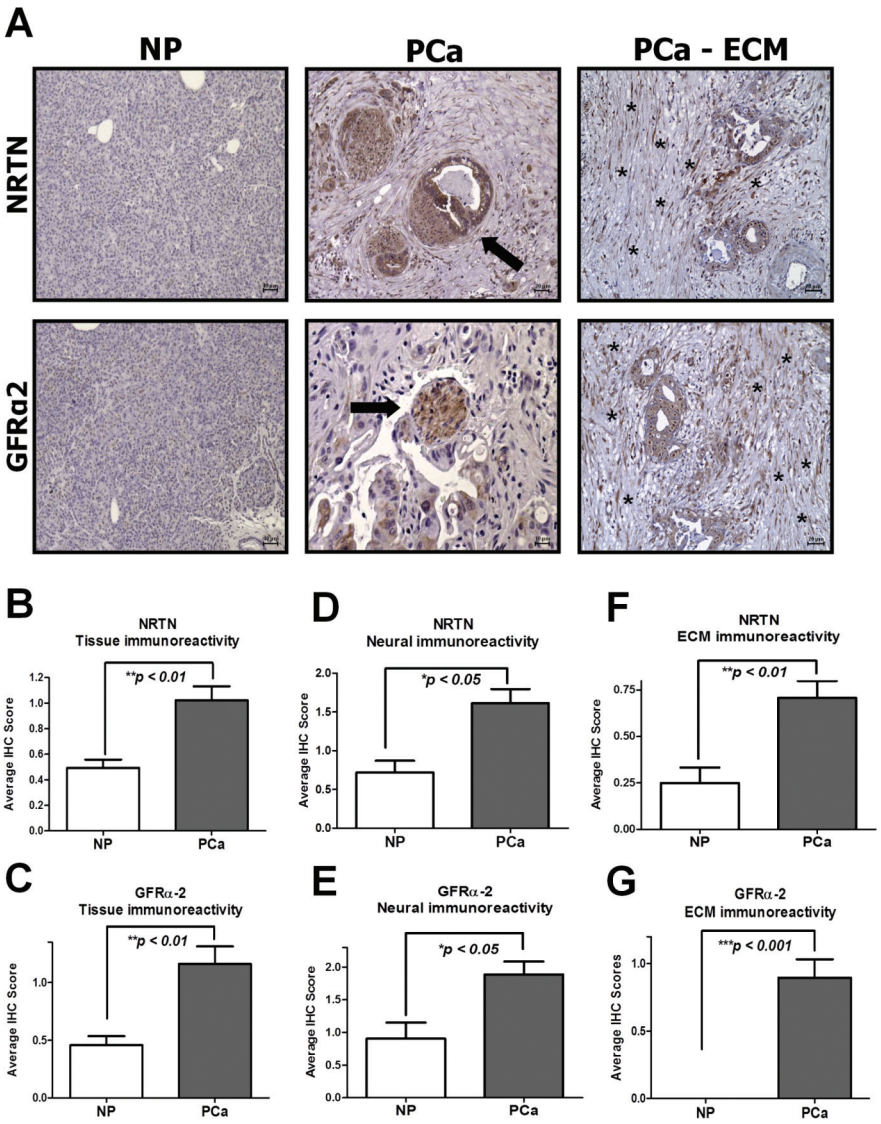

Fig. 1. Localization and site-specific upregulation of NRTN/GFRα-2 in PCa. (**A**) Representative photomicrographs of NRTN and GFRα-2 in NP ($n = 10$), PCa tissue ($n = 30$, Supplementary Table 1, available at *Carcinogenesis* Online) and PCa-associated ECM. Arrows indicate intrapancreatic nerves, which are invaded by PCC. Asterisks indicate the observed spindle-shaped ECM components immunoreactive for NRTN and GFRα-2. (**B** and **C**) The quantitative immunohistochemical scoring analysis reveals upregulation of average NRTN (NP: 0.5 ± 0.1 versus PCa: 1.0 ± 0.1) and GFRα-2 (NP: 0.5 ± 0.1 versus PCa: 1.2 ± 0.1) in PCa, particularly in intrapancreatic nerves (NP: 0.7 ± 0.2 for NRTN and 0.9 ± 0.3 for GFRα-2 versus PCa: 1.6 ± 0.2 for NRTN and 1.9 ± 0.2 for GFRα-2; **D** and **E**) and ECM (B and C; 0.2 ± 0.1 for NRTN and 0.0 ± 0.0 for GFRα-2 versus PCa: 0.7 ± 0.01 for NRTN and 0.9 ± 0.1 for GFRα-2; **F** and **G**).

