Cancer Research

LIF Drives Neural Remodeling in Pancreatic Cancer and Offers a New Candidate Biomarker

Christian Bressy[1], Sophie Lac[1], Jérémy Nigri[1], Julie Leca[1], Julie Roques[1], Marie-Nöelle Lavaut[1,2], Véronique Secq[1,2], Fabienne Guillaumond[1], Thi-Thien Bui[1], Daniel Pietrasz[3,4], Samuel Granjeaud[1], Jean-Baptiste Bachet[3,4,5], Mehdi Ouaissi[6], Juan Iovanna[1], Sophie Vasseur[1], and Richard Tomasini[1]

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is characterized by extensive stroma and pathogenic modifications to the peripheral nervous system that elevate metastatic capacity. In this study, we show that the IL6-related stem cell–promoting factor LIF supports PDAC-associated neural remodeling (PANR). LIF was overexpressed in tumor tissue compared with healthy pancreas, but its receptors LIFR and gp130 were expressed only in intratumoral nerves. Cancer cells and stromal cells in PDAC tissues both expressed LIF, but only stromal cells could secrete it. Biological investigations showed that LIF promoted the differentiation of glial nerve sheath Schwann cells and induced their migration by activating JAK/STAT3/AKT signaling. LIF also induced neuronal plasticity in dorsal root ganglia neurons by increasing the number of neurites and the soma area. Notably, injection of LIF-blocking antibody into PDAC-bearing mice reduced intratumoral nerve density, supporting a critical role for LIF function in PANR. In serum from human PDAC patients and mouse models of PDAC, we found that LIF titers positively correlated with intratumoral nerve density. Taken together, our findings suggest LIF as a candidate serum biomarker and diagnostic tool and a possible therapeutic target for limiting the impact of PANR in PDAC pathophysiology and metastatic progression.

Significance: This study suggests a target to limit neural remodeling in pancreatic cancer, which contributes to poorer quality of life and heightened metastatic progression in patients. *Cancer Res; 78(4); 909–21. ©2017 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is considered as one of the most serious cancers, with a quick and asymptomatic evolution leading to a very low survival rate in patients (1, 2) Even with recent improvements (3), current treatments, mainly based on surgery and chemotherapies, have a limited impact on the patient's fate, in part due to impaired drug perfusion provoked by the stromal reaction surrounding tumor cells (4, 5). Indeed, PDAC is characterized by the presence of a predominant stroma (intratumoral microenvironment) composed of cancer-associated fibroblasts (CAF), immune, endothelial, and nerve cells. These have all been reported as drastic modifiers of tumor cells' abilities thereby impacting on pancreatic tumor evolution and prognosis (6). However, recent advances based on the effects of the stromal compartment on PDAC are limited, and their clinical translation remains difficult (7).

In addition to evidence showing the major implication of the stroma in PDAC evolution and in therapeutic resistance, several studies have highlighted profound alterations of the neural compartment and its concrete impact on patient's fate and quality of life (8, 9). These alterations, called PDAC-associated neural remodeling (PANR), result in higher nerve densities in PDAC due to peripheral nerve fibers' infiltration and axonogenesis (10, 11). Recently, we highlighted, in a previous study, that the intratumoral microenvironment could be a cause of those profound alterations (12). Thus, deciphering the specific connection between stromal compartment and nerve system in PDAC could uncover potential therapeutic targets and clinical tools that would limit the nervous system–related impact on PDAC evolution that alter patients' fate (13, 14).

Indeed, a direct consequence of this neural remodeling in PDAC is the appearance of perineural invasion (PNI) events, marked by the cancer cell's capacity to invade pancreatic nerves present within the tumor (15, 16). In PDAC, PNI is considered as an indicator of an aggressive tumor associated with local recurrence and metastasis and acute neuropathic pain leading to bad prognosis (8, 17, 18). Interestingly, recent reports have highlighted the role of the intratumoral microenvironment (19, 20) and inflammatory processes (21) as proinflammatory cytokines like IL6 (22), in PANR. Despite this, molecular mechanisms allowing neural remodeling and PNI events in PDAC remain poorly understood. Thus, in-depth molecular studies are required to improve our knowledge in this field, which could provide new therapeutic opportunities to impair PDAC progression and

[1]CRCM, INSERM, U1068; Paoli-Calmettes Institute; Aix-Marseille University, UM 105; CNRS, UMR7258, Marseille, France. [2]Department of Pathology, Hospital North and Mediterranean University, Marseille, France. [3]INSERM UMRS 775, University PARIS DESCARTES, Paris, France. [4]Department of Hepatobiliary and Digestive Surgery, Groupe Hospitalier Pitié Salpêtrière, Paris, France. [5]Department of Hepatogastroentérology, Groupe Hospitalier Pitié Salpêtrière, Paris, France. [6]Aix-Marseille University, INSERM, CRO2, UMR 911, Marseille, France.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Richard Tomasini, CRCM, Avenue de Luminy, case 915, cedex 9, Marseille 13288, France. Phone: 3349-182-8815; Fax: 334-9182-6083; E-mail: richard.tomasini@inserm.fr

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associated symptoms that limit patients' access to chemotherapy (23, 24) and negatively influence their outcome.

