Early pancreatic cancer lesions suppress pain through CXCL12-mediated chemoattraction of Schwann cells

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Pancreatic ductal adenocarcinoma (PDAC) cells (PCC) have an exceptional propensity to metastasize early into intratumoral, chemokine-secreting nerves. However, we hypothesized the opposite process, that precancerous pancreatic cells secrete chemokines that chemoattract Schwann cells (SC) of nerves and thus induce ready-to-use routes of dissemination in early carcinogenesis. Here we show a peculiar role for the chemokine CXCL12 secreted in early PDAC and for its receptors CXCR4/CXCR7 on SC in the initiation of neural invasion in the cancer precursor stage and the resulting delay in the onset of PDAC-associated pain. SC exhibited cancer- or hypoxia-induced CXCR4/CXCR7 expression in vivo and in vitro and migrated toward CXCL12-expressing PCC. Glia-specific depletion of CXCR4/CXCR7 in mice abrogated the chemoattraction of SC to PCC. PDAC mice with pancreas-specific CXCL12 depletion exhibited diminished SC chemoattraction to pancreatic intraepithelial neoplasia and increased abdominal hypersensitivity caused by augmented spinal astroglial and microglial activity. In PDAC patients, reduced CXCR4/CXCR7 expression in nerves correlated with increased pain. Mechanistically, upon CXCL12 exposure, SC down-regulated the expression of several pain-associated targets. Therefore, PDAC-derived CXCL12 seems to induce tumor infiltration by SC during early carcinogenesis and to attenuate pain, possibly resulting in delayed diagnosis in PDAC.

Schwann cells | pancreatic cancer | CXCL12 | CXCR4 | CXCR7

The nervous system has recently been discovered to react to even the earliest stages of carcinogenesis by supplying growing tumors with nerves and with multiple modulations of tumor innervation, which constitute cancer-associated neuropathy (1–3). Pancreatic ductal adenocarcinoma (PDAC) features a pronounced neuropathy with an exceptionally high frequency of tumor cell penetration into nerves, i.e., neural invasion (NI), reaching 100% in retrospective case series (4). The pathogenesis of NI holds major translational relevance, because NI is independently associated with a dismal overall survival, local recurrence, and severe neuropathic pain during the already highly lethal course of PDAC (5, 6). Classically, cancer cells are assumed to penetrate into nerves actively and to use them as paths of dissemination (6, 7). However, we recently reported that peripheral glia cells, i.e., Schwann cells (SC), become activated in PDAC and suppress pain sensation (8). Importantly, they emerge around the premalignant precursor lesions of different human and murine gastrointestinal cancers and correlate to the frequency of NI (2). This observation led us to consider the possibility that tumor-derived chemoattractants such as chemokines may recruit neuro-glial cells very early during carcinogenesis.

The chemokine CXCL12, also known as "stromal-derived factor 1 alpha" (SDF-1α), is a CXC-class chemokine that has been shown to be widely expressed in several well-vascularized mammalian tissues and cancers and is known to regulate homing, proliferation, and survival of bone marrow-derived hematopoietic stem cells and stromal cells via its cognate receptor CXCR4 (9–11). Moreover, CXCR4 was reported to be overexpressed, to influence cancer cell proliferation and metastasis, and to modulate the tumor microenvironment in more than 25 types of human cancers including PDAC, prostate, breast, and ovarian cancer and melanoma (9, 12). Recently, CXCL12 was shown to exert its effect through an alternative receptor, CXCR7 (RDC-1). Intriguingly, CXCR7 is able to heterodimerize with CXCR4 and to fine-tune CXCR4 function (9, 13).

In the present study we hypothesized that the CXCL12/CXCR4/CXCR7 axis may mediate the chemoattraction of SC to cancer cells, thereby initiating NI and its impact on cancer dissemination (Fig. 1). Here we show that PDAC cells (PCC) harbor CXCL12, which attracts SC of peripheral nerves via CXCR4 and CXCR7. In two glia-specific murine CXCR4- and CXCR7-KO models, specific depletion of CXCR4 or CXCR7 abrogated the chemoattraction of SC to tumor cells. Correspondingly, pancreas-specific ablation of CXCL12 signaling in mice that develop PDAC disrupted the chemoattraction of glia cells to PDAC precursor lesions and resulted in increased abdominal mechanosensitivity. Accordingly, PDAC patients with pain exhibit less neural CXCR4- and CXCR7. Together with the multiple effects of CXCL12 on the transcription of pain-associated targets in activated SC, these

Significance

Pancreatic ductal adenocarcinoma cancer (PDAC) cells have an exceptional propensity to invade nerves via pronounced crosstalk between nerves and cancer cells, but the mechanisms of this early neural invasion are yet unknown. By using genetically engineered mouse models, we show that in the precursor stage PDAC induces the generation of ready-to-use nerves for dissemination by secreting the chemokine CXCL12 that attracts glia (Schwann) cells of nerves. This migration of glia cells to cancerous cells at this very early stage intriguingly attenuates cancer-associated pain via downregulation of pain-associated targets in Schwann cells and via suppression of central glia. Hence, malignant transformed cells seem to disguise cancer-associated symptoms (such as pain) actively and thereby delay the early diagnosis of cancer.

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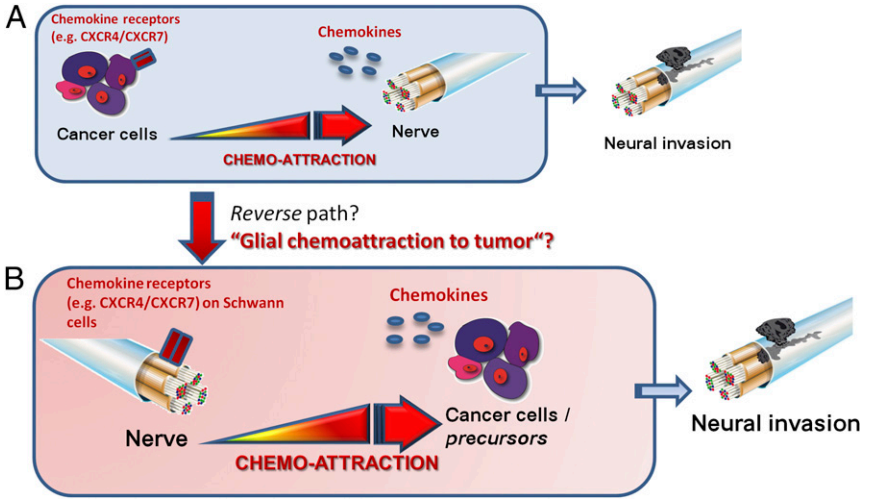

Fig. 1. The reverse pathogenesis of NI in cancer. (*A*) The classical theory of NI in cancer dictates that cancer cells that express chemokine receptors are chemoattracted to nerves that secrete chemokines. Cancer cells then actively invade nerves in the cancer-hosting organ and use the nerves as longitudinal paths for their spread. (*B*) In the present study we propose that a reverse direction of this interaction might also be possible based on the expression profile of nerves and especially SC for chemokine receptors and on the multitude of chemokines that are known to be secreted by cancer cells.

observations collectively suggest a link between chemokine-mediated SC activation, early tumor dissemination, and the suppression of pain sensation in cancer.