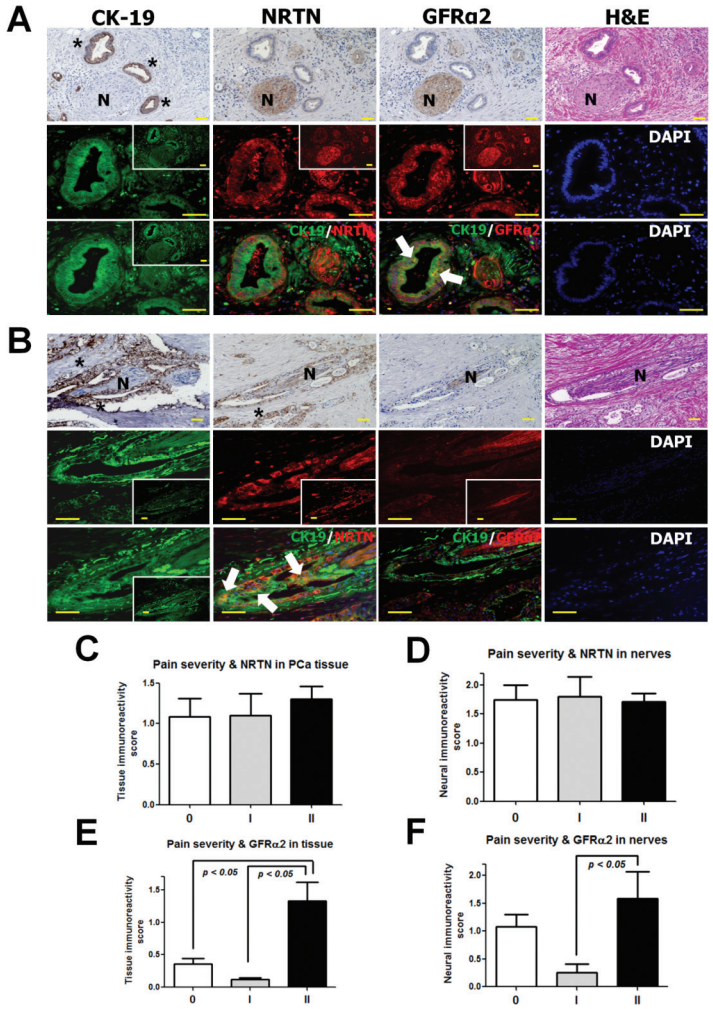

Fig. 2. Subset-specific presence of NRTN/GFRα-2 in PCC and the impact of pain upon NRTN and GFRα-2 in PCa. (**A** and **B**) Consecutive PCa tissue sections were immunostained against NRTN, GFRα-2, cytokeratin 19 or stained with hematoxylin and eosin. Double immunofluorescence labeling of PCa tissues against the PCC marker CK19 together with either NRTN or GFRα-2 revealed colocalization of CK19 with either GFRα-2 (**A**) or NRTN (**B**). Interestingly, not all, but rather a subgroup of cancer cells colocalized with either of these molecules. Nerves exhibited specific labeling by NRTN/GFR α-2. Asterisks indicate PCC colonies. 'N': nerve. White arrows indicate foci of colocalization. Insets represent the immunofluorescently labeled consecutive sections of the immunostaining images above them. Scale bars indicate 50 µm. (**C–F**) Patients in the severe pain group/II demonstrated prominently higher average tissue and neural immunoreactivity scores for GFRα-2 (1.3 ± 0.3 in tissue and 1.6 ± 0.5 in nerves), but not for NRTN, when compared with patients with no pain (group 0; 0.4 ± 0.1 in tissue and 1.1 ± 0.2 in nerves) or mild pain (group I; 0.1 ± 0.03 in tissue and 0.2 ± 0.2 in nerves).

GFRα-2 is associated with severe abdominal pain sensation in PCa patients

As neurotrophic factors have previously been reported to be associated with increased pain sensation (31), we investigated whether there is any correlation between tissue and neural immunoreactivity for NRTN/GFRα-2 and pain in PCa patients. For this purpose, we classified patients into three different pain classes based on their pain severity (19). Neither tissue nor neural immunoreactivity of NRTN had a noticeable correlation to pain sensation in PCa patients (Figure 2A and B). However, enhanced tissue ($P < 0.01$) and neural immunoreactivity ($P < 0.05$) for GFRα-2 directly correlated with a more severe pain phenotype in PCa patients (Figure 2C and D). In a multivariate analysis (binary logistic regression) including NRTN/GFRα-2 tissue/neural immunoreactivity, age, gender and Union Internationale Contre Cancer stage of patients as independent variables and the presence of pain (i.e. pain versus no pain) as the dependent variable, neither of the studied factors showed any significant independent association with the pain in PCa (Supplementary Table 4, available at *Carcinogenesis* Online).

PCa features the biologically active NRTN isoforms

The GDNF family are synthesized as pre-pro-molecules and undergo a cleavage of their 'pre-signal' peptide upon secretion (10,32). This secreted pro-form is then subject to another cleavage before yielding a monomer, which has to bind to another monomer to become a homodimer. This homodimer is the biologically active form of GDNF family of neurotrophic factors (10,32).

In PCa, NRTN was primarily detected on two bands, i.e. one which was between 25–28 kDa, and one around 50–56 kDa (Figure 3A), which shows great similarity to their previously shown isoforms in chronic pancreatitis (CP) (33). The remaining detected bands were around 40–45 kDa (Figure 3A). Considering the posttranslational processing and activation events in the GDNF family, the below 25 kDa bands in NP correspond to the pro-form of NRTN, whereas the higher band around 45 kDa representing the dimeric pro-form (32–36). In contrast, the 25–28 kDa band corresponds to the biologically active, homodimeric NRTN and 50–56 kDa bands represent the tetrameric NRTN (34,36). In other words, in PCa, there is a shift in the relative quantity of the NRTN isoforms between NP and