Here we demonstrate, in human and mice, that LIF has a direct role on PDAC-associated neural remodeling. We observed that, within PDAC, stromal cells, mainly macrophages and fibroblasts, have the ability to secrete LIF acting then on pancreatic neural compartment.

Indeed, LIF can induce migration and differentiation of Schwann cells and neural plasticity of dorsal root ganglia (DRG) neurons through modulation of the JAK/STAT3 intracellular signaling. Using endogenous mice model of PDAC treated with LIF-blocking antibody, we revealed that LIF is important for PANR. In addition, high levels of LIF were detected in sera from humans and mice with PDAC, but not that from healthy individuals or patients with benign pancreatic diseases. Altogether, our data suggest that LIF is a potent biomarker for the diagnosis of PDAC and that the therapeutic targeting of LIF-induced signaling in PDAC could limit PANR and improve patient outcome and quality of life.

Materials and Methods

Cell lines

Human pancreatic cancer cell lines (PANC-1, MIApaCa-2, BxPC-3, Capan-2) and Schwann cells (sNF96.2) as well as murine macrophage (RAW264.7) were obtained from ATCC between 2012 and 2014 and cultivated in DMEM supplemented with 10% FBS (Life Technologies) and 1% of antibiotic/antimycotic (Invitrogen, 15240-062). Human mast cells (HMC-1) were provided by Professor Michel Arock (ENS Cachan, France) in 2015 and cultivated in RPMI1640 medium, supplemented with 10% FBS and 1% of antibiotic/antimycotic. Cells were authenticated through STR profile report (LGC Standard) in 2016 and tested mycoplasma free (Lonza, LT07-318). Human primary fibroblasts (FHN) were a kind gift from Dr. Cedric Gaggioli (IRCAN, Nice, France) in 2016, and CAF cells (produced from freshly resected human PDAC; ref. 25) were cultivated in DMEM and DMEM/F12 medium, respectively. PDAC-1–4 are human pancreatic primary cancer cells derived from freshly resected PDAC samples (26). All patients gave their consent and are included in the clinical trial number 2011-A01439-32 (26). Expert clinical centers collaborated on this project after approval from their respective ethics review board (approval number 11-61).

Human samples

Chronic pancreatitis (31 samples), pancreatic benign tumor (11 samples), or PDAC (142 samples) sera or tissues used for ELISA assay, immunostaining, or immunoblots were collected in patients from Hôpital Nord and La Timone, Marseille, France and also from Hôpital La Pitié-Salpêtrière, Paris, France. In addition, 61 healthy donors' samples were amassed from Hôpital La Timone, Marseille, and from Etablissement Français du Sang (EFS), Marseille, France. All patients were recruited to participate in a translational research study of blood samples. They accepted and signed an informed consent that had been approved by the local ethics committee (agreement reference of CRO2 for tissue collection: DC-2013-1857). Concerning PDAC patients that underwent surgical resections, PDAC specimens were routinely fixed in 10% formalin, embedded in paraffin and further cut into 5-mm sections immediately stored at 4°C or stained with hematoxylin–phloxine–saffron (HPS). All tissues were collected via standardized operative procedures approved by the Institutional Ethical Board and in accordance with the Declaration of Helsinki. Informed consent was obtained for all tissue samples linked with clinical data.

Murine serum and samples

Sera from healthy and PDAC-bearing mice were obtained after intracardiac puncture and separation between plasma and blood cells by centrifugation. Mice developing PDAC were euthanized when they were moribund (average of 8.5 weeks old). To obtain sera from chronic pancreatitis in mice, caerulein (50 μg/kg/100 μL) was injected twice a week for 10 weeks intraperitoneally (i.p.). Sera from acute pancreatitis were obtained after 6 successive hourly injections intraperitoneally. All animal care and experimental procedures were performed following approval by the Animal Ethics Committee of Marseille.

Statistical analysis

The results showed are averages or medians, and error bars in graphs represent SDs. The Mann–Whitney test, recommended for the comparison of two independent groups, was performed when required. The Wilcoxon test was used, when required, to analyze two different parameters within an experimental group. Differences were considered significant if P was less than 0.05. All P values were calculated using the GraphPad Prism software. All experiments were repeated at least 3 times. LIF and IL6 concentrations were transformed using a lin-log function (namely arcsinus-hyperbolic) and scaled to cover the same range. Combining markers was carried out by the estimation of a logistic model. The rms package allowed logistic regression and models' comparison.