Results

SC in Pancreatic Nerves Express CXCR4 and CXCR7, and PCC Are a Source of CXCL12 in PDAC. The emergence of SC around the precursor lesions or cancer cells in PDAC (2) suggests that SC may express chemokine receptors that can respond to the chemokines secreted from cancer cells or their precursors (Fig. 1). Previous studies suggested overexpression of the CXCL12 receptor CXCR4 in PDAC (14, 15). Furthermore, CXCL12 was previously detected on tumor-associated fibroblasts (16) and on FAP^+ fibroblasts (17), and its receptor CXCR4 was detected on pancreatic stellate cells (18) and also on endothelial cells (14). However, no studies have focused on the distribution of CXCL12/CXCR4/CXCR7 in pancreatic nerves. In double-immunolabeling experiments using the SC marker S100, we detected the prominent and highly frequent presence of CXCR4 and CXCR7 not only in immune cells and cancer cells but also in SC of nerves within PDAC tissues (Fig. 2 *A*–*C*). CXCR4 or CXCR7 also was detectable in nerves within in the normal pancreas (NP) (Fig. 2 *D*–*F* and Tables S1–S6). In the corresponding immunolabeling experiments, CXCL12 was prominently up-regulated in the precursor pancreatic intraepithelial neoplasia (PanIN) lesions and PCC compared with NP and colocalized with the PCC marker cytokeratin-19 (CK19) (Fig. 2 *G* and *H*). In NP, CXCL12 was expressed in intrapancreatic ducts (Fig. S1 and Table S3), whereas in PDAC it was detected within the extracellular matrix (ECM), i.e., stroma, and in endothelium as well as in cancer cells (Table S3). Because of this prominent CXCL12 immunoreactivity detected in cancer precursors and cancer cells, the mean immunoreactivity score for CXCL12 in PDAC tissues (3.7 ± 3.4) was remarkably greater than in NP (0.46 ± 0.46) (Fig. 2*I*). We next performed ELISA to quantify the levels CXCL12 in lysates of eight different human PDAC cell lines. All eight PCC lines contained prominent levels of CXCL12 (average CXCL12 content = 207.5 ± 12.2 pg/mL) (Fig. 2*J*). In a comparative analysis of mRNA expression, human PDAC cell lines exhibited CXCL12 expression comparable to that of human Jurkat T cells, and the highest CXCL12 expression was observed in human pancreatic stellate cells (Fig. S1*C*). In cancer cells isolated from murine PDAC models [i.e., KC (*p48*-Cre;LSL-$Kras^{G12D}$) and KPC (*p48*-Cre;LSL-$Kras^{G12D}$; $trp53^{lox/lox}$) mice], the p53-deficient KPC cancer cells tended to have higher CXCL12 expression (Fig. S1*D*). On the other hand, the tissue expression of CXCL12 tended to be higher in 12- to 20-wk-old KC mice that have not yet progressed to overt invasive cancer than in KPC mice with overt cancer (Fig. S1*E*), indicating PanIN lesions as a potential major source of CXCL12.

In accordance with the immunohistochemical detection in human PDAC nerves, both CXCR4 and CXCR7 were detected in cultivated primary human SC (hSC) via immunolabeling (Fig. S2*A*). At the protein level, CXCR4 and CXCR7 were consistently detectable in hSC and PDAC cell lines (Fig. S2*B*). To elucidate the potential regulation of CXCR4 or CXCR7 in hSC upon confrontation with PCC, we next cocultured hSC with PCC and performed immunoblotting to detect the dynamic changes in CXCR4/CXCR7 expression in hSC (Fig. S2*C*). CXCR4 (but not CXCR7) levels were elevated in hSC when they were cocultivated with SU86.86 PCC (CXCR4: 133.1 ± 17.4% of hSC monoculture) (Fig. S2*C*). In analogy, the CXCR4 and CXCR7 content of dorsal root ganglia (DRG) was prominently enhanced in coculture with the PCC line SU86.86 (CXCR4: 181.5 ± 71.9% of DRG monoculture) (Fig. S2*C*), and a similar tendency was seen in the T3M4 PCC line (329.0 ± 444.6% of DRG monoculture).

Because of the intrinsic hypoxic trait of PDAC (19), we next investigated whether CXCR4 and CXCR7 levels in hSC are influenced by hypoxia. At the protein level, hypoxic hSC exhibited an additional, larger isoform of CXCR7 in addition to the native 42-kDa isoform (Fig. S2*D*). Accordingly, hypoxia led to the up-regulation of CXCR7 mRNA especially at 2 h (1,618 ± 1,489% of levels in normoxic hSC) and 12 h (629.3 ± 323%) of hypoxia exposure (Fig. 2*D*), and a similar tendency was seen CXCR4 expression (Fig. S2*D*). Hypoxia did not affect CXCL12 expression in hSC (Fig. S2*D*), but in both SU86.86 and T3M4 cells 6 h of hypoxia resulted in at least fivefold up-regulation of CXCL12 expression (SU86.86: 505.1 ± 310.3%; T3M4: 882.8 ± 1,180% of normoxic levels) (Fig. S2*D*). Accordingly, treatment of hSC in Boyden chambers containing the conditioned medium of hypoxia-treated PCC enhanced hSC transmigration (Fig. S2*D*, *Right*).

CXCL12/CXCR4/CXCR7 Signaling Mediates Chemoattraction of SC to Cancer Cells. To dissect the potential chemoattracting role of PCC-derived CXCL12 on hSC, we first incubated hSC in Boyden chambers containing increasing amounts of CXCL12 in the lower compartment (Fig. S2*E*). We observed a dose-dependent increase