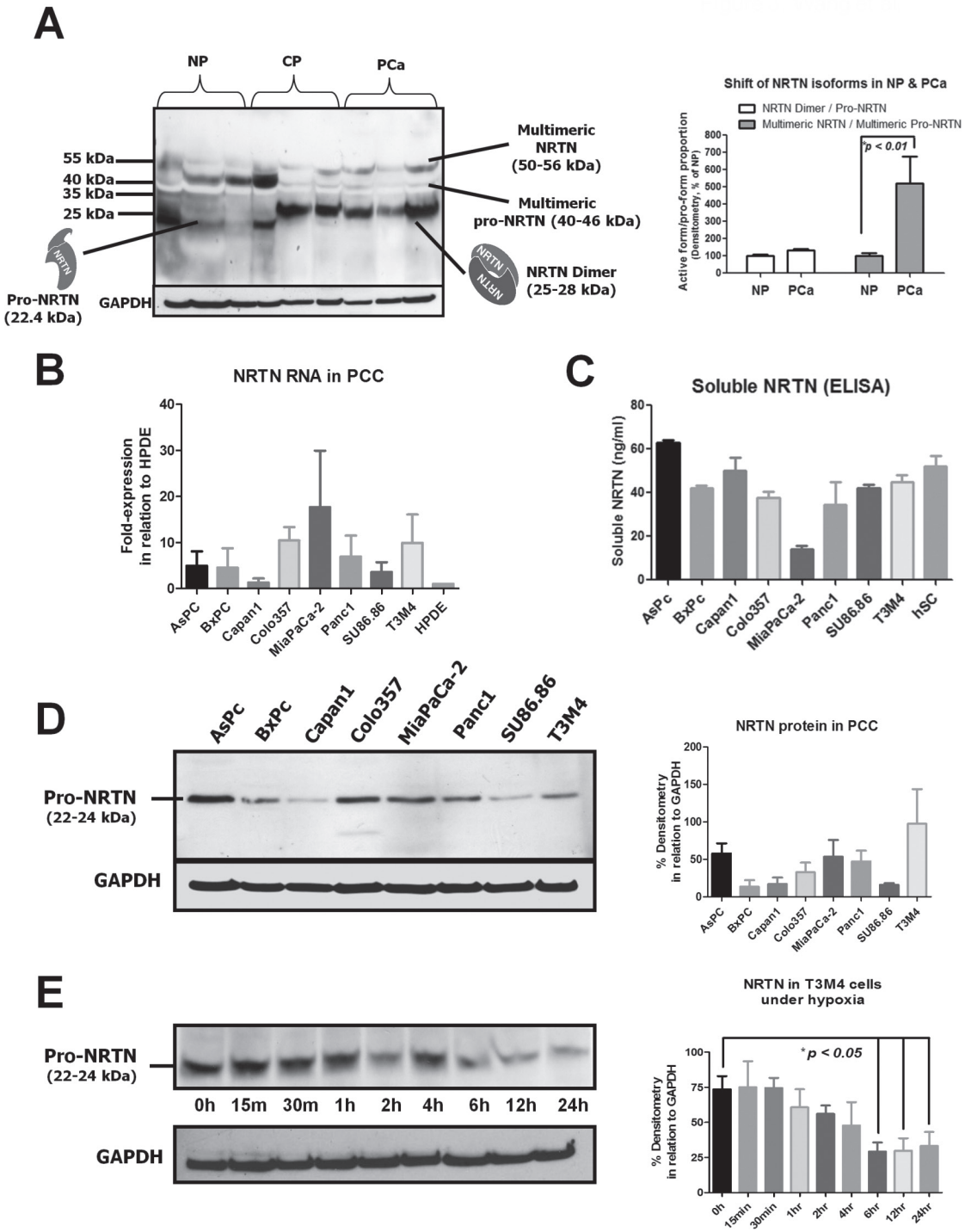

Fig. 3. PCa tissue and PCC contain biologically active NRTN. (**A**) In PCa, there was a shift toward increased presence of NRTN multimers (50–56 kDa, here tetramers) and also of the NRTN homodimer (28 kDa-sized mature NRTN homodimer) when compared with NP, as determined via relative densitometry of NRTN dimers to NRTN pro-form (22.4 kDa) and of NRTN multimers to pro-form multimers (40–46 kDa). This distribution of isoforms in PCa tissue demonstrates a major similarity to that in human CP (33). Note that the band pattern in two of the three CP patients on this blot resembles PCa patients, and that of the first one is similar to NP. Therefore, it seems that CP represents an intermediate entity between NP and PCa in terms of the presence of biologically active tissue NRTN isoforms. A total of 10 NP cases, 9 CP cases and 13 PCa cases were analyzed in the immunoblots. (**B**) PCC possess varying amounts of NRTN RNA. (**C**) PCC, but also human Schwann cells (hSC) as peripheral glia contain considerable amounts of soluble NRTN, as detected via enzyme-linked immunosorbent assay. (**D**) PCC contain pro-NRTN. (**E**) Increasing durations of hypoxia lead to a suppression of pro-NRTN in PCC.

PCa, characterized by increased relative presence of the biologically active dimeric and multimeric NRTN in PCa as compared with NP (proportion of multimeric NRTN to multimeric pro-NRTN in PCa equaling 521.7 ± 155.5% of the proportion in NP; Figure 3A).

PCC are a major source of NRTN, which is suppressed under hypoxia

In order to further elucidate the increased presence of NRTN within PCC, we analyzed NRTN in eight different PCC via quantitative

reverse transcription–polymerase chain reaction and immunoblotting analyses. At RNA level, all PCC presented NRTN, with the highest amounts in MiaPaCa-2 and T3M4 cells (Figure 3B). AsPC and T3M4 PCC were the PCC lines, which contained the largest amounts of soluble NRTN, as determined via enzyme-linked immunosorbent assay in PCC whole cell lysates (Figure 3C). Similar to RNA levels, all PCC possessed considerable amounts of intracellular pro-NRTN at protein level, where MiaPaCa-2, T3M4, Colo357 and AsPC had the highest content (Figure 3D). In addition, all PCC lines showed expression of the GFRα-2 receptor, with the highest amounts detected in T3M4, BxPc and MiaPaCa-2 cells (Supplementary Figure 2, available at *Carcinogenesis* Online).