Results

Members of the *GP130* "ligand/receptor" family are overexpressed in human and murine PDAC

We previously showed that the stromal compartment can, through its secretory ability, impact nerve system reorganization within PDAC tumors (12). Regarding recent studies revealing a role of inflammatory processes in PANR (11, 22), we hypothesized that some genes/pathways, involved in the regulation of inflammatory processes, may be upregulated in the stromal compartment of pancreatic cancer and could impact PANR. Using two sets of RNA microarray analysis previously published by our group [Gene Expression Omnibus (GEO): GSE50570 for human PDAC (12) and GSE61412 for mouse PDAC, (27)], we revealed that numerous members of the *GP130* "ligand/receptor" family, with some already associated with neuropathic disorders or regulation of the nervous system (Table 1, column c), were upregulated in the PDAC stromal compartment (Table 1, column d). Interestingly, we found that LIF (leukemia inhibitory factor) was overexpressed both in stromal compartment from human PDAC (Table 1, column d) and at late stage in spontaneous pancreatic cancer mouse model (pdx1-cre/$Kras^{G12D}$/$Ink4A^{fl/fl}$) (Table 1, column g).

While the role of the LIF–GP130 pathway is well defined within nervous system regulation and inflammation (28–30), its implication in pancreatic tumorigenesis is poorly understood (31). We first analyzed the expression of LIF in human PDAC samples and revealed that LIF expression was increased in PDAC samples in contrast to its almost complete absence in healthy pancreas (Fig. 1A–C). Interestingly, regarding the hypothetical role of LIF in neural remodeling, we observed that nerve fibers within human

Table 1. Identification of *GP130* family genes in human and murine pancreatic tumors

Gene name[a]	Symbol[b]	Neural association (+/-)[c]	Human[d]	Mice-4w[e]	Mice-6w[f]	Mice-9w[g]
Interleukin 27 receptor, alpha	IL27RA	–	1.79	2.03	1.38	1.25
Leukemia inhibitor factor receptor	LIFR	+	1.76	−1.44	−1.07	−2.85
Oncostatin-M	OSM	+	1.69	1.26	1.22	2.17
Interleukin 6 signal transducer gp1	IL6ST	+	1.68	1.35	1.24	1.51
Leukemia inhibitor factor	LIFR	+	1.64	1.45	1.26	3.08
Interleukin 11	IL11	+	1.44	1.22	1.12	2.65
Cardiotrophin-like cytokine	CLC	+	1.34	1.55	1.57	5.27
Interleukin 11 receptor, alpha	IL11Ra	+	1.2	−1.09	1.02	−1.53
PRKR interacting protein 1	PRKRIP1	+	1.1	−1.03	1.01	1.22
Oncostatin-M receptor	OSMR	+	1.06	2.25	2.26	7.07
Interleukin 6 receptor	IL6R	+	1.06	−1.04	1.17	−1.23
Ciliary neurotrophic factor receptor	CNTF	+	−1.03	1.15	1.00	1.04
Interleukin 27	IL27	–	−1.75	−1.01	−1.15	−1.33

NOTE: mRNA fold change of *GP130* family genes in transcriptomic analysis of human ($n = 4$) and mouse ($n = 9$) pancreatic samples. Values highlighted in green and red are significantly upregulated and downregulated, respectively.
[a]Gene name.
[b]Gene symbol.
[c]Genes associated with nervous system.
[d]Fold change of mRNA level in stromal versus tumor cell compartment from human PDAC samples.
[e–g] Fold change of mRNA level in spontaneous PDAC versus healthy pancreas from mouse samples.
[e]Four-week-old mice (early mPanINs).
[f]Six-week-old mice (intermediate stage).
[g]Nine-week-old mice (late PDAC).

PDAC samples commonly expressed the two LIF receptors, LIFR and GP130 (Fig. 1A and D). Indeed, LIFR proteins were present in 8 out of 12 nerves analyzed within PDAC tumors with a mean expression of 9.7% inside nerves. In addition, GP130 was found in 4 of 12 nerves but with a stronger mean expression (20%). These changes in LIF expression were confirmed in PDAC mouse model with a strong increase in *LIF* mRNA (Fig. 1E) and protein (Fig. 1F and G) level in PDAC samples compared with healthy pancreas. Altogether, these data reveal the presence of LIF and its receptors, LIFR and GP130, in PDAC samples. Moreover, the expression patterns of LIFR and GP130 support the hypothesis of LIF implication in PDAC-associated neural remodeling.

