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Phenotype screens of murine pancreatic cancer identify a Tgfa-Ccl2-paxillin axis driving human-like neural invasion

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Abstract

Solid cancers like pancreatic cancer (PDAC) frequently exploit nerves for rapid dissemination. This neural invasion (NI) is an independent prognostic factor in PDAC, but insufficiently modelled in genetically-engineered mouse models (GEMM) of PDAC. Here, we systematically screened for human-like NI in Europe's largest repository of GEMM of PDAC comprising 295 different genotypes. This phenotype screen uncovered two GEMM of PDAC with human-like NI, which are both characterized by pancreas-specific overexpression of transforming-growth-factor-alpha (TGFα) and conditional depletion of p53. Mechanistically, cancer-cell-derived TGFα upregulated CCL2 secretion from sensory neurons, which induced hyperphosphorylation of the cytoskeletal protein paxillin via CCR4 on cancer cells. This activated the cancer migration machinery and filopodia formation toward neurons. Disrupting CCR4 or paxillin activity limited NI, and dampened tumor size and tumor innervation. In human PDAC, phospho-paxillin and TGFα-expression constituted strong prognostic factors. Therefore, TGFα-CCL2-CCR4-p-paxillin axis is a clinically actionable target for constraining NI and tumor progression in PDAC.

Keywords: Phenotype screen, neural invasion, pancreatic cancer, TGFα, CCL2, CCR4, phospho-paxillin, cytoskeleton.
Introduction

Pancreatic ductal adenocarcinoma (PDAC) is currently the third leading cause of cancer-associated death worldwide, and is projected to become the second by 2030 (1). Remarkable progress has been made in the last five years in disentangling the complex genetic and molecular drivers, and subtypes of PDAC. Oncogenic Kras-driven, genetically engineered mouse models (GEMMs) of PDAC have uncovered several aspects of the co-evolution of cancer lesions and tumor microenvironment during carcinogenesis (2). As faithful models, they are powerful in modelling the molecular events that lead to metastasis, intra-tumoral heterogeneity, and several defining features of the tumor microenvironment such as local immunosuppression (3).

Human PDAC also exhibits yet an unparalleled high frequency of neural invasion (NI) and neuroplastic alterations in the pancreas and in the central nervous system (4-6). NI was shown to be present in up to 100% of classical ductal adenocarcinomas of the pancreas (7). NI in human PDAC typically manifests as “perineural” invasion, which implies the circular alignment of cancer cells along the epineural sheaths (8, 9) (Figure 1A). Importantly, in PDAC, the severity of NI, i.e., the penetration depth of cancer cells into the intrapancreatic nerves, is an independent prognostic factor for overall and disease-free survival, as well as local recurrence (10). Indeed, nerve-invading cancer cells use these as highways for rapid spread, which results in massive local tumor invasion, surgical irresectability, and severe pain (4, 11-13). In human PDAC, NI, starts, however, at the earliest stages of cancer, e.g. in T1 tumors (14). In fact, Schwann cells of peripheral nerves were shown to emerge already around the precursor lesions of PDAC, i.e. PanINs (pancreatic intraepithelial neoplasia), which suggests that it is nerves, and not cancer cells, that first migrate to initiate NI (15). Neural and extrapancreatic tumor invasion toward the spinal cord have been previously reported to be present in the oncogenic Kras-driven $p48$-Cre;LSL-Kras$^{G12D};p53^{lox/}$ (KPC) GEMM of PDAC (16, 17). However, a direct comparison of NI in mouse PDAC to the morphology and molecular drivers of NI in human PDAC, has not yet been performed.

Considering this gap and the cardinal importance of NI for the course of human PDAC, we performed a systematic investigation of NI and neuro-morphology in a large cohort of PDAC GEMMs comprising nearly 300 different allele combinations of oncogenes and tumor suppressors. Here, we uncover PDAC genotypes with “human-like” NI and point out the underlying molecular mechanisms that promote these human-like
neuro-phenotypes. These models will serve as valuable tools for preclinical trials that aim to target NI in PDAC.
Results

Phenotype screens of GEMMs of PDAC reveal human-like neuro-invasive models

The Collaborative Research Centre 1321 (CRC1321) in Munich has generated, cataloged and characterized 295 different allele combinations of oncogenes and tumor suppressors in GEMMs of PDACs in the past four years. These invaluable tools that enable a profound mechanistic understanding of the complex genetics and molecular drivers of PDAC, have served here as a unique platform for a phenotype screen for NI. The mouse alleles in this source comprise, among others, mutants of key genes and signaling pathways involved in pancreatic carcinogenesis, such as tumor suppressors (e.g. \( p16^{lox/lox} \), \( Ink4a^{lox/lox} \), \( p53^{lox/lox} \)), regulators of epithelial-mesenchymal-transition (EMT) (\( LSL-Snail^{+/−} \), \( Cdh1^{lox/lox} \), \( Tnc^{lox/lox} \)), TGFbeta signaling (\( Tgfbr2^{lox/lox} \), \( Smad4^{lox/lox} \)), genetic reporters (\( R26dTo^{lox/lox} \), \( dsRED-eGFP^{lox/lox} \)), viral targeting systems (\( Tva \) transgene), drivers of endodermal lineage specification (\( Hnf4a^{lox/lox} \)), cell ablation alleles (DTA), and dual recombinase system alleles (\( Pdx-Flp \), \( FSF-Kras^{G12D} \), \( FSF-Rosa26-Cre^{ER} \), \( trp53^{frt/frt} \), \( LSL-Kras^{G12D} \)). From each, we screened two hematoxylin- and eosin-stained slides, as well as two immunostained slides against the neuronal marker protein-gene-product 9.5 (PGP9.5) per mouse in each of a total of 295 different genotypes in the GEMMs from CRC1321. All genotypes were analysed at an age at which they had developed overt invasive cancer (for a list of the analysed age ranges and the number of mice per genotype, please refer to the Table S1-2).

In our screen, we detected a remarkably low frequency of human-like “peri-”neural invasion in the primary tumor, which is defined as perineural alignment of cancer cells along the neural sheath (Figure 1A). In fact, the widely used oncogenic \( Kras \)-driven GEMMs of PDAC such as KC (\( p48-Cre; LSL-Kras^{G12D} \)) or KPC (\( p48-Cre; LSL-Kras^{G12D}; trp53^{lox/lox} \)) did not exhibit perineural invasion, although they harbored several, enlarged nerves that were diffusely scattered in the tumor-infiltrated stroma. The lack of a “targeted” invasion of nerves by cancer cells in these important GEMMs of PDAC motivated us to further search for genotypes with exact human-like appearance of perineural invasion. In our screen, we detected two genotypes with overt PDAC that fulfilled this criterion. Interestingly, both were driven by a pancreas-specific, transgenic overexpression of the transforming growth factor alpha (TGFα) under the control of the elastase-1 promoter (\( Ela1-Tgfα \)): 1)
Ela1-Tgfa; p48-Cre; trp53lox/lox (herein termed “TPC”) and 2) Ela1-Tgfa; p48-Cre; trp53lox/lox, RelAlox/lox (herein termed “TPAC”). The latter genotype was originally generated by Mar.L. and H.A. for studying the influence of canonical NF-kB signaling on the growth dynamics of PDAC.

In the TPC genotype, we detected long-distance perineural contact between cancer cells and the enlarged pancreatic nerves, yet the cancer cells did not fully encircle the nerves as in human PDAC (Figure S1E). In the TPAC genotype, however, the nerves were fully engulfed by cancer cells, which represents the typical appearance of perineural invasion of human PDAC (Fig 1B, Figure S1E). Accordingly, the histological severity of NI, assessed by a clinically established scoring system (10, 18, 19) (“score 0” – the tumor cells in no contact with nerves; “score 1” – the tumor cells perineurally touch the nerves, and “score 2” – the tumor cells invade inside the nerve, Figure S1D), was markedly higher in TPAC and TPC mice as compared to the Kras-driven KC and KPC mutants (TPAC: 0.26 ± 0.10, TPC: 0.14 ± 0.09, KPC: 0.0 ± 0.0, KC: 0.0 ± 0.0) (Figure 1C, Figure S1D). Interestingly, the liver and lung metastases of TPAC mice did not exhibit NI (Figure S2). To our knowledge, NI has also not yet been previously described in the metastases of human PDAC.