Since PCa is a hypoxic tumor (21,37), we studied the influence of hypoxia upon NRTN production by PCC. For this purpose, we exposed T3M4 cells to hypoxia for varying periods, and plotted the time course of intracellular pro-NRTN amounts. As shown in Figure 3E, increasing durations of hypoxia noticeably suppressed the amount of pro-NRTN in T3M4 cells, where the highest reduction was observed around 6–12 h of hypoxia.

Intrinsic NRTN ensures sustained proliferation of PCC

As the first step to assess the role of NRTN in PCa biology, we cultivated all PCC with increasing amounts of NRTN and assessed their viability via 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay. None of the eight PCC demonstrated any changes in their proliferation rate under increasing concentrations of NRTN (Supplementary Figure 1, available at *Carcinogenesis* Online). However, the suppression of intrinsic NRTN in PCC via specific siRNA silencing, as verified via quantitative reverse transcription–polymerase chain reaction and immunoblotting analysis (Figure 4A), led to decreased proliferation in SU86.86 (72.9 ± 2.6% of controls at 10 nM siRNA and 66.4 ± 5.3% at 30 nM siRNA) and T3M4 (54.7 ± 14.3% at 10 nM, 33.0 ± 7.2% at 30 nM) over 72 h when compared with control (Figure 4B).

NRTN promotes PCa invasiveness

The high invasion potential of PCa is a cardinal feature of its aggressiveness. For this reason, we studied the invasiveness of the PCC SU86.86 and T3M4 under the influence of external NRTN and also following silencing of NRTN via specific siRNA. When PCC were treated with increasing doses of NRTN (i.e. 10 ng/ml; 100 ng/ml), the invasiveness of both tested PCC was noticeably enhanced (Figure 4C). T3M4 showed a response already at 10 ng/ml of NRTN (472.2 ± 230.9 versus 278.7 ± 87.3% at 100 ng/ml), whereas in SU86.86, the response was most pronounced at 100 ng/ml (373.4 ± 84.1 versus 225.8 ± 54.2% at 10 ng/ml; Figure 4C).

In harmony with the effect of external NRTN, the specific siRNA silencing of intrinsic NRTN led to a significant decrease in the number of invading SU86.86 (39.9 ± 15.1%) and T3M4 cells (55.42 ± 14.07%) compared with control-transfected cells; Figure 4D.

NRTN induces increased neural density in PCa

Thereafter, we explored the potential of NRTN to induce the typical neuroplastic alterations in PCa. For this, we depleted NRTN in PCa tissue extracts and PCC supernatants via a specific NRTN-blocking antibody (anti-NRTN) and measured the neurite density of DRG neurons. When treated with recombinant human NRTN (2.0 ± 0.1) or PCa (1.9 ± 0.2), DRG neurons showed highest neurite density, surpassing that for NP (Figure 5A–F). Strikingly, neurite density was severely diminished when they were cultured in PCa extracts supplied with anti-NRTN (1.2 ± 0.1, Figure 5D), but not with non-immunized mouse IgG_1 isotype antibody (2.2 ± 0.3). In contrast, when anti-NRTN was added into NP tissue extracts, there was no major change in the neurite density of DRG neurons (1.1 ± 0.2 versus 0.9 ± 0.1, Figure 5B and F). Similarly, neurite density of untreated DRG (NC: 1.2 ± 0.1) did not reach the level of PCa extract treatment (Figure 5F). Treatment of DRG neurons only with anti-NRTN (+anti-NRTN), in the absence of pancreatic tissue extracts, did not influence DRG neurite density (0.9 ± 0.2; Figure 5F).

A similar effect was observed when DRG neurons were cultured with PCC supernatants. Although PCC supernatants are not as neurotrophic as PCa tissue extracts (29), increased neurite density which was induced by supernatants of AsPC (1.1 ± 0.1), Capan1 (1.0. ± 0.1), Colo357 (1.2 ± 0.1), MiaPaCa-2 (1.0 ± 0.1), Panc1 (1.2 ± 0.1) and T3M4 (1.1 ± 0.1) was significantly reduced when DRG neurons were treated with anti-NRTN (Figure 5G; AsPC: 0.8 ± 0.1, Capan1: 0.7 ± 0.1, Colo357: 0.7 ± 0.1, MiaPaCa-2: 0.8 ± 0.1, Panc1: 0.8 ± 0.1, T3M4: 0.8 ± 0.1). BxPC and SU86.86 cells showed a similar effect, but statistically not significant (data not shown).

NRTN does not influence glial density in PCa

In order to elucidate the role of NRTN on pancreatic neuropathic pain, we studied its impact on satellite glia of DRG neurons. For this purpose, glia was immunolabeled with glial fibrillary acidic protein and density was quantified in DRG cultures treated with NP or PCa tissue extracts under the influence of anti-NRTN. Here the blockade of NRTN from NP (NP+anti-NRTN) or from PCa tissue extracts (PCa+anti-NRTN) did not influence the density of DRG glia (data not shown).