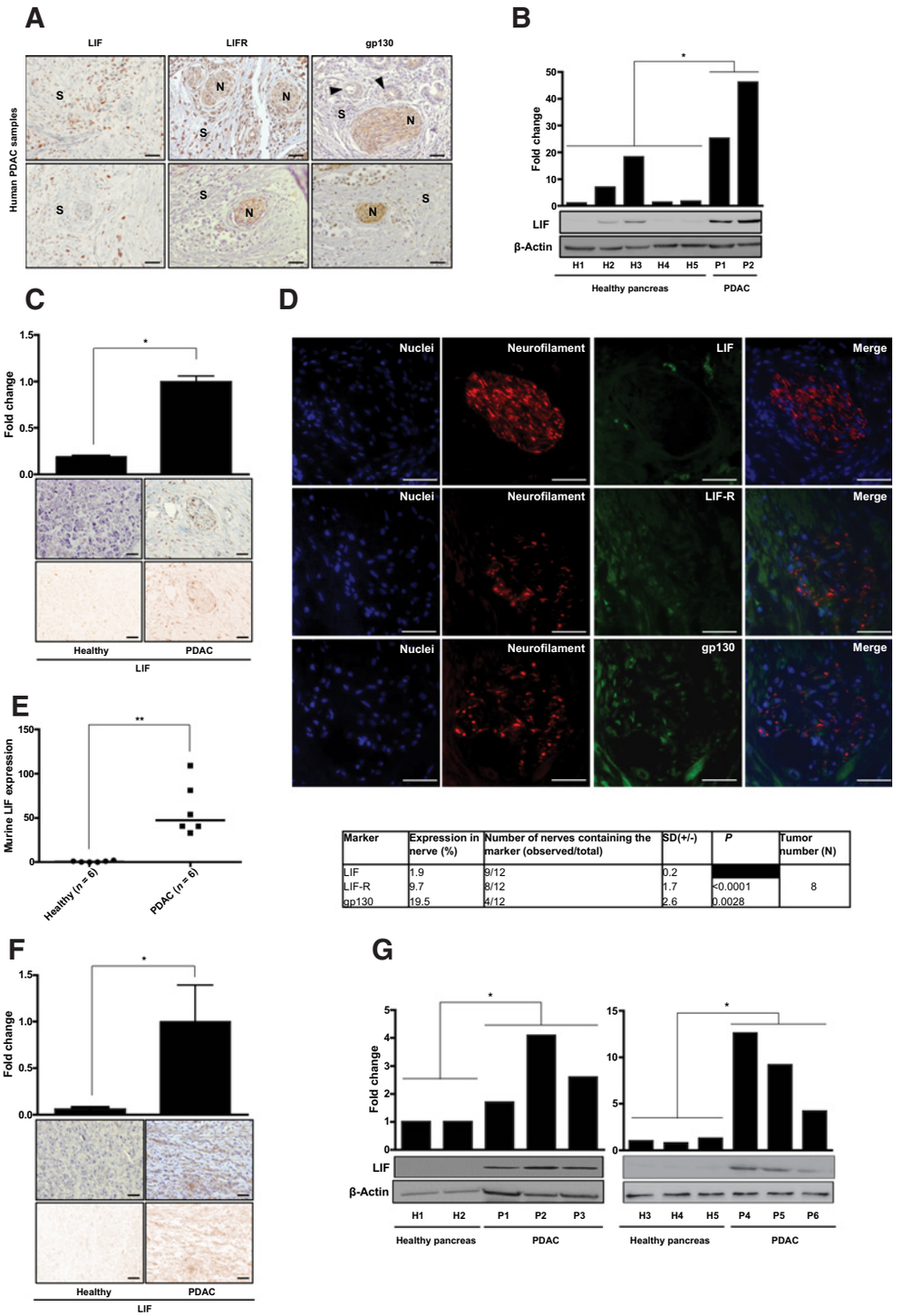
In PDAC, secretion of LIF is mediated by the stromal compartment *in vitro* and *in vivo*

Regarding above data, we next sought to determine which cell types within PDAC produced LIF. Using tissue microarray of various human PDAC samples, we observed that few epithelial cancer cells (cytokeratin-19) expressed LIF, whereas higher percentages of macrophages (CD68), CAFs (αSMA), and mast cells (CD117) were labeled with LIF staining (Fig. 2A).Such analysis on 8 different human PDACs showed that only 6% of cancer cells expressed LIF, whereas mast cells, macrophages, and CAFs expressed LIF at 21%, 34%, and 47.5%, respectively. This was confirmed by measurement of *LIF* mRNA expression *in vitro*, where fibroblasts (FHN) expressed higher amount of *LIF* mRNA than macrophages (RAW) or mast cells (HMC-1; Fig. 2B). Interestingly, when cocultured with RAW or HMC-1 or RAW+HMC-1, the fibroblasts showed an increased *LIF* mRNA expression (Fig. 2B). This level of *LIF* mRNA expression is similar with the one observed in primary CAFs from PDAC patients (Supplementary Fig. S1). These data suggest, as shown previously (11), that FHN cocultivated with RAW reach a similar threshold of activation than primary CAFs.

Comparison of *LIF* mRNA levels in fibroblasts cocultured with macrophages versus various established (PANC-1 and MIA-PaCa-2) or primary PDAC tumor cell lines (PDAC#1–4) revealed that stromal cells express the highest amount of *LIF* mRNA (Fig. 2C). Surprisingly, the increased level of *LIF* mRNA in PDAC cocultured stromal cells did not result in higher LIF protein levels compared with single stromal or tumoral cell cultures (Fig. 2D and E). Considering this discrepancy between mRNA production and intracellular protein levels, we hypothesized that there was a change either in translation machinery, in LIF degradation, or in LIF secretion. As LIF is referenced as a secreted cytokine, we measured by ELISA the amount of LIF secreted in media, and observed a higher LIF concentration in media from fibroblasts cocultured with macrophages compared with other stromal cell cultures (Fig. 2F; Supplementary Fig. S2A). Interestingly, whereas amount of LIF secreted by CAF was higher than by FHN, we observed that amounts of LIF secreted were not different between CAF and FHN when cocultured with RAW. Importantly, LIF was either undetectable or present in small amount in media from various tumor cells (Fig. 2G). Altogether, these data revealed that while numerous cell types within PDAC potentially express *LIF*, the ability to secrete it seems restricted to the stromal compartment and in particular mostly to activated fibroblasts, a major cell component of PDAC microenvironment that we have recently linked to PANR (12).