As the TPAC genotype exhibited a rather higher severity of NI and a more pronounced perineural invasion than the TPC genotype, we also considered the possibility that the conditional/pancreas-specific loss of RelA/p65 (p65lox/lox) and the entailing suppression of the canonical NFkappaB signaling is linked to perineural invasion. Therefore, we also analysed the nerve morphology in another genotype with pancreas-specific loss of RelA/p65 (p65lox/lox) together with conditional activation of the oncogenic Kras and bi-allelic loss of p53 (p48-Cre; LSL-KrasG12D; trp53lox/lox, RelAlox/lox, herein termed “KPAC”). We found that the KPAC mice did not harbor perineural invasion and therefore displayed much lower neural invasion severity scores (data not shown). Hence, this observation suggested that it is the TGFA hypersignalling, rather than the loss of the canonical NFkappaB signaling, that promotes human-like NI in the TPAC and TPC models. We also dissected the pathways that are differentially regulated in the TPAC- vs. TPC-derived cancer cells with the aim to uncover signaling events that may result in the more severe and human-like NI of TPAC vs. TPC mice.

In this transcriptome analysis, we found that the overwhelming majority, i.e. 50% of all differentially regulated pathways, were related to metabolic events, such as fructose/mannose, histidine, sphingolipid, pentose
phosphate pathway (Figure S3). This suggests that metabolic alterations can further aggravate the human-like perineural invasive phenotype of murine pancreatic cancer.

Both genotypes were associated with extensive desmoplasia / fibrosis, more pronounced than in KPC mice. The TPC and TPAC tumors displayed a ductal-like phenotype and, accordingly, strongly expressed the ductal cancer cell marker cytokeratin 19 (CK-19) (Figure S4A), yet the TPAC genotype with loss of RelA/p65 and p53 had an even more ductal-like appearance (Figure S4A&C), more desmoplasia (Figure S4C), a lower cancer cell proliferation rate (Figure S4B) than the TPC mice. TGFα is a known driver of acinar-to-ductal metaplasia (ADM). Importantly, the rate of ADM in the TPAC genotype was more pronounced both in vivo and in vitro than in TPC mice (not shown). Overall, these data suggested a more ductal, fibrotic, and slowly growing tumor phenotype in TPAC mice than in TPC or KPC mice. Accordingly, the overall survival of analyzed TPAC mice was significantly longer than that of TPC mice. TPAC mice had a median survival of 370 days compared to 297 days in TPC cohort (P < 0.0001, Figure S1B). To further study the biological consequences of RelA loss in TPC mouse model, 46 TPC or TPAC tumors were analyzed. A remarkably high incidence of pancreatic ductal adenocarcinoma (PDAC) was observed in TPAC compared with TPC mice (75% vs 9.1%). In addition, RelA deficiency in TPC model resulted in a significant decrease in metastasis rate (31.8% in TPC vs 8.3% in TPAC, Figure S4D).

These survival times clearly surpassed the lifespan of KPC mice (median 61 days), but were comparable to the lifespan of KC mice, which do not harbor a priori loss of p53 in carcinogenesis (Figure S1A). However, KC mice, despite their slower tumorigenesis, did not exhibit human-like perineural invasion. Interestingly, neural hypertrophy was present in all analysed genotypes, independent of NI (Figure S1F). To exclude the presence of mutant Kras in the TPAC mouse model, we performed targeted sequencing of the Kras locus in the spleen of the TPAC mice, as well as in cancer cells isolated from the TPAC mice. As predicted, the tissues and cells from TPAC mice did not harbor Kras mutations and were thus Kras-wildtype (Table S3). Despite the absence of the oncogenic Kras mutation, we explored whether the TPAC cancer cells also exhibited elevated Ras activity. In Ras activity assays, we detected the Ras activity to be even higher in the isolated TPAC cancer cells than in KPC cancer cells, which confirms the strong Ras-activating capacity of TGFα hypersignalling. (Fig S4E). Altogether, these data strongly suggested that TGFα signaling, without the
need for mutant Kras, gives rise to slowly growing, highly fibrotic, ductal pancreatic cancer, which seem to be necessary for the emergence of human-like perineural invasion in murine PDAC.

TGFa-associated perineural invasion in murine PDAC genotypes correlates with neuro-invasiveness and Schwann cell chemoattraction ex vivo

For functional analyses on neural invasion, we employed a series of ex vivo 3D culture setups. We first explored whether the enhanced neuro-invasiveness of the TPAC genotype is due to a superior chemoattraction of cancer cells of this genotype toward DRG neurons, which are the main source of the sensory innervation in the murine pancreas. For this, we placed primary cancer cells of each genotype in ECM droplets connected via bridges to droplets containing neurons isolated from dorsal root ganglia of P1-P3 C57Bl/6J mice and analysed cell migration through time-lapse microscopy (Figure 1D). We did not identify any differences in the velocity of migrating cancer cells of all genotypes toward neurons (Figure S1G). However, the forward migration index (FMI), which indicates directional chemotaxis, was much higher in TPAC cancer cells compared to the primary cancer cells of all other studied genotypes (TPAC: 0.32 ± 0.03; KC: 0.09 ± 0.04, KPC: -0.22 ± 0.05, TPC: 0.09 ± 0.03) (Figure 1E). We then analysed whether the cancer cells of these genotypes also differ with regard to their neurotrophic attributes. Interestingly, conditioned media/CM from cultured primary cancer cells from TPAC mice augmented neurite outgrowth similarly to positive control media supplemented with 10ng/ml nerve growth factor/NGF (TPAC: 1.822; SFM: 1.05 neurites per 2500µm²) (Figure 1F, G), whereas the number of neurites in cultures with CM from the remaining genotypes did not differ from negative control (KPC: 1.235, TPC: 1.411 neurites per 2500µm²) (Figure1F-G). To explore molecular factors that drive a genotype-specific induction of neurite formation, we performed transcriptome arrays with the primary cancer cells from the different genotypes. Here, we found prominent upregulation of several neurotrophic and neurogenic factors in the TPAC cancer cells as compared to KPC cancer cells. For example, Fos (33-fold), which is required for neurite elongation and crucial for neuronal differentiation (20, 21), and neuropeptide Y/Npy (31-fold), which plays an important role in axonogenesis (22), were the two most prominently upregulated neurotrophic genes (Figure 1H). In addition, TPAC cancer cells overexpressed Fgf9, which promotes proliferation of neuronal precursors (23), and Nrg1, which has
been shown to drive proliferation and/or induce myelin differentiation (24); and Nrg4, which is involved in the establishment of early sensory innervation in the skin (25) (Figure 1H).

Another recently discovered aspect of nerve-cancer interactions is the appearance of Schwann cells (SCs) around the pre-malignant lesions of pancreatic cancer, which contrary to the traditional assumption, implies that the nerves and not the cancer cells migrate first during NI (15). Therefore, we analysed the cancer tropism of SCs toward primary cancer cells isolated from KC, KPC, TPC and TPAC genotypes in our Schwann cell outgrowth assay (15). For this purpose, we placed freshly isolated sciatic nerves from C57BL/6J mice and connected them via bridges to droplets containing cancer cells of the respective genotype on one side and empty ECM droplets on the other side. (Figure 1I). The velocity and distance of migrated SCs was unchanged toward the four analysed mutated cancer cells (velocity: KC-0.44 ± 0.05 μm/min, KPC-0.47 ± 0.07 μm/min TPC-0.41 ± 0.04 TPAC-0.54 ± 0.07 μm/min; distance: KC-104.6 ± 14.48 μm; KPC- 67.11 ± 9.16 μm; TPC- 96.36 ± 10.33 μm; TPAC-106.6 ± 15.75 μm) (Figure S1G). However, the FMI of Schwann cells was increased towards TPAC cells (0.33 ± 0.07) when compared to KPC and KC cells with negative FMI (KPC: -0.3 ± 0.04; KC: -0.13 ± 0.12), indicating that there was not the same extent of directional migration of SC towards the KC and KPC cells as compared to TPAC cells (Figure 1J).