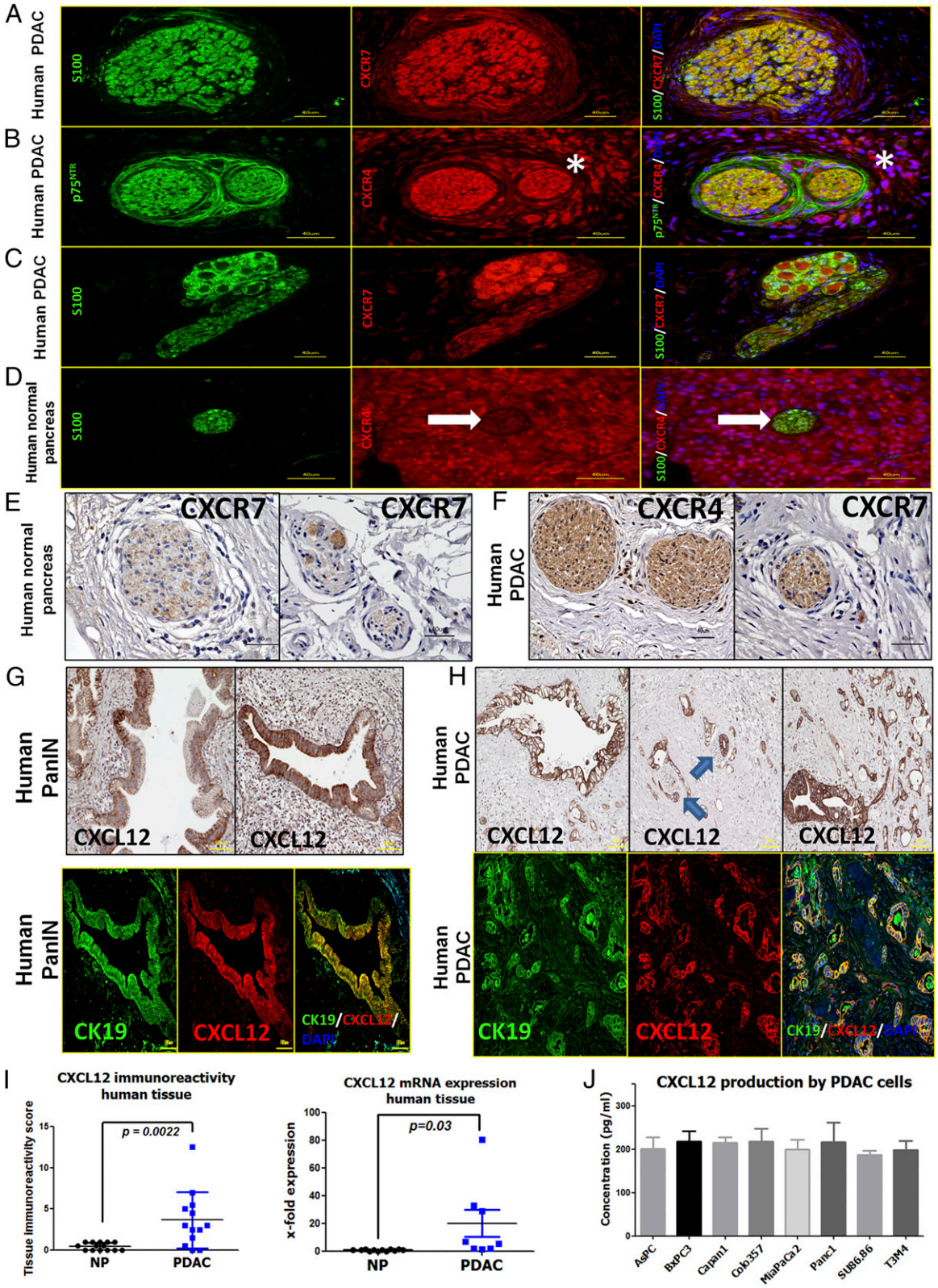

Fig. 2. SC in human PDAC harbor the chemokine receptors CXCR4 and CXCR7, whereas PanIN lesions and established human PCC lines contain CXCL12. (*A*–*C*) Coimmunolabeling of nerves in PDAC tissues with the SC markers S100/p75NTR demonstrates the presence of CXCR4 and CXCR7 in nerves (*A* and *B*) and also in intrapancreatic ganglia (*C*). The asterisks indicate the immunostained perineural inflammatory cells. (*D* and *E*) In normal human pancreas the chemokine receptors are only faintly detectable in SC. The white arrow points to the nerve embedded in the exocrine tissue. (*F*) However, several nerves in PDAC tissues exhibited immunoreactivity for CXCR4 and CXCR7. (*G* and *H*) Immunolabeling of PDAC tissues against CXCL12 and colabeling with the PCC marker CK19 revealed PanIN lesions (*G*) and PCC (*H*) as the major source of this chemokine in PDAC. The blue arrows point to nerves that are invaded by CXCL12-expressing cancer cells. (*I*) Accordingly, the mean immunoreactivity of PDAC tissues for CXCL12 (*Left*) and the mean tissue CXCL12 mRNA expression (*Right*) was prominently greater in PDAC than in NP. For the mRNA expression, the levels of NP tissues were normalized to 1 (Mann–Whitney *u* test). (*J*) Human PCC lines synthesize substantial amounts of CXCL12 as detected via ELISA. Experiments were repeated three times. (Scale bars: *A*–*F*, 40 μm; *G*–*H*, 100 μm.)

in the amount of transmigrating hSC (10 ng/mL: 156.4 ± 86.4%; 50 ng/mL: 160.4 ± 66.4%; 100 ng/mL: 193.8 ± 110.5% of 0 ng/mL control hSC) (Fig. S2*E*). We next placed three different human PCC lines (T3M4, MiaPaCa-2, and Panc1) into the lower chamber of the Transwell migration assays and pretreated the hSC in the upper chamber with either the CXCR4 small-molecule inhibitor AMD3100 or with a CXCR7-blocking antibody. Blockade of CXCR4 did not affect hSC transmigration toward the PCC lines (Fig. S2*E*); however, when a CXCR7-neutralizing antibody (9C4) was added to the hSC, there was a significant dose-dependent attenuation of hSC transmigration toward PCC (toward T3M4 at 30 μg/mL: 56.1 ± 15.4% of control; toward MiaPaCa-2 at 30 μg/mL: 58.4 ± 29.1% of control; toward Panc1 at 30 μg/mL: 71.5 ± 21.8% of control) (Fig. S2*E*).

To test this impact of CXCL12 on hSC migration toward PCC further, we used a more complex 3D migration assay in which the respective cell types were resuspended in ECM to simulate the in vivo situation more closely. Here, PCC were simultaneously confronted with control hSC (pretreated with the solvent for AMD3100 or with a nonimmunized mouse IgG_1 antibody) and with hSC pretreated with inhibitor (either AMD3100 for CXCR4 or with anti-CXCR7 antibody), and the cancer-directed migration of hSC was tracked by digital time-lapse microscopy (Fig. 3*A*). In this setting, hSC that were pretreated with AMD3100 or with anti-CXCR7 exhibited a much less targeted, shorter, and slower migration toward T3M4 or SU86.86 PDAC cells than control hSC [forward migration index (FMI) of hSC+AMD3100: 0.20 ± 0.39 vs. control hSC: 0.31 ± 0.38; FMI of hSC+anti-CXCR7: 0.15 ± 0.38 vs. control hSC: 0.35 ± 0.30) (Fig. 3 *B* and *C*). In additional 3D migration assays we also evaluated whether inhibition of CXCR4 or CXCR7 on PCCs influenced their targeted migration toward DRG. Inhibition of CXCR4 or CXCR7 on PCC did not inhibit the migration of these cells to DRG (Fig. 3*D*). In fact, inhibition of CXCR4 via AMD3100 even augmented the T3M4 cancer cell migration toward DRG (Fig. 3*D*). Overall, these observations suggest that CXCR4 and CXCR7 are mediators of hSC migration toward PCC, whereas CXCR4 seems to exert this effect in an ECM-containing environment.