LIF enhances the migratory capacity of peripheral nerve Schwann cells

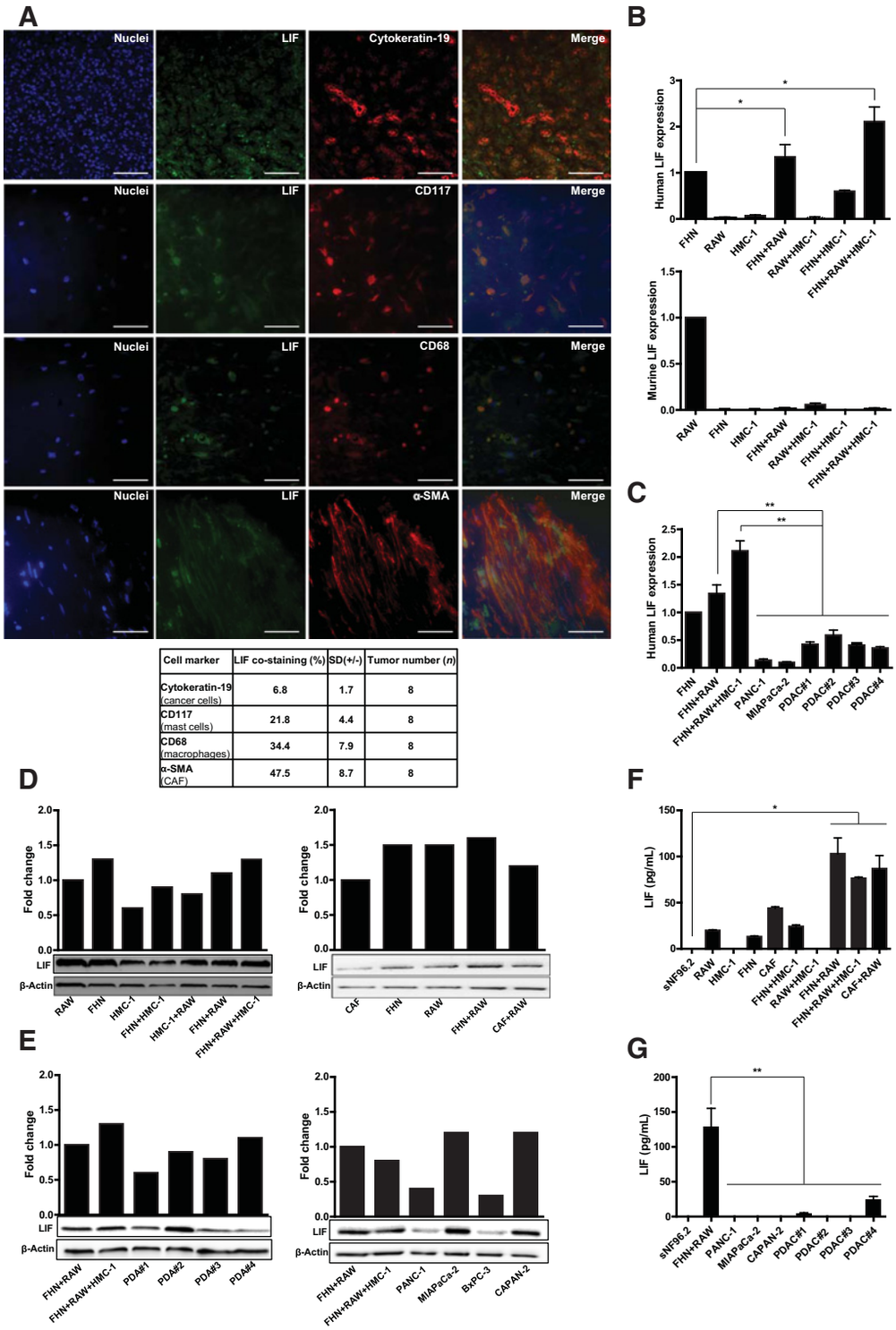
We sought to determine whether the presence of secreted LIF in PDAC could modulate nerve cells' abilities, and therefore have an impact on PANR. We investigated the effect of stromal conditioned media (CM) with the highest LIF titer (FHN+RAW and FHN+RAW+HMC1) on the migratory ability of peripheral nerve Schwann cells and we observed a 2-fold increase of Schwann cell migration after 4 hours (Fig. 3A; Supplementary Fig. S2B). To assess whether LIF from those stromal CM was the mediator of the observed migratory improvement, we blocked LIFR and consequently impaired LIF signaling with a blocking LIFR antibody (Ab-LIFR) and observed that migration ability is restored to control level (Fig. 3B, top). Similar results were obtained using CM from CAF + macrophages and inhibition of LIFR compared with the use of a control antibody (Fig. 3B, bottom). The use of



Marker	Expression in nerve (%)	Number of nerves containing the marker (observed/total)	SD(+/-)	*P*	Tumor number (N)
LIF	1.9	9/12	0.2		
LIF-R	9.7	8/12	1.7	<0.0001	8
gp130	19.5	4/12	2.6	0.0028	

Figure 1.