NRTN deficiency drives PCC to NI

Finally, we tried to shed light on the potential contribution of NRTN to the cardinal feature of pancreatic neuropathy in PCa, i.e. NI of cancer cells. Here, we made use of our recently demonstrated 3D ECM-based neural migration assay (7) and confronted isolated DRG with control-transfected and NRTN-silenced T3M4 cells simultaneously, and recorded their neuron-targeted migration via digital time-lapse microscopy (Figure 6A). Although both control and silenced PCC migrated a comparable total ('accumulated') distance (171.3 ± 6.1 μm in silenced versus 148.6 ± 4.4 μm control cells) at a similar velocity toward DRG (0.21 ± 0.01 μm/min in silenced versus 0.24 ± 0.01 μm/min control cells), the migration of the NRTN-silenced PCC was characterized by a longer direct linear ('Euclidean') distance (31.6 ± 2.5 μm) and a greater FMI (0.1 ± 0.01) than in control cells (Euclidean distance: 21.5 ± 1.4 μm and FMI: 0.06 ± 0.01), implying that the NRTN-silenced cells migrated in a more targeted fashion toward neurons than non-silenced ones (Figure 6B–E).

Discussion

The present study was designed to elucidate the role of the NRTN/GFRα-2 axis in the pathophysiology of PCa and especially in pancreatic neuropathy. It demonstrates the site-specific upregulation of NRTN and GFRα-2 in PCa and patients with severe pain, where PCC emerge as a major source of NRTN. Importantly, the neurotrophic factor NRTN contributes toward sustained proliferation and increased invasiveness in PCa. Furthermore, it is evident that NRTN influences the generation of neuroplastic alterations in PCa and that NRTN deficiency in PCC, e.g. induced by hypoxia, can lead to an enhanced targeted invasion of nerves.

The site-specific upregulation of NRTN in PCC and nerves, and the increased presence of GFRα-2 in intrapancreatic nerves suggest a reactive upregulation of this axis in PCa. A similar upregulation of artemin and its receptor GFRα-3, but also of NGF and its receptor TrkA was previously demonstrated in PCa (4,5). In contrast, a previous study did not show any immunoreactivity of NRTN in intrapancreatic nerves in PCa, possibly due to the specific and limited focus of that study on invaded nerves (38). Looking at this altered expression pattern in PCa, it is possible that nerves upregulate NRTN and GFRα-2 to compensate in an autocrine loop the neural damage, which is a frequent phenomenon in PCa (6). Such a reactive upregulation of NRTN and the autocrine reparative mechanism were previously demonstrated in brain insult models (39). In this context, it is conceivable that NRTN may be originating from DRG neurons and be retrogradely transported in sensory axons to restore intrapancreatic neural integrity, as previously demonstrated for NRTN, GDNF and persephin (4,40).

Another dimension of this finding lies in its contribution to neuropathic pain. As recently demonstrated, PCa is often characterized by severe abdominal pain sensation, which seems to be associated with