To further explore the temporal relationship between TGFα overexpression and NI, we also compared the expression of TGFα in whole tissue lysates derived from KC mice with pre-invasive PanIN lesions, as compared to KPC, TPC and TPAC mice with over cancer. Here, we found that KC cells indeed had a lower expression level of TGFα as compared to KPC and TPAC cells (KC: 100.0±53.7%, KPC: 256.5±53.1%, TPC: 389.6±49.5%, TPAC: 413.1±56.5%, Figure S4F). At the protein level, TPAC tissues had higher TGFα levels than KPC tissues (KPC: 432.3±22.1ng/ml, TPC: 648.4±75.2ng/ml, TPAC: 830.5±27.5ng/ml), which suggests the gradual increase of TGFα during “perineurally invasive” pancreatic carcinogenesis. We also analysed the expression of TGFα in human pancreatic lysates and found that in human chronic pancreatitis CP), which is a potential precursor of human PDAC, the tissue levels of TGFα were markedly lower than in human PDAC tissues (delta-delta Ct/2-ΔΔCt of CP: 0.14±0.06 vs. PDAC: 0.62±0.18, p=0.017, Mann Whitney U test, Figure S4F).

**Cancer-derived TGFα upregulates CCL2 expression in pancreatic nerves**
To confirm the relevance of cancer-derived TGFα in human PDAC, we analysed three different publicly available single cell RNA sequencing datasets of human PDAC and searched for the prime source of TGFα in the human PDAC tissue. In line with our expectations, we detected cancer cells as the main source of TGFα in human PDAC (Figure S5), expressing 19.7 times higher amounts of TGFα when compared to other cells in the tumor microenvironment, which had negligible amounts of TGFα expression (Fig S5).

In the next step, we analyzed the transcriptome of pancreatic cancer cells from KPC and TPAC mutants using the Affymetrix Mouse Gene ST1.0 array with subsequent bioinformatics analysis at the GSEA platform. We identified 211 up-regulated and 55 down-regulated genes with a log2 fold change above 0.58 and below -0.58 and an FDR-adjusted p-value (q-value) ≤ 0.05 within 28,941 genes by comparing TPAC with KPC cancer cells (Figure 2A, B). Among the top 10 upregulated genes, we identified the Cxcl15 chemokine involved in lung-specific neutrophil trafficking under normal and inflammatory conditions (26); serine palmitoyltransferase (Sptssb), which catalyses the first step of sphingolipid biosynthesis; secreted frizzled-related protein 2 (Sfrp2), a member of WNT signaling that promotes the neuronal differentiation potential of apical papilla stem cells (27), Decorin (Dcn), a small leucine-rich proteoglycan that plays a role in the assembly of collagen fibrils, coiled-coil domain-containing protein 80 (Ccdc80), which promotes melanoma cell migration via the FAK/E-cadherin pathway (28) and aldehyde dehydrogenase 3 family member A1 (Aldh3a1), which together with ALDH1A1 is a biomarker for stem cell formation in pancreatic cancer (Figure 2A). Many of the upregulated genes in the TPAC cancer cells are involved in extracellular matrix formation (ECM) and related intracellular pathways. Interestingly, we also found strong up-regulation of Cxcl15, Sfrp2, Dcn and Ccdc80 genes, when we compared TPC with KPC cancer cells (Figure S6A). In addition, the expression of genes that support neuritogenesis, namely Npy and Fos, was also increased in TPC as compared to KPC derived cancer cells (Figure S6B). We also found an enrichment of genes encoding the extracellular matrix, including ECM glycoproteins, collagens and proteoglycans involved in biological oxidation, nuclear receptors, and proteins encoding drug metabolism via cytochrome P450 and retinoic acid signaling (Figure 2B). Overall, these analyses showed that the TGFα-driven PDAC GEMMs TPAC and TPC showed enhanced expression of neurotrophic and neurogenic factors as compared to the KPC genotype-derived cancer cells.
Next, we performed a literature search for soluble factors that are known to be overexpressed under the influence of TGFα. We found that in primary chondrocytes, TGFα was shown to up-regulate secretion of the chemokine CCL2 (29). Furthermore, CCL2 released by DRGs was shown to facilitate perineural invasion of prostate cancer cells in vitro (30). In order to explore a possible link between TGFα and CCL2 in our neuro-cancer interaction setups, we treated murine DRG cultures with recombinant TGFα (rTGFα). Here, we detected increased expression of the Ccl2 gene within the DRG. In addition, Npy mRNA content was also upregulated (Figure 2D, E). Accordingly, we found increased CCL2 protein in the supernatant of co-cultured neurons and human cancer cell lines SU.86.86, T3M4, and DLD-1 (Figure S3C, D). Thus, CCL2 emerged here as a secreted molecule, which was induced by TGFα, and potentially involved in the interactions between neurons and cancer cells.

To find out the potential role of CCL2 in neural invasion (NI) in our GEMMs, we analyzed CCL2 content in the nerves within primary pancreatic tumors from KPC and TPAC mice (Figure 2F-G). Here, we detected increased CCL2 protein immunoreactivity in the nerves of pancreatic tumors of TPAC mice as compared with KPC. Similarly, we detected a more pronounced increase in CCL2 content in nerves in patient-derived PDAC samples with neural invasion (NI group) compared with patient-derived samples without NI (No-NI group) (NI group: 20.78%; No-NI group: 0.3067%) (Figure 2H, I).

To verify the DRG neurons as a major source of CCL2 in the PDAC context, we analyzed Ccl2 expression levels in available single-cell RNA sequencing datasets of mouse DRG (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7307422/) (Figure S7). Here, we detected DRG neurons, especially CGRP-Alpha+ and nonpeptidergic nociceptor subtype neurons, as the main source of CCL2 in DRG ganglia compared to lower expression of CCL2 in other cells in the ganglion (Figure S7D). Next, we analysed the CCL2 content in the PDAC tissue by semi-quantitative scoring analysis of CCL2 immunoreactivity in 14 PDAC patients (Figure S7A-C). We found that the CCL2 content was highest in nerves, which scored much higher when compared to acinus cells (p<0.0001), tumor cells (p<0.0001), immune cells (p<0.0001), and fibroblasts (p<0.001). In parallel, CCL2 immunostaining scoring in 10 TPAC mice showed that the nerves scored again highest when compared with acinus (p<0.0001), tumor (p<0.0001), immune cells (p<0.0001), and fibroblasts (p<0.0001) (Figure S7A-C).
In addition, we sorted out murine neurons out of DRG of TPAC mice and other cell types (cancer cells, myeloid cells, lymphoid cells) from the primary tumors of TPAC mice and KPC and cross-compared the expression levels of CCL2 (Figure S7E). In line with above, we again detected the highest expression in the DRG neurons, which was much higher in TPAC-derived DRG neurons (610.9±157.8% of KPC-derived neurons, p=0.03), as compared to KPC-derived DRG neurons (Figure S7E).

We also quantified the mRNA content of the Ccl2 gene in granulocytes, monocytes, endothelial cells, mesenchymal cells and cancer cells isolated from KPC and TPAC pancreatic tumours (n=3). Populations were identified as shown in the Figure S8 (A and B), incuding granulocytes as Cd45+Gr1+Cd11b+, monocytes as Cd45+Gr1medCd11b+, endothelial cells as Cd45-CD31+Cd326-, and cancer cells as Cd45-Cd31+Cd326+ cells. With the exception of a lower proportion of monocytes in the pancreatic tumours of TPAC mice compared to KPC mice, other sorted cell populations were unchanged (Figure S8C). Therefore, all these sort data underlined the DRG neurons and nerves as the leading and most dynamic source of CCL2 in PDAC.