Theoretically, for SC migration to occur in PDAC, SC would need to grow out of the damaged intrapancreatic nerves toward PCC. Therefore in the current study, dissociated, SC-containing DRG of genetically engineered mice were confronted with PCC in the 3D migration assay (Fig. 4 *A* and *B*). After induction of the Cre recombinase with 4-hydroxytamoxifen (4-OHT) in the growth medium, SC from the DRG of GCE;$CXCR4^{lox/lox}$ and GCE;$CXCR7^{lox/lox}$ (KO DRG) mice (Fig. 4*B*) did not exhibit the same extent of targeted migration to PCC as those from $CXCR4^{lox/lox}$ and $CXCR7^{lox/lox}$ mice with no Cre recombinase (nonrecombined; NR DRG) (Fig. 4 *B* and *C*) or SC from KO DRG mice in the absence of 4-OHT (Fig. 4*C* and Table S7). Overall, these multiple models provided evidence for the CXCR4- and CXCR7-mediated specific migration of SC toward PCC. Mechanistically, upon siRNA-mediated silencing of CXCR4, the expression of CXCR7 was also prominently down-regulated to a mere 7.3% of control hSC. Conversely, when CXCR7 was silenced via siRNA, the expression of CXCR4 was strongly up-regulated to 322% of control levels (Fig. S3 and Table S8). Thus, it seems that CXCR7 expression is coupled to CXCR4 expression in a repressive-feedback mechanism. The absence of CXCR4 seems to obviate the expression of CXCR7, and the absence of CXCR7 seems to release the brake on the CXCR4 expression. This interaction is thus comparable to the previously described fine-tuning of CXCR4 activity (20), because in both cases CXCR7 seems to reduce excessive signaling from CXCL12 to CXCR4. Therefore, genetic CXCR4 depletion seems to decrease SC migration more potently than CXCR7 depletion.

Ablation of Cancer Cell-Derived CXCL12 in PDAC Abrogates the SC Affinity Toward PanIN Lesions. In the next step we investigated the impact of the disruption of CXCL12 signaling on glial activity in vivo in the murine KC model of PDAC (Fig. 5*A*). For this purpose, we generated mice in which CXCL12 secretion by pancreatic cells, including (pre)cancerous cells, was abrogated in a conditional manner (*p48*-Cre;LSL-$Kras^{G12D}$;$CXCL12^{lox/+}$, termed "$KC12^{lox/+}$") (Fig. 5*A*). $KC12^{lox/+}$ mice exhibited distorted lymph node architecture and clusters of altered acinar cells that had a "ballooning" appearance (Fig. 5*B* and Fig. S4). Control *p48*-Cre;$CXCL12^{lox/+}$ mice did not exhibit any obvious histological abnormality, and their pancreata did not differ from normal wild-type pancreas (Fig. S4). The decrease in CXCL12 content of PanIN lesions and the comparability of PanIN degree and stromal activity in KC and $KC12^{lox/+}$ mice was confirmed by immunohistochemistry (Fig. S5). A typical feature of the KC model is the emergence of SC around the PanIN lesions, an observation that is assumed to be the precursor of NI (2). Here, SC can be detected by the expression of different glial markers ($GFAP^+Sox10^+S100^+$-expressing cells) (Fig. 5*C*). In comparison with KC mice, we observed a prominent reduction in the number of SC that surrounded PanIN lesions in $KC12^{lox/+}$ mice (KC: 75.9 ± 10.3% and $KC12^{lox/+}$: 11.9 ± 9.9% for GFAP/S100 coexpression; KC: 78.5 ± 8.7% and $KC12^{lox/+}$: 23.5 ± 11.0% for GFAP/Sox10 coexpression) (Fig. 5*D*). Hence, this model suggested that CXCL12 from the transformed PanIN lesions mediates the emergence of SC around these precursor lesions.

Disruption of the CXCL12-Mediated SC Recruitment Toward PDAC Increases Pain Sensation Through Reactivation of Spinal Glia and Up-Regulation of Multiple Nociceptive Mediators in SC. We next evaluated the possibility that disruption of CXCL12 signaling between PCC and SC may affect the clinical course of PDAC. For this purpose, we assessed the extent of mechanical sensitivity (8) as an indirect measure of potential pain sensation in the abdominal area of KC and $KC12^{lox/+}$ mice (Fig. 6*A*). Intriguingly, $KC12^{lox/+}$ mice had much greater abdominal hypersensitivity scores (8.6 ± 3.4) than KC mice (4.6 ± 2.8), as assessed by means of von Frey filaments. Interestingly, the mechanosensitivity of $KC12^{lox/+}$ mice was comparable to wild-type C57BL6/J mice (von Frey score: 8.4 ± 2.9), implying the suppression of pain in KC mice rather than the promotion of pain in $KC12^{lox/+}$ mice. This observation would suggest that PDAC patients with active CXCL12–CXCR4/CXCR7 signaling in SC would suffer less pain. We therefore analyzed the immunoreactivity of nerves among PDAC patients subgrouped into those with or without pain. In this analysis, we indeed detected a decreased mean neural immunoreactivity for CXCR7 among patients with pain as compared with patients without pain (neural CXCR7: no pain = 0.4 ± 0.5 vs. pain = 0.06 ± 02) (Fig. 6*B*), with a similar tendency for tissue CXCR7 immunoreactivity (tissue CXCR7: no pain = 4.4 ± 4.0 vs. pain = 1.3 ± 1.7) (Fig. 6*B*). The average neural CXCR4 immunoreactivity also appeared weaker among patients with pain than in patients without pain (neural CXCR4: no pain = 0.5 ± 0.9 vs. pain = 0.02 ± 0.004) (Fig. 6*B*). The tissue CXCR4 immunoreactivity did not differ between the no pain and pain groups (tissue CXCR4: no pain = 6.2 ± 3.0 vs. pain = 4.5 ± 2.3) (Fig. 6*B*). We also counted the nuclei that colocalized with S100/GFAP within the intrapancreatic nerves of PDAC patients and compared these counts in patients with or without pain. The average number of SC tended to be lower in intrapancreatic nerves of PDAC patients with pain than of patients without pain (Fig. S6). This observation is in harmony with our previous findings on the decreasing GFAP content of nerves of PDAC patients with pain (8).