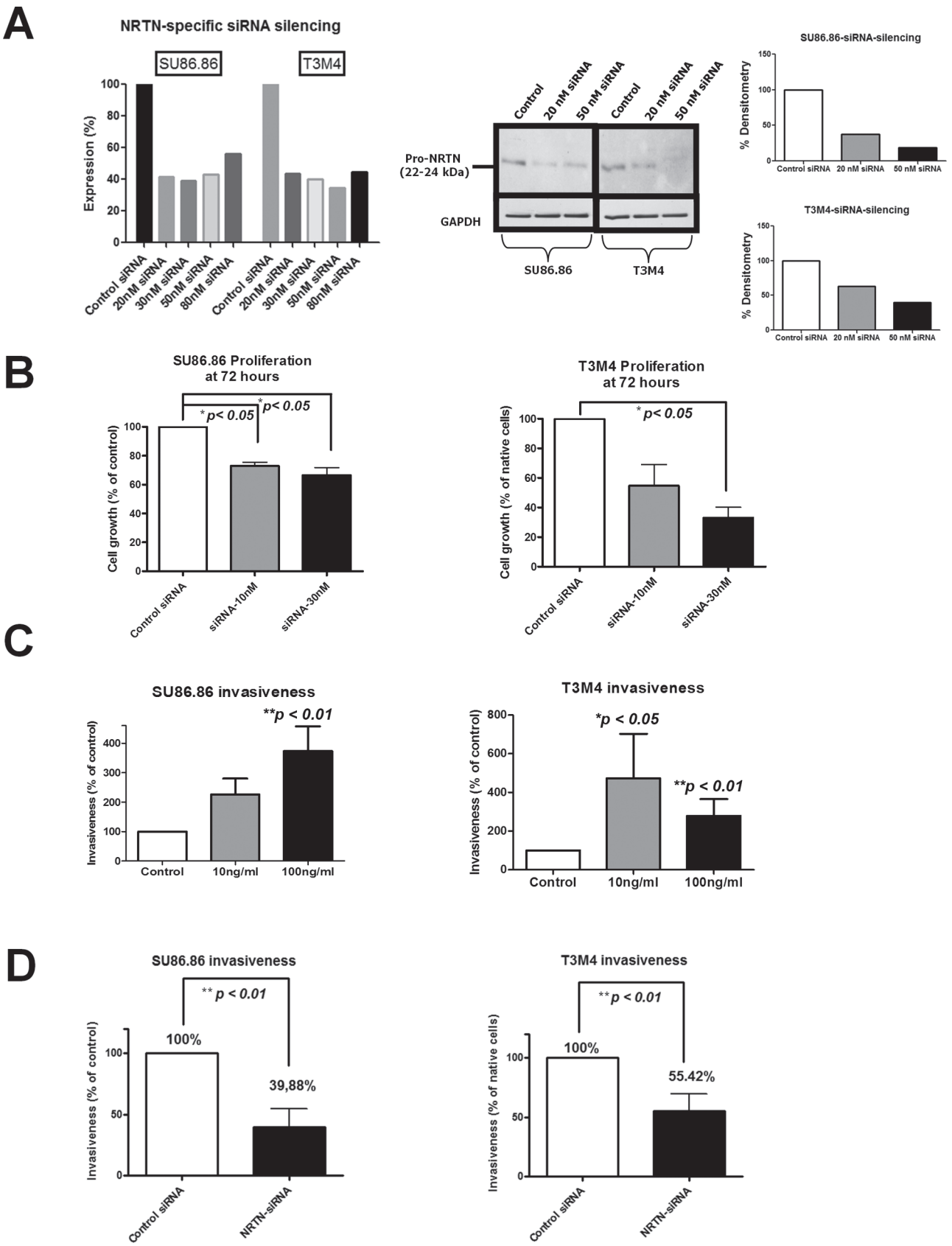

Fig. 4. NRTN and its impact on PCa biology. (**A**) NRTN-specific siRNA silencing in SU86.86 and T3M4 cells was confirmed by quantitative reverse transcription–polymerase chain reaction and immunoblotting in conjunction with densitometry. (**B**) Specific siRNA silencing of NRTN limited their proliferation over 72 h. (**C**) Strikingly, NRTN increased the invasiveness of the SU86.86 and T3M4. (**D**) Correspondingly, siRNA silencing of NRTN significantly restricted their invasive capability, as opposed to the non-silencing control siRNA group.

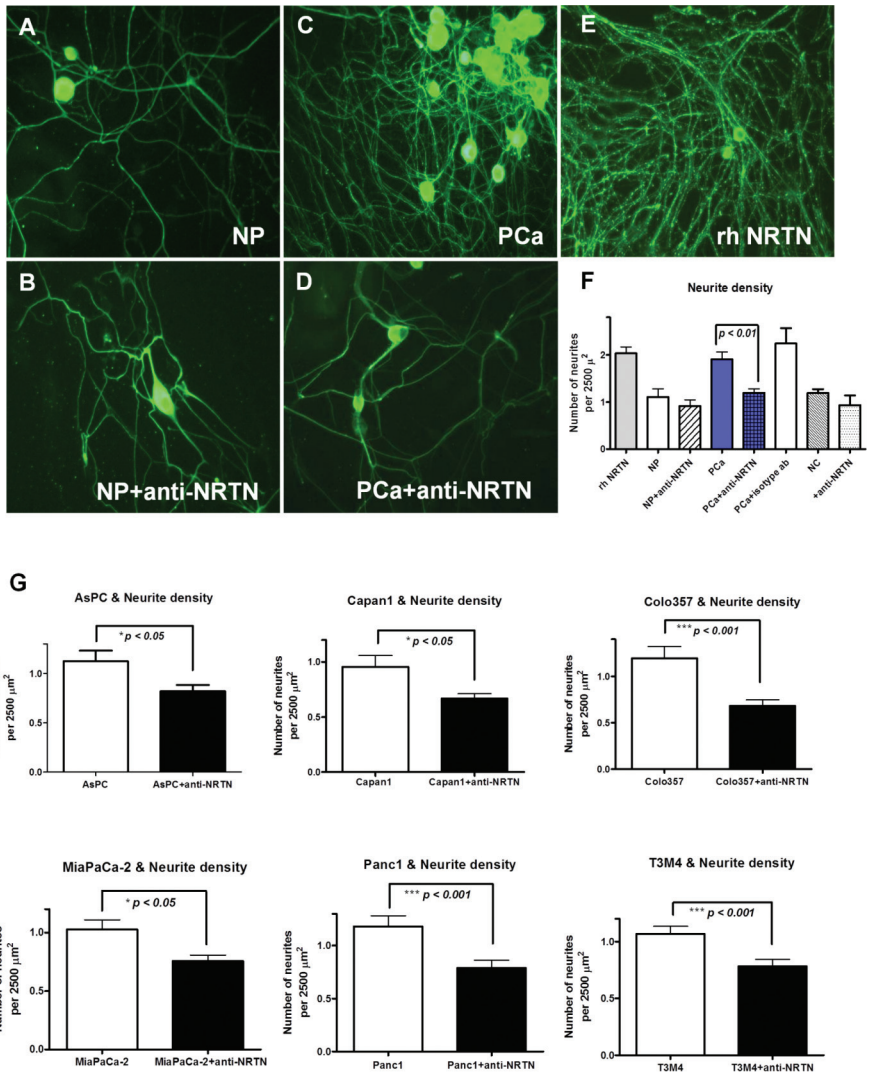

Fig. 5. NRTN mediates neuroplasticity in PCa. DRG cultured in PCa tissue extracts (**C**) or with recombinant human NRTN (rhNRTN; **E**) revealed a much higher neurite density than those cultivated in NP tissue extracts (**A**). Importantly, the depletion of NRTN from the PCa tissue extracts, but from the NP extracts (**B**), via a specific NRTN-blocking antibody (PCa+anti-NRTN, **D**) significantly diminished neurite density of DRG cultures (**F**). This effect was not present when PCa tissue extracts were treated with a non-immunized mouse IgG_1 isotype antibody (PCa+isotype antibody, F) or with the regular growth medium of the DRG neurons (NC). A similar effect was observed when the high DRG neurite density as induced by the AsPC, Capan1, Colo357, MiaPaCa-2, Panc1 and T3M4 was reduced when the supernatants were additionally supplied with anti-NRTN (**G**).