The upregulation of CCL2 in DRG neurons upon exposition to TGFα derived from cancer cells would necessitate the presence of EGFR receptor on DRG neurons. To prove the presence of this receptor on DRG neurons, we employed the spatial transcriptome technology (Nanostring GeoMx® DSP) platform to quantify and to compare the expression of the whole mouse transcriptome in the DRG of our TPAC model in simultaneous comparison with oncogenic Kras-based mouse models of pancreatic cancer and with wild type (WT) mice. This analysis allowed us to capture the expression of the whole transcriptome at single cell and spatial resolution in DRG neurons of different genotypes. Using this technology, we indeed detected EGFR receptor expression on the DRG neurons of the mice, and EGFR expression was significantly greater in the DRG neurons of TPAC mice (133.2±14.0%), when compared to the oncogenic Kras-based KC mice (p48-Cre;LSL-KrasG12D; 100±15.8%), which might explain the receptivity of TPAC-derived DRG from cancer cells (Fig S9A-B). We complemented these analyses on murine DRG with the spatial transcriptome analysis of nerves within human pancreatic cancer specimens to explore whether we can detect the EGFR also in human nerves at the periphery that would bind TGFα (Fig S9C-D). In the Nanostring GeoMx® DSP spatial transcriptome-based analysis of EGFR expression, we found that the levels of EGFR expression in nerves with versus no neural invasion did not vary, but EGFR was detectable in the nerves in all analysed
human PDAC specimens (Fig S9C-D). We confirmed the expression of EGFR in FACS-sorted DRG of TPAC, KPC and wildtype mice (Fig S9E), in nerves of human PDAC specimens (Fig S9F) and in the DRG of TPAC mice (Fig S9G) also via immunostaining. Therefore, these three lines of evidence further strengthened the notion of TGFα responsiveness of DRG neurons in neuro-invasive PDAC.

**CCL2 activates the cancer cell cytoskeleton and enhances the migratory properties of pancreatic cancer cells via CCR4**

NI was originally believed to follow along paths of lowest physical resistance in the perineural area. However, numerous recent studies have convincingly shown that cancer cells, neurites and Schwann cells are chemoattracted very early during carcinogenesis to each other, which is orchestrated by soluble factors such as chemokines and neurotrophic factors (12, 31). Reorganization of the cytoskeleton is essential for the acquisition of migratory and invasive properties of cancer cells (32). One of the central molecules in the regulation of cytoskeletal reorganization is the multidomain scaffold protein paxillin (33). In this regard, phosphorylation of paxillin at tyrosine residues 31 (Tyr-31) and 118 (Tyr-118) by the nonreceptor protein tyrosine kinase SRC and focal adhesion kinase 1 (FAK1) is essential for cytoskeleton assembly and cancer cell migration (34-37).

To elucidate the cytoskeletal adaptation in cancer cells that are confronted with neurons, we investigated the morphology of filopodia and lamellipodia of cancer cells in the 3D migration assays with neurons. We quantified filopodia formation during migration toward DRGs using the FiloQuant® software (Figure 3A, B). Interestingly, cancer cells at the migration front showed a significant increase in filopodia numbers and length (Figure 3B, C). In addition, we detected an increased number of p-paxillin-positive spots in the cytoskeleton of cancer cells in the migration front (MF) toward DRGs compared with the back front (BF) that faces the empty ECM gel (Figure 3D, E). These results suggested that cytoskeleton reorganization and microfilament formation in cancer cells are triggered by neurons.

Next, we asked whether CCL2, secreted by neural cells, affects the cytoskeletal mediators of migration in cancer cells. To this end, we treated human pancreatic cancer cells SU.86.86 and T3M4 for 15 and 25
minutes with conditioned media of murine DRG neurons (Figure S6E). After 25 minutes of treatment, we observed increased phosphorylation of paxillin at Y118 in both cell lines, suggesting that DRG neurons secrete molecules that induce phosphorylation of paxillin. We next tested whether CCL2 directly induces phosphorylation of paxillin, and treated SU.86.86 and T3M4 pancreatic cancer cells with 100 ng/ml of rCCL2 for 5, 15 and 25 minutes. Indeed, treatment of both patient-derived cancer cell lines with rCCL2 significantly enhanced the phosphorylation of paxillin already after 15 minutes of treatment (Figure 3F, G), as well as of its downstream mediators Src and Erk (Figure S6G-J).

In cancer bone metastasis, CCL2 signaling is activated via binding of CCL2 ligand to the CCR2 and CCR4 receptors (38). In prostate cancer, CCL2-CCR2 is associated with perineural invasion (30). However, in our experiments, the CCR2 antagonist RS-504393 had no effect on paxillin phosphorylation in either of tested pancreatic cancer cell lines (Figure S6F). Remarkably, though, treatment of both cancer cell lines SU.86.86 and T3M4 with the CCR4 antagonist (C021) decreased paxillin phosphorylation in a time-dependent manner (Figure6H, I).

To test a functional effect of the CCL2-CCR4 pathway on migration, we pre-treated cancer cells with rCCL2 or C021 for 15 minutes and used them in the migration assay in the presence of DRG neurons (Figure 3J). Vehicle pre-treated cancer cells served as a negative control. Here, treatment with rCCL2 increased cancer cell motility compared to vehicle-treated cells with a corresponding elevation in FMI (vehicle: 0.25 vs. rCCL2 treatment: 0.33), higher velocity (vehicle: 0.075µm/min vs. rCCL2 treatment: 0.095µm/min), and greater distance (vehicle: 41.61µm vs. rCCL2 treatment: 46.71µm) (Figure 3K). Accordingly, CCR4 blockade with its inhibitor C021 resulted in decreased FMI (vehicle: 0.25 vs. C021 treatment: 017), decreased velocity (vehicle: 0.067 vs. C021 treatment: 0.051µm/min), and shorter distance compared to vehicle control (vehicle: 40.12µm vs. C021 treatment: 31.03µm) (Figure 3K). These results suggested that the CCL2-CCR4 cytokine signalling pathway plays an essential role in the affinity of pancreatic cancer cells towards neurons.

P-paxillin is a prognostic factor in PDAC

We next interrogated whether paxillin phosphorylation is relevantly increased in human pancreatic cancer cells that are physically adjacent to nerves in the pancreas. To this end, we stained tumor paraffin sections
of PDAC patients with neural invasion (NI group) and without neural invasion (no NI group) with PGP9.5, pan-CK (for labeling cancer cells) and p-paxillin. We found that cancer cells located close to nerves (within 200µm diameter) expressed more p-paxillin (15.57% of total tissue area) than cancer cells located in the pancreas distant from nerves (4.23% of total tissue area) (Figure 4A, B). As NI is a strong and independent prognostic factor for overall survival in PDAC (39), we then analyzed whether p-paxillin content also relates to patient survival. First, we divided the patient samples into low p-paxillin (n=31) and high p-paxillin (n=23) groups based on a cut-off value of 3.77%, which corresponded to the median of the p-paxillin/paxillin ratio (Figure 4B). Here, the mean staining intensity value of the high p-paxillin group (16.72%) was significantly increased compared with the low p-paxillin group (0.62%) (Figure 4C, D). In contrast, the total paxillin concentration in the tumors of both groups were comparable (Figure 4C, D). As shown on the Kaplan-Meier curves, the survival time of patients from the low p-paxillin group (n=31; median survival rate: 22.8%) tended to be longer than that of patients from the high p-paxillin group (n=28; median survival rate: 18.1%) (Figure 4E). We also found that CCL2 immunoreactivity was increased in the high p-paxillin expression group (Figure 4F), suggesting that CCL2 overexpression is indeed linked to paxillin phosphorylation in human PDAC tissue.