To decipher the implications of suppressed pain sensation in KC versus $KC12^{lox/+}$ mice, we analyzed the activity status of the central spinal glia, i.e., astrocytes and microglia, which are well-established actors in chronic and neuropathic pain states (Fig. 6*C*) (8, 21, 22). A comparison of the proportion of activated

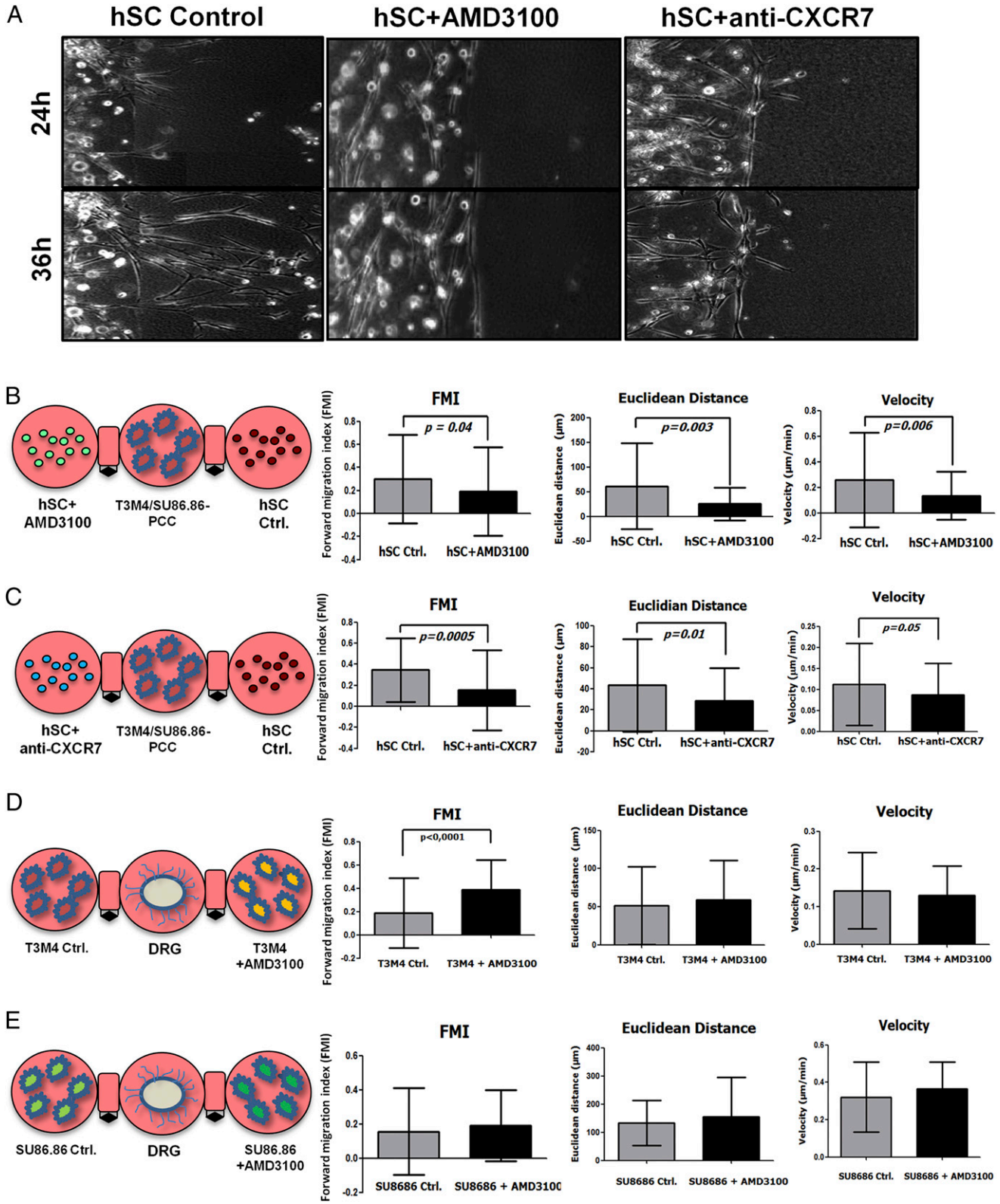

Fig. 3. Impact of CXCR4/CXCR7 on hSC migration to PCC in a 3D migration assay. (*A*–*C*) hSC pretreated with AMD3100 or CXCR7-blocking antibody were resuspended in an ECM drop, placed next to PCC, and recoded via live-cell imaging. Although solvent- or IgG_1-treated (Control) hSC rapidly extend protrusions and migrate toward PCC (which do not possess any affinity to hSC) (*A*), hSC in which CXCR4 or CXCR7 was inhibited did not demonstrate any 3D migration, as evidenced by their prominently reduced FMI (*B* and *C*). (Magnification: *A*, 100×.) (*D* and *E*) In comparison, the pretreatment of the human PCC lines T3M4 or SU86.86 with AMD3100 or CXCR7-blocking antibody did not reduce their migration toward DRG neurons (Mann–Whitney *u* test for the FMI, unpaired *t* test for the remaining parameters). Experiments were repeated three times.