LIF, LIFR, and GP130 expression levels in human and murine PDAC. **A,** Representative images of LIF, LIFR, or GP130 staining on human PDAC (scale bars, 50 μm). N, nerve sections; S, stroma; black arrowhead, tumoral cells. **B,** LIF immunoblot in human healthy pancreas (H, $n = 5$) or PDAC (P, $n = 2$). Quantifications noted are expressed as fold increase compared with H1. **C,** Representative images following color deconvolution of LIF staining in human healthy pancreas ($n = 6$) or PDAC ($n = 6$; scale bars, 50 μm), with corresponding quantifications done on 10 images per tissues (mean ± SD). **D,** Representative images of colocalization of LIF, LIFR, or GP130 with neurofilament on human PDAC (scale bars, 50 μm, $n = 8$ tumors). The table indicates the percentage of expression of these markers in nerves, and the fraction of nerves containing them. SD (+/−); *P* values compared LIF-R or GP130 expression in nerves compared with LIF. **E,** Fold change of *LIF* mRNA expression level in mouse healthy pancreas ($n = 6$) or PDAC ($n = 6$); each dot is representative from one mouse. **F,** Representative images following color deconvolution of LIF staining in mouse healthy pancreas ($n = 6$) or PDAC ($n = 6$; scale bars, 100 μm), with corresponding quantifications done on 10 images per tissues (mean ± SD). **G,** LIF immunoblot in mouse healthy pancreas (H, $n = 5$) or PDAC (P, $n = 6$). Quantifications noted are expressed as fold changes compared with H1. Each experiment was reproduced at least three times. *, $P < 0.05$; **, $P < 0.01$.



Cell marker	LIF co-staining (%)	SD(+/-)	Tumor number (*n*)
Cytokeratin-19 (cancer cells)	6.8	1.7	8
CD117 (mast cells)	21.8	4.4	8
CD68 (macrophages)	34.4	7.9	8
α-SMA (CAF)	47.5	8.7	8

Figure 2.

In PDAC, LIF secretion is driven by stromal compartment and mainly by CAFs. **A,** Colocalization of LIF with cytokeratin-19, CD117, CD68, or α—SMA on human PDAC sections (scale bars, 50 μm). The table indicates the percentage of LIF colocalization with these markers on human PDAC ($n = 8$). **B,** Human (top) and mouse (bottom) *LIF* mRNA expression levels (mean ± SD). **C,** Human *LIF* mRNA expression levels (mean ± SD). PDAC#1 to 4 represents human PDAC primary tumor cells. **D,** LIF immunoblots. Quantifications noted are expressed as fold changes compared with macrophages (Raw) or fibroblasts (CAF). **E,** LIF immunoblots. Quantifications noted are expressed as fold changes compared with fibroblasts cocultivated with macrophages (FHN+RAW). **F,** Quantification of secreted LIF, by ELISA assay, in various stromal cells conditioned media (mean ± SD). This experiment was reproduced four times, using four different CAFs. **G,** Quantification of secreted LIF, by ELISA assay, in conditioned media from stromal and tumor cells (mean ± SD). Each experiment was reproduced at least three times. *, $P < 0.05$; **, $P < 0.01$.