Analyzing the clinical parameters, we found that the high p-paxillin group had a higher percentage of patients with large tumors (T4>6 cm) than the low p-paxillin group (chi-square test: p=0.01, Figure S10A, C). In addition, the percentage of moderately differentiated tumors (G2: 76%) was increased, whereas differentiated G1 tumors were not detected in the high p-paxillin group compared with the low p-paxillin group (G2: 63%, G1: 9%) (chi-square test: p=0.006, Figure S10A, C). We also observed a marked, but not significant, increase in the proportion of affected lymph nodes with tumour cells (Figure 4G). These data point out that the tumor size and higher tumor grading were indeed more prevalent in the high-paxillin group, which suggest that the survival difference of high vs low-paxillin groups might be indirect due to other clinico-pathological parameters. Overall, these results also suggested that p-paxillin is a prognostic factor and may predict poor prognosis and aggressive tumor biology in human PDAC.

**Targeting of the CCL2-CCR4 axis in vivo limits neural invasion and tumor innervation**

To understand the role of the CCL2-CCR4 axis in an organismal context, we modulated CCL2-CCR4 signalling in vivo by intraperitoneal administration of recombinant CCL2 protein (50µg/kg i.p.) or of the CCR4
receptor antagonist C021 (1mg/kg), starting with KPC mice that exhibit hyperinnervation (Figure S1F), yet no perineural invasion (Figure 5A). No metastases were detected in the brain, lung, heart, liver, kidney, jejunum, and colon in the studied groups (Figure S11). Here, the amounts of nerves as detected through intratumoral PGP9.5 immunostaining in the pancreas of KPC mice was increased after treatment with rCCL2 (mean 1.76) and dramatically decreased after treatment with C021 (mean 0.137) compared with KPC control animals (mean 0.647) (Figure 5B, D). Although these mice lack human-likeNI, we scrutinized whether this treatment resulted in a change in the physical proximity between nerves and cancer cells in the primary tumor. Indeed, the index for proximity between cancer and nerves was significantly increased after treatment with rCCL2 and decreased after CCR4 inhibition compared with control mice (mean index: rCLL2: 0.64, C021: 0.2, control: 0.43; mean scores: rCCL2: 28.2, C021: 8.8, control: 16.00) (Figure 5B, E). Moreover, treatment with rCCL2 increased paxillin phosphorylation in tumor cells (mean: 3.13), while CCR4 inhibition decreased p-paxillin amounts (mean: 1.06) (Figure 5C, F). Taken together, these results, in agreement with our in vitro observations, suggest that the CCL2-CCR4 pathway affects paxillin phosphorylation in cancer cells and the proximity of the cancer to neural cells, even in the absence of perineural lining of cancer cells.

To further confirm the in vivo relevance of CCR4 signalling on NI in the herein described TPAC model, we treated TPAC mice (aged 42 weeks) with the CCR4 inhibitor C021 three times a week for 3 weeks and subsequently analysed the severity of NI in the primary tumor via histology. Accordingly, the CCR4 inhibition remarkably reduced the neural invasion severity in the TPAC mice (mean NI score of Control/ctrl: 0.4±0.08 vs. CCR4 inhibitor-treated: 0.1±0.06), as assessed by the neural invasion severity score (Figure 5G). CCR4 is known to exhibit relatively high expression levels also in T lymphocytes and macrophages (https://www.proteinatlas.org/ENSG00000183813-CCR4/single+cell+type). We therefore also explored the possibility that the amounts of these two immune cell subsets in the TPAC mice might be affected when the TPAC mic are treated with the CCR4 inhibitor. Here, we found that CCR4 inhibition did not alter the amounts of CD4+ T lymphocytes in the TPAC tumors (Control: 1.11±0.28 vs. CCR4-inhibited: 0.68±0.20 cells/mm2) but, interestingly, also reduced the number of F4/80+ macrophages (Control: 7.26±0.48 vs. CCR4-inhibited: 2.56±0.35 cells/mm2, Figure S14A-B), underlining an additional role for macrophages in the modulation of neural invasion in murine PDAC.
Inhibition of paxillin-Src-Erk signalosome reduces neural invasion, innervation and tumor size

In a next step, we investigated whether inhibition of the paxillin-Src-Erk signalosome has effects on cancer cell migration toward neurons and tumor severity. We used the small molecule inhibitor of paxillin protein disruptor 6-B345TTQ, which inhibits the binding of paxillin to integrin4-alpha and regulates cell migration (40-42). As expected, we found a significant decrease in paxillin phosphorylation at Y118 in SU.86.86 and T3M4 cells treated with 6-B345TTQ by Western blots (Figure S12A, B). Ex vivo, we found that cells pre-treated with 6-B345TTQ performed poorly in the migration assay toward DRG for every parameter examined: forward migration index (vehicle: 0.2136; treated with 6-B345TTQ: 0.1469), speed (vehicle: 0.08442; treated with 6-B345TTQ: 0.05425), and distance (vehicle: 42.08; treated with 6-B345TTQ: 31.73) (Figure 6A, B). We observed similar effects after pre-treatment of cancer cells with the ERK1/2 phosphorylation inhibitor AZD8330 for 1 hour (Figure S12D). The decreased phosphorylation of ERK1/2 in treated cells was verified by immunoblotting (Figure S12C-D).

Next, we tested the effects of paxillin inhibition with 6-B345TTQ on pancreatic cancer progression in vivo. To this end, we treated 5-month-old TPAC animals with 6-B345TTQ (1mg/kg) 5 days per week for 4 weeks and analyzed the pancreas for tumor growth (Figure 6C). All animals developed tumors with comparable pancreatic weights in both groups (Figure S13A). Remarkably, the tumor innervation as measured through the PGP9.5 content was decreased in the tumors of TPAC mice treated with 6-B345TTQ (mean 0.31% of total area) compared with control TPAC mice treated with DMSO (mean 0.64% of total area) (Figure 6D, E). Moreover, a reduction in p-paxillin was detected in the pancreas of the treated TPAC mice (mean 4.72% of total area) compared with the DMSO controls (mean 9.79% of total area) (Figure 6D, F). These results strongly suggested that blockade of paxillin phosphorylation leads to a rapid decrease in tumor innervation.

In addition, we tested the activity of highly proliferating preselected in vitro clones from TPAC tumors by implanting them orthotopically into the pancreas of 8-10 weeks old wild type mice (129xC57BL/6) (Figure 6G). We treated transplanted mice for 4 weeks with 6-B345TTQ (1mg/kg) by applying it for 5 days weekly and treated the control animals with DMSO (Figure 6G). We did not detect any changes in body weight of recipient animals transplanted with TPAC cancer cells (Figure S13B). Interestingly, pancreatic weight of recipients implanted with TPAC cells decreased after treatment with 6B345TTQ (0.138 g) compared with...
DMSO (0.168 g) (Figure S13C) due to strong reduction of the primary tumor area in mice treated with 6B345TTQ (0.95% of total area) compared with DMSO-treated controls (3.43% of total area) (Figure 6H, I). Furthermore, we identified a strong reduction of paxillin phosphorylation in cancer cells of recipients treated with 6B345TTQ (4.72% of total area) compared with control mice treated with DMSO (9.79% of total area) (Figure 6H, J). These results confirmed paxillin phosphorylation as a key actor in tumor progression and a promising target for therapeutic approaches.