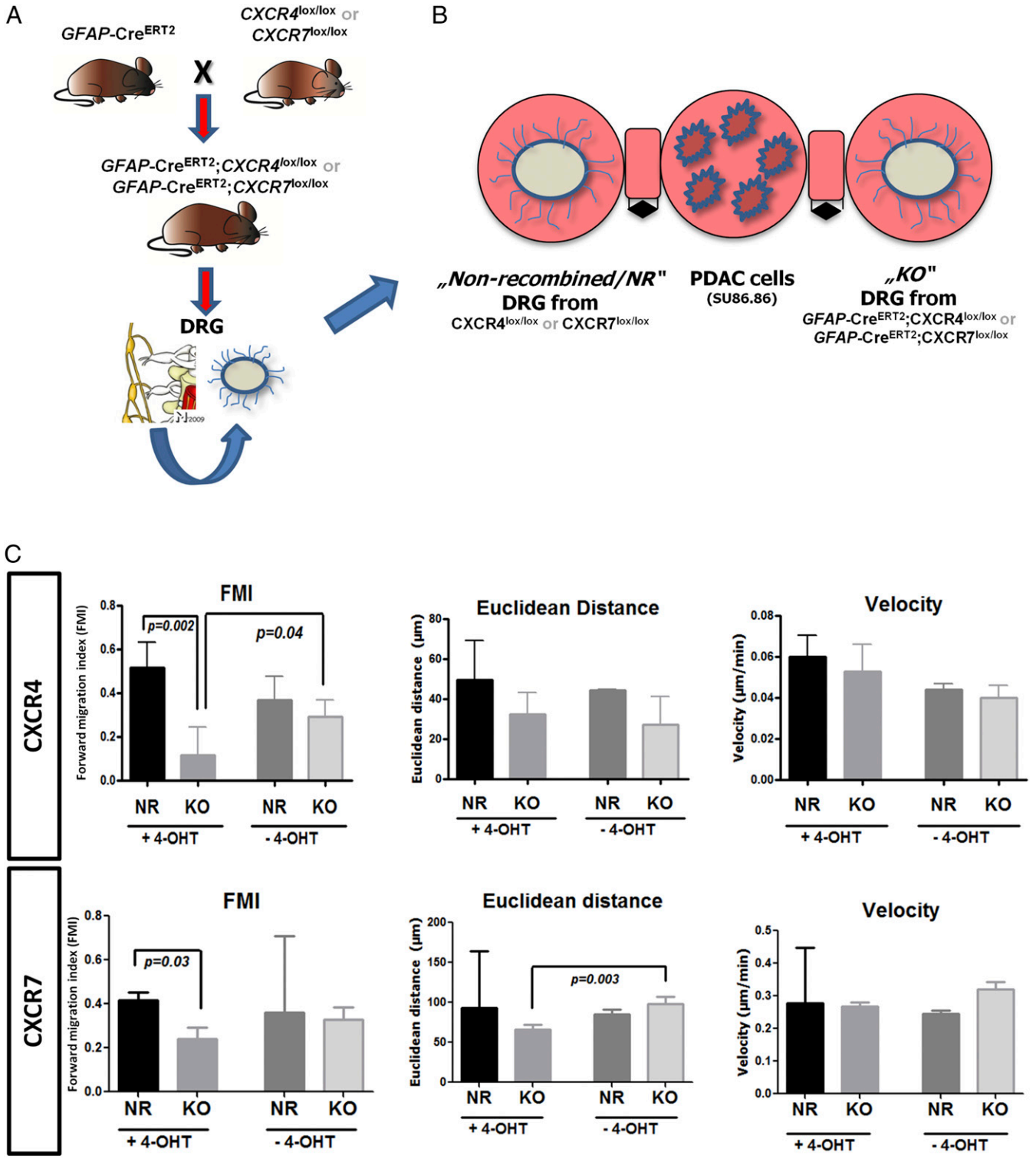

Fig. 4. Glia-specific knockout of CXCR4 or CXCR7 abrogates SC migration to cancer cells. (*A*) *GFAP*-Cre^{ERT2} mice were interbred with *CXCR4*$^{lox/lox}$ or *CXCR7*$^{lox/lox}$ mice to generate *GFAP*-Cre^{ERT2};*CXCR4*$^{lox/lox}$ (n = 3) or *GFAP*-Cre^{ERT2};*CXCR7*$^{lox/lox}$ (n = 3) mice. The DRG of these mice were explanted. (*B*) After 4-OHT supplementation of their medium, SC of dissociated DRG from *GFAP*-Cre^{ERT2};*CXCR4*$^{ox/lox}$ or *GFAP*-Cre^{ERT2};*CXCR7*$^{lox/lox}$ (collectively, KO) mice were compared with SC of dissociated DRG from *CXCR4*$^{ox/lox}$ or *CXCR7*$^{lox/lox}$ (NR) mice for their migration to PDAC cells. (*C*) The addition of 4-OHT significantly impaired the migration of SC from KO animals to human PCC as compared with SC from NR mice; this difference was not seen in the absence of 4-OHT (unpaired t test). Experiments were repeated three times.

astrocytes (coexpressing p75NTR^{+}GFAP^{+}) and microglia (coexpressing p-p38^{+}Iba-1^{+}) in the pancreas-innervating thoracic segments of the spinal cord of KC and KC12$^{lox/+}$ mice revealed a considerably higher proportion of activated astrocytes and microglia in the dorsal, i.e., sensory, part of the spinal cord of KC12$^{lox/+}$ mice (active astrocytes: 69.2 ± 20.5%; active microglia:

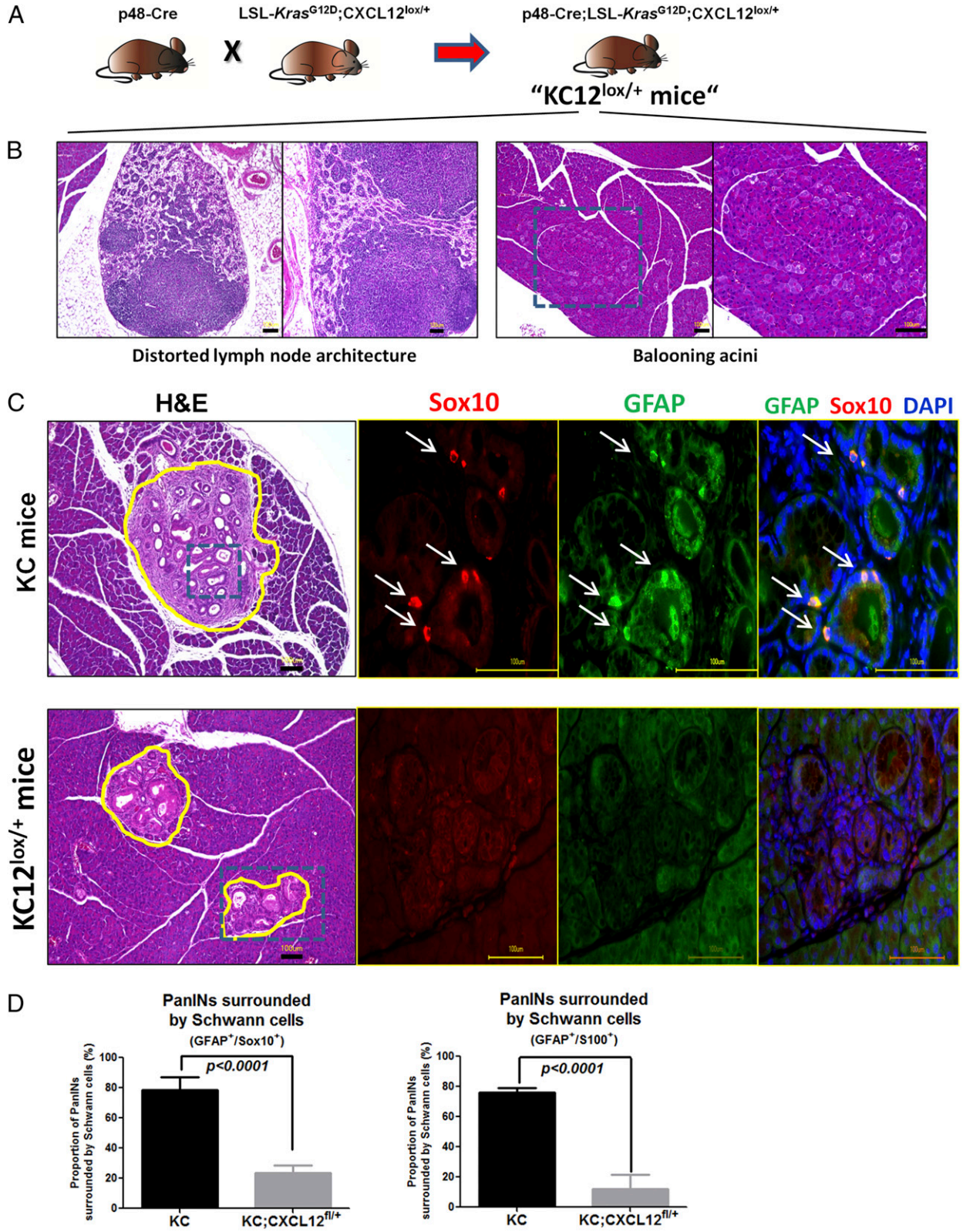

Fig. 5. Pancreas-specific ablation of CXCL12 production disrupts SC accumulation around PanIN/precursor lesions. (*A*) *p48*-Cre mice were interbred with LSL-*Kras*^{G12D};*CXCL12*$^{fl/+}$ mice to generate the KC12$^{lox/+}$ mice with pancreas-specific reduction of the CXCL12 production. (*B*) The KC12$^{lox/+}$ mice exhibited alterations in their lymph nodes, which appeared distorted, and ballooning of pancreatic acini. (*C*) These mice were killed between 12–22 wk of age when KC mice mice typically exhibit PanIN lesions (n = 9 mice). Immunolabeling against different glial markers (here Sox10/GFAP) revealed the presence of glial marker-expressing cells around PanIN lesions. (*D*) Quantification of glia-surrounded PanINs revealed that such cells were widely absent around the PanINs of KC12$^{lox/+}$ mice (n = 5 mice). The number of analyzed PanINs was n = 289 in the KC12$^{lox/+}$ mice and n = 423 in the KC mice (unpaired t test). (Scale bars: 100 μm.)

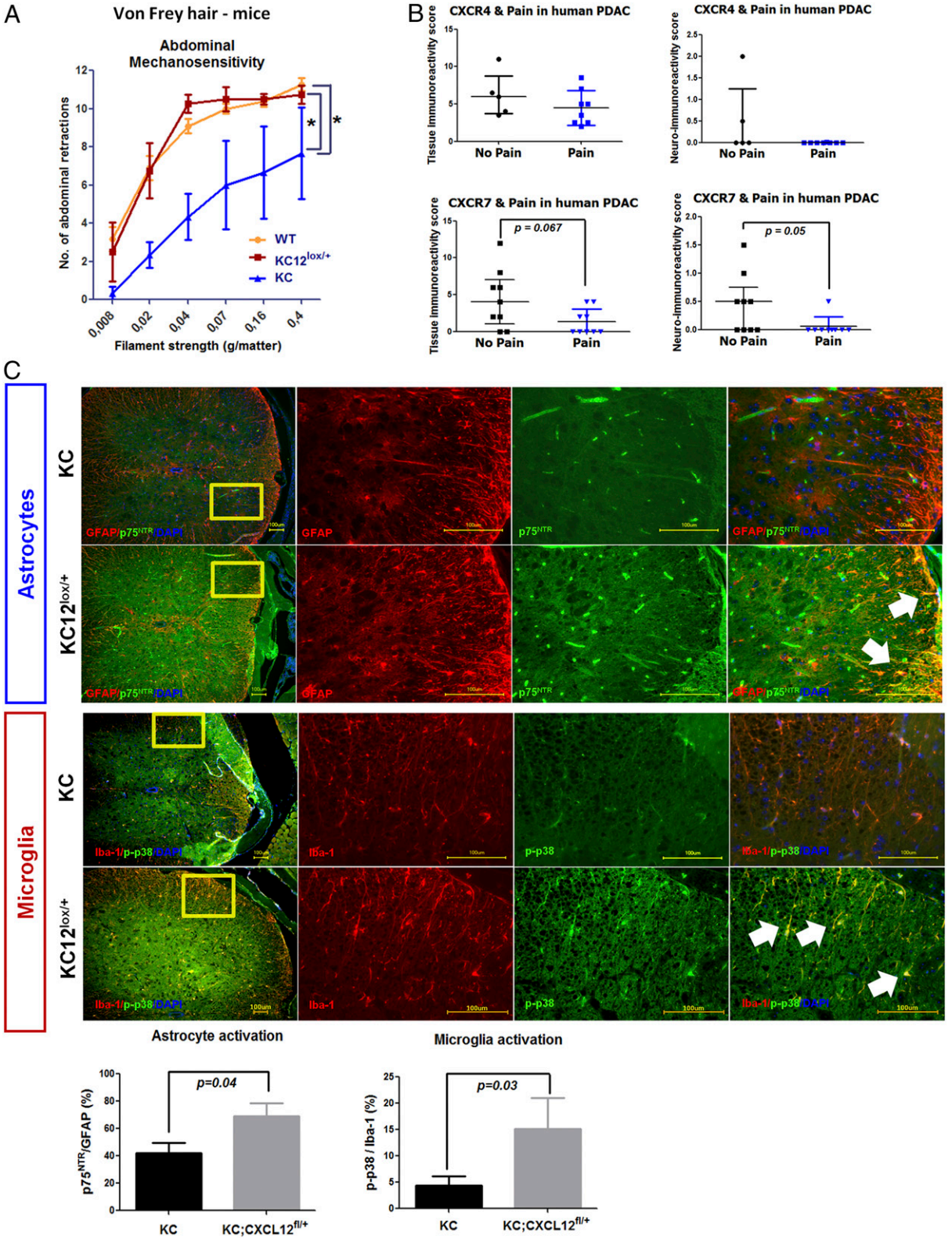

Fig. 6. Active CXCL12 signaling in the pancreas diminishes pain by suppressing spinal glial activity. (*A*) Abdominal mechanosensitivity to von Frey hairs was compared in KC ($n = 3$) and KC12$^{lox/+}$ ($n = 4$) mice. The number of abdominal retractions when the abdomen was touched was quantified and compared with wild-type C57BL6/J mice. The enhanced SC activity in KC mice was associated with the suppression of abdominal retractions and thus less mechanosensitivity (unpaired *t* test). (*B*) The immunoreactivity of tumor tissue and tumor nerves for CXCR4 and CXCR7 was compared in PDAC patients with ($n = 10$) and without pain ($n = 11$). The neural immunoreactivity for CXCR7 was prominently lower in patients with painful disease (unpaired *t* test). (*C*) In the spinal cord of KC and KC12$^{lox/+}$ mice, the proportion of activated dorsal horn astrocytes (p75^{NTR+} proportioned to GFAP^{+} cells) and microglia (p-p38^{+} proportioned to Iba-1^{+} cells) was compared via immunolabeling of the thoracic spinal segments 8–11 (three sections per spinal cord). Diminished CXCL12-signaling was associated with enhanced astrocyte and microglia activity in the dorsal horn (unpaired *t* test). The white arrows point toward double-stained astrocytes or microglia. (Scale bars: 100 μm.)