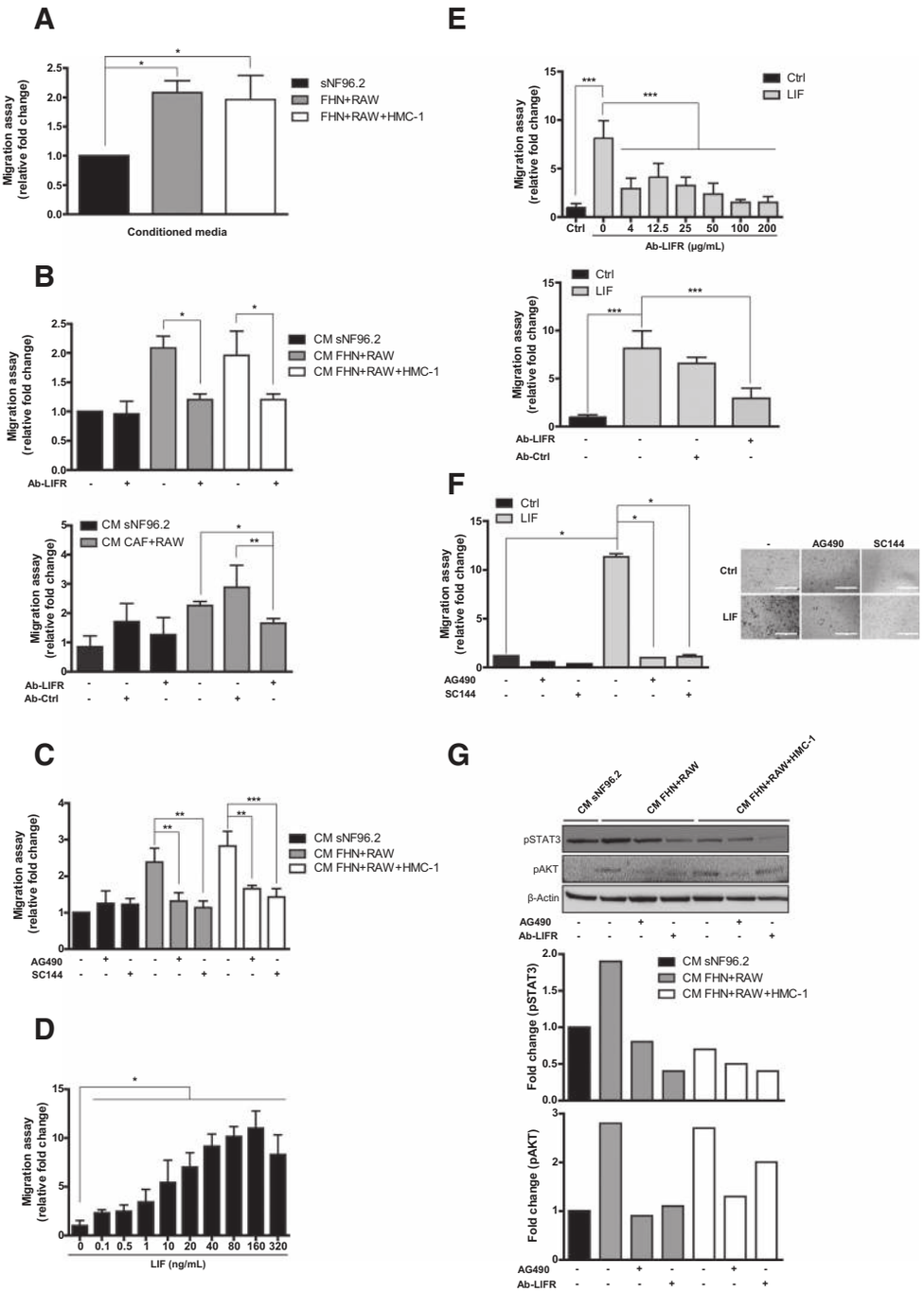


Figure 3.

LIF-triggered signaling enhances migratory capacities of Schwann nerve cells. **A–C,** Effects of stromal cells conditioned media on sNF96.2 migration ability **(A**; mean ± SD) using Ab-LIFR/Ab-Ctrl (**B**) or AG490/SC144 (30 μmol/L/2 μmol/L, respectively, preincubation for 2 hours; **C**). **D,** Effects of various doses (0–320 ng/mL) of LIF recombinant protein on sNF96.2 migration (mean ± SD). **E,** Impact of Ab-LIFR on sNF96.2 migration (mean ± SD), using 50 ng/mL of LIF and various doses of Ab-LIFR (top) or 4 μg/mL of Ab-LIFR compared with Ab-Ctrl (bottom). **F,** Impact of AG490/SC144 on sNF96.2 migration (mean ± SD), using 50 ng/mL of LIF. **G,** pSTAT3 and pAKT immunoblots in sNF96.2 cells following CM incubations and AG490/SC144 treatments. Quantifications noted are expressed as fold changes compared with sNF96.2 cells under sNF96.2 media. Each experiment was reproduced at least three times except for **B** (bottom), which was reproduced four times, using four different CAFs. *, P < 0.05; **, *P* < 0.01; ***, P < 0.001.

SC144 and AG490, two chemical inhibitors that block GP130 (the coreceptor of LIF), or JAK2 signaling, the specific pathway activated after GP130/LIFR induction (32, 33), respectively, confirmed previous data (Fig. 3C). Finally, LIF's specific ability to enhance the migration of peripheral nerve Schwann cells was confirmed with human recombinant LIF protein at a dose of 50 ng/mL, determined as the lowest dose inducing the higher migration improvement (Fig. 3D). Such induced migration ability with LIF recombinant protein was inhibited using LIFR blocking antibody at the lower dose of 4 μg/mL (Fig. 3E, top and bottom) but also using AG490 and SC144 (Fig. 3F).

Regarding intracellular signaling induced by LIF stimulation through its receptors, LIFR and gp130, STAT3 and AKT are two of the main pathways known to be induced (34). First, we confirmed that in SNF96.2 cells, LIF can trigger STAT3 and AKT phosphorylation/activation (Supplementary Fig. S3A and S3B). We further confirmed that such signaling activation is mediated by LIF receptors, LIFR and GP130, as AG490 or LIFR blocking antibody were able to inhibit STAT3 and AKT phosphorylation (Supplementary Fig. S3C). As reported in Fig. 3G, stromal cells CM could induce STAT3 or AKT phosphorylation/activation. However, the use of AG490 or LIFR blocking antibody inhibited stromal cells CM effects on intracellular signaling, suggesting that the CM-derived LIF could no longer activate LIFR/GP130 signaling. Altogether, our results revealed that LIF from stromal cell conditioned media is able to induce Schwann cell migration through LIFR/GP130 signaling then STAT3/AKT phosphorylation/activation.