Finally, we interrogated whether TGFα, which is the prime driver of carcinogenesis in the neuro-invasive TPAC and TPC genotypes, is also prognostically relevant in human PDAC. Analysis of survival data from the TCGA database uncovered TGFα as a very strong prognostic factor in human PDAC. Here, PDAC patients with a low level of TGFα expression had a remarkably high 5-year survival rate of 60%, whereas the 5-year survival rate dropped to 18% for patients with high intratumoral TGFα content (https://www.proteinatlas.org/ENSG00000163235-TGFA/pathology/pancreatic+cancer). We also explored whether the tissue expression levels of Tgfa correlate with the emergence of neural invasion in human PDAC. For this purpose, we scrutinized the TCGA for information on the perineural invasion (PNI) status of the PDAC patients. The metadata and data repository of the TCGA-PDAC database includes digitized pathology reports that included the information on the perineural invasion status for 159 samples, 134 of which has been reported to show PNI, and 25 were described as non-PNI. This way, we could compare expression of TGFα in the PNI vs. non-PNI groups and discovered a significantly higher expression of TGFα in the PNI group, as compared to the non-PNI group (Figure S14C). There was no correlation between the PNI status and the tumor tissue bulk RNA expression levels of CCl2, CCR4 or paxillin (Figure S14C).
Discussion

Neural invasion (NI) is an extremely frequent mode of tumor spread in human PDAC, and its severity is a clinching determinant of prognosis (10, 16, 17). However, NI is not sufficiently modelled by the commonly used, oncogenic Kras-driven GEMMs of PDAC, as they do not exhibit the extent of the typical perineural invasion of human PDAC. In this study, we performed the largest phenotypic screen of GEMMs of PDAC to date for detecting models with genuine perineural invasion. This way, we found that mutants that overexpressed TGFα, i.e. the TPAC and TPC GEMMs, harboured “human-like” perineural invasion. In the Kras-based mutants, on the other hand, the cancer cells were present in close proximity to the nerves, but this occurred more or less sporadically. Functionally, cancer cells isolated from the primary tumors of TGFα-driven GEMMs mice showed higher neuroaffinity, and induced stronger neuritogenesis than oncogenic Kras-based models, possibly due to increased secretion of neurogenic/neurotrophic factors. We also show that TGFα promotes the secretion of CCL2 from DRG neurons, which in turn activates the cancer cell cytoskeleton through CCR4, paxillin phosphorylation and cell protrusion formation. Targeting of the CCR4-phospho-paxillin axis was sufficient in reducing tumor progression and neuro-affinity of cancer cells in vivo.

Our study has several implications. First, we provide examples for GEMM of PDAC that are more similar to human PDAC with respect to perineural invasion. Although oncogenic Kras-driven PDAC also exhibit a rich innervation and close spatial relation between nerves and cancer cells in the primary tumor, the typical perineural lining of cancer cells as seen in human PDAC was not as prominent and frequent as in the TPAC model. We believe that GEMM like TPAC can serve as valuable tools for future studies that aim to analyse mechanisms of NI in PDAC and for preclinical trials that aim to target NI. One of first events in the emergence of NI in PDAC is Schwann cell activation by pre-invasive lesions, i.e. PanIN lesions (15, 43, 44). On the other hand, once fully transformed, invasive cancer cells seem to upregulate TGFα, which augments NI over induction of CCL2 secretion from neurons, which acts upon CCR4 on cancer cells. The gradual increase of TGFα levels from KC to the KPC, TPC and TPAC mice hence supports the notion that TGFα aggravates the perineurally invasive phenotype of pancreatic cancer.

Second, our observations imply that, despite anatomic differences between the murine and human pancreas, neuropathic phenomena like NI are reproducible in GEMM of PDAC. Human pancreas is a compact organ in
the retroperitoneum, whereas the murine pancreas is less demarcated, quite diffusely distributed in the
mesentery, and is therefore termed an intraperitoneal, “mesenteric type” pancreas (45). Furthermore, nerve
trunks in the human pancreas are widely distributed over the parenchyma and present both within and around
the lobes (45). In contrast, the normal mouse pancreas contains nerve trunks only around extralobular
vessels and in the vicinity of lymphoid sites (45). In the light of this knowledge, it was interesting for us to
detect enlarged nerves and perineural invasion in the tumor core that was embedded in the lobes of the
pancreas. Hence, the genetic features of GEMMs are sufficient to overcome the anatomic differences
between the human and murine pancreas and to generate human-like neuro-phenotypes.

Third, our study points out a mechanism that generates human-like neural invasion (NI). NI is believed to be
initiated and orchestrated by chemotactic factors released from neurons that chemoattract cancer cells (31,
46). In our study, we screened for chemokines that were increasingly secreted from DRG neurons when
confronted with cancer cells, and detected increased production of CCL2. As the TGFα-based GEMMs
harboured human-like neural invasion, we explored and found that TGFα can induce CCL2 expression in
DRG neurons. In studies with prostate cancer, which is another prime example for a neuro-invasive cancer,
He et al. demonstrated that DRG neurons express CCL2, which mediated the nerve-derived migration
capacity of cancer cells (30). Neural invasion was significantly hampered in migration assays using DRG
neurons isolated from CCL2−/− mice (30). Moreover, we show a previously unknown link between TGFα, Ccl2
and neural invasion in pancreatic cancer, using the herein reported, human-like neuro-invasive TPAC model
and human data. Here, we also show that invaded nerves in human PDAC contain visibly higher amounts of
CCL2 than non-invaded nerves. Although chemokines and neurotrophic factors with chemoattractive
properties such as NGF, GDNF, artemin or neurturin have long been studied in the context of neural invasion
in PDAC (31, 47-50), the cytoskeletal adaptations of cancer cells during their nerve-directed migration have
not been in the focus. We detected a 4.03-fold overexpression of the high-affinity NGF receptor TrkA (Ngfr).
In addition, we found a 2.53-fold overexpression of Gdnf in the TPAC cancer cells when compared to KPC
cancer cells. It should be noted that, in the present study, the expression of neurotrophic and neurogenic
factors (like Gdnf) was compared between the TPAC and KPC genotypes. Neurotrophic factors that we found
to be overexpressed (like GDNF) are thus likely to be even more prominently upregulated when compared
to the healthy normal pancreas. In addition to this classical neurotrophic molecule, we demonstrate that CCL2
derived from DRG neurons activates the cancer cell cytoskeleton through the CCR4 receptor and the entailing paxillin phosphorylation. Paxillin is one of the major components of focal adhesions and is involved in the transmission of signals, regulation of cell morphology and control of cell spreading and migration (51). Recent studies have shown that phosphorylation of paxillin by FAK or SRC at Tyr118 and Ser178 is necessary for the stimulation of cancer cell migration (52). Hence, we provide a mechanistic explanation for the enhanced motility of cancer cells at the cytoskeletal level when chemoattracted by neuron-derived chemokines. Cytoskeletal adaptations in pancreatic cancer have recently also been shown to be induced by Schwann cells, which, by applying forces on cancer cells, were shown to alter the cancer cell cytoskeleton and promote the migration capacity of cancer cells (53).

Interestingly, administration of rCCL2 resulted in increased nerve density in the murine pancreas, which was reversible upon blockade of the CCR4 receptor. This suggests that the CCL2/CCR4 signalling pathway affects not only the chemoattraction and the migration machinery of cancer cells, but additionally modulates the neural ingrowth and innervation of cancer.

Our findings also underline some clinically relevant aspects of the identified molecular players. We found TGFα content of the primary tumor, and p-paxillin in cancer cells within PDAC patient samples to be unfavourable prognostic markers for overall survival. Furthermore, inhibition of paxillin phosphorylation with the inhibitor 6B345TTQ significantly reduced nerve density and tumor size in TPAC mice, and cancer-nerve affinity in KPC mice. We propose that interference with the TGFα-CCL2-paxillin axis holds potential for prognostic improvement and should be considered in upcoming clinical trials.

Our analyses of single cell RNAseq expression data of human PDAC from publicly available sources showed cancer cells as the most prominent source of TGFα in human PDAC tissues. Other cell types like myeloid cells or endocrine cells also turned out to exhibit TGFα expression, which was, however, much lower when compared to the TGFα expression levels in cancer cells. Therefore, although we cannot exclude a similar effect of TGFα derived from such non-cancerous cells, cancer-cell-derived TGFα is most likely to generate the observed effects.

We also questioned the genetic similarity of the TPAC model to human PDAC, when we saw the presence of nerve invasion but the lack of any Kras mutation in the TPAC model. However, based on our results, TGFα overexpression seems to be a typical feature of human PDAC, as shown in the TCGA database, although
we cannot know for sure how relevant this overexpression is in the earliest phases of human pancreatic
cancer. We believe that the solution to this seeming discrepancy lies in the fact that the TGFalpha-EGFR
axis activates those pathways that have been repeatedly shown to be overrepresented and hyperactive in
RNAseq studies of human PDAC, such as Ras-Raf-MAPK and PI3K-Akt-mTOR signaling. Indeed, we could
show here a higher Ras activity in TPAC cancer cells, as compared to that in KPC cancer cells. As such, TGFa
overexpression imitates the signaling events induced by mutant (oncogenic) Kras as known from human
PDAC as well as from the KPC model. This is the most likely reason why the Ela1-TGFa mouse model was
one of the earliest autochthonous murine transgenic models of PDAC (54).