15.1 ± 13.4%) than in KC mice (active astrocytes: 42.3 ± 7.4%; active microglia: 4.3 ± 1.8%) (Fig. 6*C*). Collectively, these observations suggested that SC recruitment via cancer cell-derived CXCL12 attenuates pain by suppressing spinal glial activity.

Diminished mechanosensitivity or pain resulting from CXCL12-mediated SC chemoattraction implied that CXCL12 also may suppress some pain-associated pathways in SC. To test this possibility, we analyzed the transcriptomic alterations in hSC treated with recombinant CXCL12 with a particular focus on the expression of 84 pain-associated targets. Here, 17 of 84 pain-associated genes were found to be differentially expressed (i.e., at least threefold up-regulated) in hSC after CXCL12 treatment. Among these, 6 of the 17 targets were up-regulated and thus were associated with potential pain-promoting consequences (Fig. S7). However, the larger portion, 11 of 17, were down-regulated and included major pain-associated targets such as the tachykinin receptor 1 (TACR1/NK1R, down-regulated 12.2-fold), the purinergic receptor P2RX3 (down-regulated 9.4-fold), the metabotropic glutamate receptor 1 (GRM1) (down-regulated eightfold), and the calcitonin gene-related peptide alpha (CGRPa/CALCA) (down-regulated 5.1-fold). Hence, these target alterations suggest that chemokine-mediated SC recruitment and the resulting masking of pain may be caused by the diminished transcription of pain-associated pathways in SC.

Discussion

Cancer cells readily exploit intrinsic developmental and defense mechanisms in their microenvironment to boost their growth and spread. In the present study, we show another example of the exploitation of the microenvironment by tumor cells, namely, the exploitation of glial cells, a cell type that had not previously been subject to investigation in this context. We show that PCC can chemoattract SC in vivo and in vitro in a CXCL12-dependent mechanism that seems to result in decreased pain sensation resulting from the suppression of SC-intrinsic molecular pain pathways and of spinal astrocytes and microglia in vivo. These observations add dimensions to the very recent investigations of cancer-associated neuropathy and provide a mechanistic perspective on the very early dissemination of PDAC.

Previous studies showed that CXCL12 and CXCR4 actively participate in nociception via direct stimulation of nociceptive neurons (23), via induction of mechanical hypersensitivity in rats (24), or via desensitization of opioid receptors, but all these effects are in the central nervous system (25). In the present study, we show this chemokine–receptor axis has a role in the activation of peripheral glia, i.e., SC, in the tumor microenvironment, which seems to entail an analgesic effect in the early disease course. Accordingly, we recently demonstrated decreased pain sensation among PDAC patients who bear increased pancreatic gliosis with cellular hypertrophy of pancreatic glia (8). In the light of the previously reported pro-nociceptive roles of CXCR4 and CXCL12, our observations suggest that peripheral and central CXCL12-mediated signaling exert contrasting effects for nociception, i.e., CXCL12 mediating analgesia via modulation of SC activity.

The tumorigenic effects of CXCL12–CXCR4 in PDAC include promotion of PCC invasiveness, migration, proliferation, epithelial–mesenchymal transition, and metastasis via Akt-, Erk-, and sonic hedgehog-dependent pathways (11, 14, 15, 26–31). High levels of tissue CXCL12 and CXCR4 were also shown to be associated with poor survival of PDAC patients (15, 26, 32). These studies thus uncovered the autocrine trophic effects of CXCL12 on cancer cells and disease progression and on the cancer-driven modeling of inflammation via selective chemoattraction of growth-promoting immune cell subtypes to the tumor (33). The present study expands this knowledge of chemokine-driven cancer progression by unraveling the chemoattraction of glia to cancer via CXCL12–CXCR4–CXCR7 as the initiator of nerve–cancer interactions and as a factor that delays the onset of symptoms such as pain.

This pathomechanistic concept for the generation of NI in PDAC turns the classical view of the active invasion of nerves by cancer cells on its head and provides an explanation for the very early dissemination of PDAC (2). As the peripheral glial cells, SC previously were shown to express both CXCR4 and CXCR7 in a cAMP- and TNFα-dependent manner (34, 35). In the current study, we show that this expression is subject to up-regulation by glia–cancer confrontation and especially by hypoxia as encountered in PDAC tissues. Both these receptors are essential for regulating SC survival, migration, and communication (e.g., with DRG neurons) (34, 35). However, beyond SC biology, CXCL12–CXCR4 interactions regulate multiple aspects of glial biology in general, including astrocytic and microglial migration and survival (36), neural recovery after ischemia or damage (23, 37), and neuron–glia communication (23). CXCR4 was further shown to be increasingly expressed on astrocytes after stimulation by interleukin-6 (38), which we recently demonstrated to be a key factor that generates and maintains reactive gliosis in PDAC (8). Therefore, one can assume that all the above attributes of CXCR4 and CXCR7 in glia biology may also influence the extent of nerve–cancer interactions in PDAC.

Specifically, we propose that CXCR4- and CXCR7-expressing SC are chemoattracted to PCC in the cancer precursor stage and, similar to their role in nerve regeneration, can serve as paths of axonal guidance toward cancer cells and physically initiate nerve–cancer cell contact. In a recent study, Pukrop et al. demonstrated a very similar cancer-promoting function for microglia in the central nervous system (39). In brain slice cocultures with breast cancer cells, microglia emerged at cancer infiltration zones and actively migrated toward breast cancer cells before the entrance of breast cancer cells into the brain. Upon establishment of physical contact, microglia "pulled" clusters of cancer cells into the brain and thereby initiated metastasis. Moreover, these transport interactions and brain metastases could be disrupted via the microglia inhibitor clodronate (39). Therefore, it is conceivable that CXCR4- and CXCR7-mediated SC chemoattraction of cancer cells may represent a corresponding example of the misuse of glia by cancer cells in PDAC and may serve as a future therapeutic target.

In conclusion, CXCL12 secretion from PCC can induce SC carcinotropism via both CXCR4 and CXCR7 and contribute to central hypoalgesia in PDAC. Modulating this chemokine–receptor axis thus may turn out to be a double-edged sword, limiting the initiation of NI in PDAC at the cost of potentially increased pain sensation. Although these findings need to be extended by studies in advanced stages of cancer, this mechanistic demonstration of the role of CXCL12–CXCR4–CXCR7 axis in reactive gliosis in PDAC may have implications for understanding NI, pain, and nerve–cancer interactions in several malignancies.

Materials and Methods

All animal experiments were carried out in accordance with the regulations of the governmental commission for animal protection of the Government of Upper Bavaria. All patients provided informed written consent for tissue collection. The study of the collected tissue was approved by the ethics committee of the Technische Universität München (approval no. 1926/07) and the University of Heidelberg (approval no. 301/2001). For details on the experimental methods, please refer to *SI Materials and Methods*.

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