neuropathic alterations (5,19). In recent studies, NGF and its receptors were suggested as potential mediators of pancreatic neuropathic pain (12,41), whereas such an effect could not be demonstrated for the neurotrophic factor artemin (4). Based on our findings, one cannot exclude a role for the NRTN-GFRα-2 axis in neuropathic pain generation in PCa. It is known that neurotrophic factors act on their corresponding receptors on peripheral nociceptive nerve endings (42). Here, it seems that not the level of NRTN *per se*, but rather its receptor GFRα-2 mediates the proalgesic effect of the NRTN/GFRα-2 axis via the corresponding nociceptors. It is conceivable that increased amounts of GFRα-2 are responsible for enhanced neurotransmission and thus pain sensation in the NRTN-rich microenvironment in PCa tissues. The results of our multivariate analysis did not support an independent role for the presence of neuropathic pain in PCa; however, due to sample size restrictions, this analysis could not consider the independent impact of the NRTN/GFRα-2 axis on pain severity. Therefore, NRTN and its receptor GFRα-2 should be subject to more intense investigation in larger scale studies that aim at characterizing the association between pain severity and NRTN/GFRα-2 in a mutiparameter approach and also in multifunctional PCa models, which are characterized by neuropathic abdominal pain.

The current study departed from the fact that neurotrophic factors have a major impact in PCa biology. A few recent studies could demonstrate the contribution of NGF and artemin to the aggressiveness of PCa (4,14,43). What originally motivated researchers to investigate neurotrophic factors in a non-neural malignancy is the extremely high frequency of NI in PCa (3). However, it turned out that, independently from nerves, PCC produce neurotrophic factors for their own benefit, i.e. for enhanced proliferation and invasiveness (4,14). Based on our findings, NRTN plays at least an important role for PCC proliferation and invasion as the previously reported factors. To our knowledge, this study is the first one to demonstrate the production of NRTN in a non-neural crest-derived human malignancy (44,45). The signals and molecular mechanisms, which can in general trigger such a switch from pro-NRTN to active NRTN, are unknown and should therefore be an interesting subject for future neuroscientific studies.

The production of active NRTN in PCa bears a crucial implication for the generation of neuroplastic alterations in PCa. Based on our findings, nerves have two concomitant 'drives' to undergo plastic alterations. Following neural damage in PCa, nerves upregulate the NRTN/GFRα-2 axis for their own repair and regeneration, whereas PCC represent another rich source of NRTN. Hence, it may be of no surprise to