It should be noted that in our study, not all TPAC or TPC mice exhibited perineural invasion (PNI: 60% of
TPAC mice), which suggests the presence of tumor heterogeneity in animals of identical genotype. Genomic
sequencing of outlier animals of the same genotype may here provide insight into further genetic alterations
that are necessary for a human-like neuro-invasive phenotype.

In conclusion, the present study demonstrates the power of large-scale phenotype screens for identifying
GEMMs of PDAC with human-like neuropathic characteristics. Our TPAC mouse model of NI in PDAC
represents a fundamental tool for studying the molecular mechanisms behind the cancer-neuron cross-talk.
Moreover, the TGFa-CCL2-CCR4-p-paxillin axis represents an NI-inducing, actionable axis that deserves
further testing in preclinical and clinical settings for prognostic improvement.
Methods

For further details on methods, please refer to the “supplementary methods”.

Mice

We used GEMM that are available in the repository of the The Collaborative Research Centre 1321 (CRC1321) in Munich (“Modeling and Targeting Pancreatic Cancer”). The CRC1321 has generated, cataloged and characterized 295 different allele combinations of oncogenes and tumor suppressors in GEMMs of PDACs over the past four years. For a full list of the genotypes, please refer to the Table S1. For the list of the analysed age ranges and the number of mice per genotype, please refer to the Table S2. The following abbreviations have been used for the genotypes of particular interest for this study: TPAC (Ela1-Tgfa; p48-Cre; trp53lox/lox; RelAlox/lox), TPC (Ela1-Tgfa; p48-Cre; trp53lox/lox), KPC (p48-Cre; LSL-KrasG12D; trp53lox/lox), and KC (p48-Cre; LSL-KrasG12D). For orthotopic and i.v. transplantations we used 129X57BL/6 and C57BL/6N wild type mice. All animals were housed in IVC cages under SOPF conditions at ZPF, Klinikum rechts der Isar, Technical University Munich, Munich Germany.

Patient samples

Tumor samples were collected from 59 patients with pancreatic cancer who underwent resection at the Department of Surgery at the Technical University Munich, Germany. Tissues were fixed in 4% paraformaldehyde in 1xPBS overnight and embedded in paraffin. 2 mm thin cuts were prepared using a rotary microtome (HM355S, ThermoFisher Scientific, Waltham, USA). Patients were grouped into p-paxillin low-expression group (p-paxillin expression/paxillin expression) and p-paxillin high expression group (p-paxillin expression/paxillin expression) based on immunostaining density. The survival data of the patients in The Cancer Genome Atlas (TCGA) was extracted from the following link: https://www.proteinatlas.org/ENSG00000163235-TGFA/pathology/pancreatic+cancer. For comparison of the survival of the high vs. low expression groups, the median expression was chosen as cut-off based on the following information provided by the TCGA: The survival data in the TCGA database were retrieved using the median expression based on the FKPM (number Fragments Per Kilobase of exon per Million reads) value calculated based on the gene expression data from all patients in the dataset.
Statistics

All results in graphs are shown as mean value ± SEM. For the statistical analyses, we used t-test, Mann-Whitney test, ordinary one-way ANOVA, Dunnett’s multiple comparisons test and, for the distribution, Shapiro-Wilk normality test using GraphPad Prism 5 (La Jolla, CA, USA). The P-value < 0.05 was considered to have significance.

Study approval

All animal experiments were approved by the governmental commission for animal protection of the Government of Upper Bavaria (Regierung von Oberbayern, no. 55.2-2532-Vet-02-16-165). The study was approved by the ethics committee of the Technical University of Munich (589/19s).

Data availability

The microarray data are publicly available at the Gene Expression Omnibus (GEO) with the accession number GSE201994 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201994). The spatial transcriptomics data are publicly available at the Gene Expression Omnibus (GEO) with the accession number GSE201994 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201994). All primary data are available as supplementary material online as uploaded on the journal website.
Author contributions
I.E.D., G.O.C., and R.I. designed the study. X.W., L.Y, S.T., R.I., R.Ö., C.J., K.C, C.M.R., Ü.Y., I.G., Q.L., M.S., S.E.Y., O.U.S, S.C. and G.S. performed the experiments and/or analysed the data. M.L. supported IF and analysis of the 3D-Migration assay. K.N.D., K.G., M.L. and H.A. supported the transplantation experiments and generated the TPAC model; B.V. and M.K. performed bioinformatical analysis of RNAseq data; K.S. and A.M. assisted in the histopathological analyses; X.W., R.I and I.E.D. wrote manuscript. M.R., A.K., R.R. critically read and reviewed the manuscript. D.S. and S.B. provided the majority of the mouse models. H.F., G.O.C. and I.E.D. supervised the study. All authors have approved the final version of the manuscript.

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Figure 1. Mutant TPAC mice represent a model for human-like perineural invasion

Representative images of genuine perineural invasion in PDAC resection specimens (A) and in TPAC mouse mutants (B; please also see Figure S1E for a broader view of the tumor area with perineural invasion); pan-neural marker: PGP9.5 (brown), cancer cell marker: CK-19 (pink) and Aniline blue (for collagen). (C) Graphs showing the severity of neural invasion (NI) in genetically engineered mouse models (GEMM) of PDAC. (D) Scheme of migration assay of cancer cells and neurons from dorsal root ganglia (DRGs). (E) Graphs show the forward migration index (FMI) of primary cancer cells isolated from KC, KPC, TPC and TPAC mice in the 3D migration assay. (F) Representative images of DRG neurons treated with conditioned medium from KPC, TPC and TPAC cancer cells, stained with neuronal marker beta3 Tubulin (Tubb3) antibody. (G) Results of the in vitro neuroplasticity assay of DRG neurons treated with supernatants of the primary cancer cells from the analysed mouse genotypes. (H) Transcriptome analysis of TPAC- vs. KPC-derived primary cancer cells. (I) Schematic representation of the 3D Schwann cell outgrowth assay. Sciatic nerves isolated from WT mice were placed between bridges connected to ECM gel drops containing primary murine cancer cells (KC, KPC, TPC, TPAC) and empty ECM gel drops. The test was performed for 72 hours in culture media with CO₂ supply. (J) Graphs showing the FMI of Schwann cells.

All results in the graphs are shown as mean ± SEM. For statistical analyses, Mann-Whitney U test (C,E), ordinary one-way ANOVA (G), Kruskal-Wallis test, Dunnett’s test for multiple comparisons (G,H) and Shapiro-Wilk normality test (all panels) for distribution were used.
Figure 2. CCL2 induced by TGFα is enriched in co-cultures of cancer cells and DRG neurons, TPAC neurons and in patient derived PDAC samples with neural invasion.

(A) Volcano plot of differentially expressed genes (DEGs) for the comparison of murine cancer cells derived from TPAC vs. KPC mice. (B) Bar plot displaying a selected set of over-represented pathways among the DEGs for the comparison groups TPAC vs. KPC. (C) Pathway-based interaction networks of DEGs for the comparison groups TPAC vs. KPC, where network line thickness indicates the confidence of the interaction. (D) Experimental set-up – DRGs from neonatal mice were cultured for 2 days, and rTGFA was added at concentrations of 1, 10, 25, 50 and 100 ng/ml for 24 hours. After the treatment, cells were used for qRT-PCR. (E) Graphs representing Ccl2 and Npy mRNA content in DRGs after treatment with rTGFα. (F) Representative images of pancreatic tumors from TPAC and KPC mice stained with PGP9.5 and CCL2 (both in brown). (G) Plots showing the colorimetric CCL2 content in nerves from TPAC and KPC tumors. (H) Representative images of consecutive sections of PDAC patient samples stained with CCL2 and S100 (both in brown) and counterstained with HE. (I) Plots showing the colorimetric CCL2 content in the nerves of patient samples measured with the QuPath software. Scale bars: 50 μm. All results in the graphs are shown as mean ± SEM. For statistical analyses, ordinary one-way ANOVA (E), Kruskal-Wallis test (E), Dunnett’s test for multiple comparisons (E), Mann-Whitney U test (G, I), and Shapiro-Wilk normality test for distribution (all panels) were used.
**Figure 3. The migration behaviour of cancer cells triggered by neurons is regulated by the CCL2/CCR4 axis**

(A) Schematic representation of the 3D migration assay. Arrows indicate the direction of migrating cells. (B) Representative images of migrating SU.86.86 pancreatic cancer cells in the migration front (MF) (toward DRGs) and back front (BF) (opposite to DRGs) analysed by confocal microscopy and labelled filopodia (pink lines). (C) Diagrams showing the number and length of filopodia in the cancer cells in BF and MF, quantified with the FiloQuant® software. (D) Representative images of SU.86.86 cancer cells from the migration assay, stained with phalloidin (red) and phospho-paxillinY118 (green) and counterstained with DAPI (blue). (E) Diagram showing the number of p-paxillin-positive points per 10x magnification. (F) Representative Western blots of SU.86.86 and T3M4 cancer cells treated with recombinant CCL2 (100ng/ml). (G) Graphs with relative content of proteins identified by Western blot measured with the ImageJ software (n=3 biological replicates). (H) Western blots of SU.86.86 and T3M4 cancer cells treated with CCR4 inhibitor C021 (140nM). (I) Graphs with relative content of proteins identified by Western blot measured with the ImageJ software. (J) Scheme of experiment: cancer cells were pre-treated with rCCL2 or C021 for 15 minutes and placed into the 3D migration assay with DRG neurons. As control, cells pre-treated with vehicle were used. (K) Graphs indicating forward migration index (FMI), velocity and migrated distance of cancer cells in the 3D migration assay.

All results in graphs are shown as a mean value ± SEM. For the statistical analyses we used unpaired t-test (B,E), one-way ANOVA (G,H,K), Dunnett’s multiple comparisons test (G,I,K) and for the distribution - Shapiro-Wilk normality test (all panels). The $P$-value < 0.05 was considered to have significance. Scale bars: 20 μm.
A  no Neural invasion  Neural invasion

B  p-Paxillin content

C  Low pPaxillin group  High pPaxillin group

D  p-Paxillin content

E  Kaplan-Meier curves

F  CCL2 content

G  Lymph nodes with tumour

Survival analysis p-paxillin
Cut off: 3.77
Median survival low p-Paxillin: 22.8 %
Median survival high p-Paxillin: 18.1 %
Figure 4. Paxillin phosphorylation in cancer cells is associated with poorer survival in patients with PDAC

(A) Representative images of consecutive sections from human PDAC resection specimens stained for p-paxillin (brown), the neural marker PGP9.5 (pink), cancer cell marker pan-CK (brown) and counterstained with haematoxylin.

(B) Graphs showing the percentage of p-paxillin content in cancer cells located distal and proximal to nerves. (C) Representative images of consecutive sections from patient derived PDAC samples stained with paxillin and p-paxillin (brown) and counterstained with hematoxylin. (D) Graphs indicating relative content of p-paxillin to paxillin in “low-p-paxillin” and “high p-paxillin” groups of PDAC patients. (E) Kaplan-Meier curves showing percentage survival of patients with low p-paxillin content (black line) and high p-paxillin content (red line). The cut-off value for p-paxillin content was set at four percent stained cells in all analysed areas. (F) Graphs showing the percentage of CCL2 content in “low-p-paxillin” and “high p-paxillin” groups of PDAC patients. (G) Graphs showing the percentage of lymph nodes infiltrated with tumor cells to all lymph nodes analyzed in “low-p-paxillin” and “high p-paxillin” groups of PDAC patients.

Scale bars: 20 μm. All results in graphs are shown as a mean value ± SEM. For the statistical analyses we used Mann-Whitney test (B,D,F), and the Mantel-Cox test (E). The P-value < 0.05 was considered to have significance.
Figure 5. CCL2/CCR4 axis regulates paxillin phosphorylation and innervation in KPC mice.

(A) Experimental design of rCCL2 and C021 inhibitor treatment: 12-week-old KPC mice (n=5) were injected i.p. with rCCL2 or C021 every other day for 3 weeks and normal saline as control. (B, C) Representative images of consecutive sections from PDAC samples of KPC mice treated with rCCL2, C021 and control groups stained with the neural marker PGP9.5 (pink), cancer cell marker CK-19 (brown), p-paxillin (brown) and counterstained with haematoxylin. Plots show (D) PGP9.5 content, (E) score of cancer cell proximity to neurons and (F) p-paxillin content in PDAC sections from treated KPC mice. (G) The neural invasion score in the pancreatic tumors of TPAC mice treated with the CCR4 inhibitor vs. control (solvent) substance.

All results in graphs are shown as a mean value ± SEM. For the statistical analyses, we used ordinary one-way ANOVA (D-F), Dunnett’s multiple comparisons test (D-F) and for the distribution, t-test (G), Shapiro-Wilk normality test (all panels). The P-value < 0.05 was considered to have significance. Scale bars: 20 μm.
A. Diagram showing cancer cells treated with 6B345TTQ or vehicle.

B. Bar graphs showing forward migration index, velocity, and distance.

C. Graph showing TPAC 5 months and treatment with 6B345TTQ or DMSO.

D. TPAC mice treated with DMSO or 6B345TTQ.

E. Graph showing PGP9.5 content percentage area.

F. Graph showing p-Paxillin content percentage area.

G. TPAC cells i.v. and orthot. Tx with 1x10^6 cells treated with 6B345TTQ or DMSO.

H. TPAC cells derived allografts treated with DMSO or 6B345TTQ.

I. Graph showing pancreatic tumors percentage area.

J. Graph showing p-Paxillin content percentage area.
Figure 6. Inhibition of Paxillin-Src-Erk signalosome in vitro and in vivo.

(A) Experimental design: SU86.86 cancer cells pretreated with the paxillin phosphorylation inhibitor 6-B345TTQ for 1 h were used for a migration assay with murine DRG neurons. Cells pre-treated with vehicle were used as control. (B) Graphs showing the forward migration index/FMI, speed and distance of cells migrating to DRGs. (C) Scheme of in vivo treatment with 6-B345TTQ. 5-month-old TPAC mice were treated with 1mg/kg of the inhibitor daily for 5 days for a total of 4 weeks. The control group was treated with DMSO. (D) Representative images of pancreatic tumors from TPAC mice treated with 6-B345TTQ and the control group, stained with the neural marker PGP9.5 (pink), cancer cell marker CK-19 (brown), p-paxillin (brown) and counterstained with haematoxilin (blue). The tumor injection bed is marked by the yellow border lines. Graphs show PGP9.5 and CK-19 content (E) and p-paxillin (F) as percentage of positively stained cells stained in the total area. (G) Scheme of treatment in mice allografted with TPAC cancer cells: 1x10^6 cultured primary TPAC cancer cells were orthotopically transplanted into the pancreas of 129xC57Bl6 mice. Three weeks after transplantation, the mice were treated according to the regimen. (H) Representative images of pancreatic tumors of transplanted TPAC mice treated according to the regimen, stained with haematoxylin/eosin (blue/pink), p-paxillin (brown) and counterstained with haematoxilin (blue). (I) Diagrams showing the percentage of tumor area out of the total analysed area of transplanted mice after treatment. (J) Diagrams showing the p-paxillin content as percentage of positively stained cells in relation to the total area.

All results in graphs are shown as a mean value ± SEM. For the statistical analyses we used Mann-Whitney U test (E,F, I,J), t-test (B,E), and for the distribution, Shapiro-Wilk normality test (all panels). The P-value < 0.05 was considered to have significance. Scale bars: 20 μm.