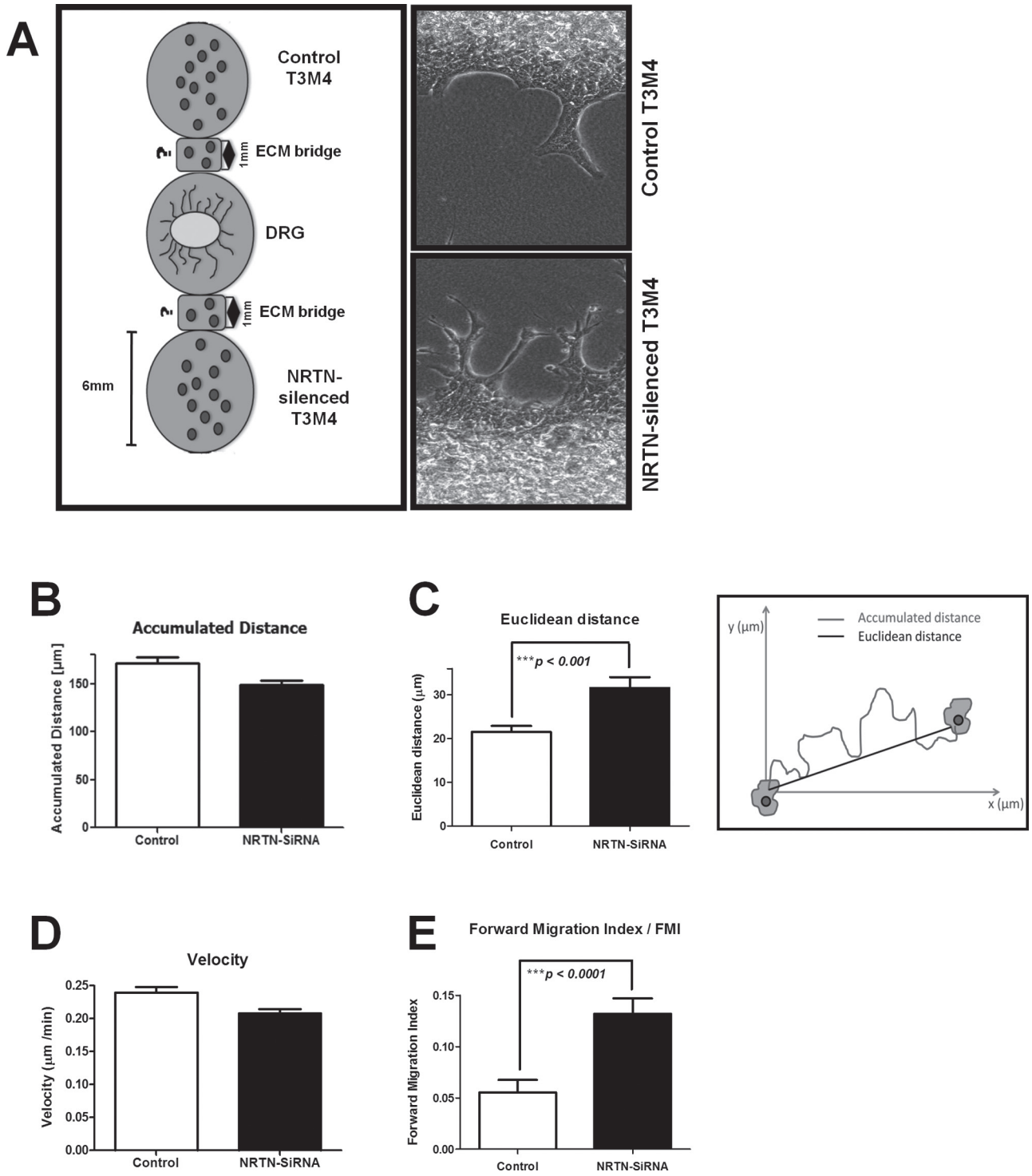

Fig. 6. NRTN deficiency triggers NI. (**A**) DRG were simultaneously cocultivated with control-transfected T3M4 and NRTN-silenced T3M4 cells. Migratory behavior of T3M4 facing the DRG was recorded via digital time-lapse microscopy. As an indicator of increased chemoattraction and migration, NRTN-silenced T3M4 cells (right lower panel) demonstrated increased spike-like pseudopods toward DRG than control T3M4 cells (right upper panel). (**B**–**E**) Interestingly, although both types of cells migrated a comparable total ('accumulated') distance at a similar velocity, the direct linear ('Euclidean') distance toward DRG covered by silenced T3M4 cells was longer, and the directionality of their migration was more pronounced (higher FMI) when compared with control T3M4 cells. Note that the accumulated distance stands for the total path of migration of a cell from the starting toward the end point of its migration (gray line), and that the Euclidean distance indicates the linear, direct distance between this starting and end point (black line).

observe numerous neuroplastic alterations in the presence of so many trophic signals in PCa microenvironment. Evidence for this explanation is derived from the *in-vitro* neuroplasticity assay: The blockade of NRTN from both PCa tissue extracts and PCC supernatants results in a prominent reduction in the initial high neurite density of DRG neurons. Here, it should be considered that DRG neurons lie in the nociceptive pathway of pain transmission from the pancreas toward the central nervous system and thus represent key neurons in pain sensation. The ability of NRTN to ensure (e.g. dopaminergic) neuronal survival is a fascinating niche of research, since it is currently employed to treat

patients with advanced Parkinson's disease via viral gene delivery in phase I trials (46). Therefore, in addition to its crucial role for PCC biology, NRTN may turn out to be an important factor for the regenerative capacity of intrapancreatic or extrinsic neurons in PCa.

In terms of understanding nerve–cancer interactions, our findings on the reinforcement of NI following suppression of intrinsic NRTN should deserve attention. Although NRTN can obviously support PCC invasiveness, it has a divergent role in the migration process toward nerves. In this context, it should first be considered that invasion, as studied in a matrigel-based assay, reflects the breaching of the basement membrane by cancer cells, and not necessarily the migration process that follows (47). Second, the 3D neural migration model is a heterotypic culture system, which includes more variables than the invasion assay. Therefore, it seems that silencing of NRTN in PCC in the migration assay activates neuron-targeting mechanisms. It is well conceivable that in the absence of NRTN which PCC need for their proliferation and invasion ability, they turn even more to nerves, which are *per se* a richer source of neurotrophic factors. Furthermore, there may also be a link between hypoxia-induced NRTN suppression and NI, since hypoxia was shown previously to increase PCC motility (48). Overall, NI may therefore be a compensatory mechanism for PCC to turn to more abundant sources in their own hypoxia-induced intrinsic deficiency of neurotrophic factors.

In summary, the present study demonstrates the reactive alterations of the NRTN/GFRα-2 axis in a non-neural crest-derived, gastrointestinal malignancy. The presence of active NRTN in PCa points to the reinforcement of biological properties of PCC by neurotrophic factors. By secreting NRTN, PCC themselves may trigger neuroplastic alterations in PCa. Finally, suppression of intrinsic neurotrophic factors like NRTN in PCa microenvironment and the associated increased neuron-targeted motility may be considered as novel mechanisms in our understanding of nerve–cancer interactions.

Supplementary material

Supplementary Tables 1–4 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Acknowledgements

The authors would like to thank Mrs Ulrike Bourquain for her tireless technical assistance. This work is part of K.W.'s MD thesis. G.O.C. is the guarantor of the study. I.E.D., G.O.C., B.X. and H.F. designed the study. K.W., I.E.D., J.G.D., E.T., K.K., S.S. and T.K. performed the experiments. K.W. and I.E.D. analyzed the data. I.E.D., K.W., T.K. and G.O.C. wrote manuscript. B.X., H.F., T.K. and G.O.C. supervised the study. All authors have approved the final version of the manuscript.

Conflict of Interest Statement: The authors have no conflicts of interest.

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Received March 25, 2013; revised August 5, 2013; accepted August 25, 2013