LIF inhibits Schwann cell proliferation

We next examined the effects of stromal cell CM on Schwann cell proliferation and revealed that 48-hour incubation with the highest LIF-titrated CM (FHN+RAW or FHN+RAW+HMC-1) decreased cell proliferation by 16% (Fig. 4A) without affecting cell survival (Supplementary Fig. S4A). We validated that this decreased cell proliferation was due to the presence of LIF by adding the LIFR blocking antibody to the CM, which restored cell proliferation to the control level (Fig. 4B). We obtained similar results using LIF recombinant protein with a cell growth reduction of about 17% (Fig. 4C) without modification of cell survival (Supplementary Fig. S4B).

In agreement with previous report linking JAK/STAT3 pathway activation and cell growth arrest (35), we observed an increase in *P21* mRNA level by 24 hours post-LIF treatment (Fig. 4D) and an increase in P21 protein level by 36 and 48 hours (Fig. 4E). Interestingly, P21 protein level is restored with AG490 or SC144 treatments on cells incubated with LIF recombinant protein (Fig. 4F) or with stromal cells CM (Fig. 4G). These data highlight the impact of LIF secreted by PDAC stromal cells on the reduction of Schwann cell proliferation, which occurs concordantly with their enhanced migratory abilities.

LIF induces Schwann cell differentiation and neuronal plasticity

Interestingly, JAK/STAT3 pathway is known to induce cell differentiation, a crucial process for nerve cells involved in PANR. Thus, we analyzed Pou3F2 and S100, two independent markers of Schwann cell differentiation (36, 37) that we found expressed in human PDAC nerve fibers (Supplementary Fig. S5A). Interestingly, we observed an induction of both markers in Schwann cells after 48 hours of incubation with stromal cell CM (Fig. 5A and B, left). Such increase was lost when stromal cells CM was supplemented either with LIFR blocking antibody (Fig. 5A and B, right), AG490 or SC144 (Supplementary Fig. S5B). Moreover, we showed that incubation with LIF recombinant protein was able to induce *POU3F2* and *S100* expression in Schwann cells at both mRNA (Fig. 5C) and protein levels (Fig. 5D and E). Besides its impact on Schwann cells, we wondered whether LIF may affect neuronal plasticity associated to PANR (38). As suspected, we found that recombinant LIF could induce neuronal plasticity with increased neurite outgrowth (Fig. 5F) and soma area (Fig. 5G). Those data reveal that LIF, secreted by PDAC stromal cells, is able to induce Schwann cell differentiation and neuronal plasticity. In addition to data shown in previous parts, our study firmly support the potent impact of LIF in the neural remodeling observed in PDAC tumors.

LIF titer in serum as a diagnostic and prognostic biomarker for PDAC patients

To definitively assess whether LIF is an inductor of PANR *in vivo*, we first analyzed LIF titer in sera from PDAC bearing mice compared with LIF titer in sera from healthy mice and mice developing acute or chronic pancreatitis. Interestingly, not only we found a significant increase in LIF titer sera in PDAC-bearing mice compared with control or benign pancreatic diseases (Fig. 6A) but we found that LIF titer in sera from PDAC-bearing mice is positively correlated with intratumoral nerve density ($R^2 = 0.82$, $n = 12$; Fig. 6B). Finally, we assessed *in vivo* whether LIF was directly influencing intratumoral nerve density by using a LIF-neutralizing antibody in mice developing PDAC. As shown in Fig. 6c, control mice (treated with a control antibody) displaying a low level of LIF in serum (<124 pg/mL) exhibit few intratumoral nerves while control mice displaying a higher level of LIF in serum (>124 pg/mL) showed a significant increase in the intratumoral nerve density. Interestingly, mice treated with the LIF-neutralizing antibody showed a significant reduction of intratumoral nerves in spite of the presence of a high LIF quantity in serum (>124 pg/mL). Those data revealed that LIF is directly enhancing intratumoral nerve density in PDAC and that LIF titration in serum could serve as a biomarker to predict PANR.

Using a cohort including human sera from healthy donors ($n = 61$), patients with chronic pancreatitis ($n = 31$), or benign pancreatic tumor ($n = 11$) with cystic adenomas and IPMN (intraductal papillary mucinous neoplams) and PDAC patients ($n = 142$), we confirmed previous mice data (Fig. 6A) and showed that LIF titer was only increased in sera from PDAC patients compared with other groups (Fig. 6D). Also, we confirmed in sera from PDAC patients the positive correlation between LIF titer and intra-PDAC nerve density ($R^2 = 0.74$, $n = 10$; Fig. 6E). Above data suggest that LIF titer in serum is associated to PANR and could help in classifying PDAC patients in terms of PANR grade.

Altogether, these data support the use of LIF titer as a diagnostic marker for all stages pancreatic cancer and as a biomarker to discriminate PANR level in PDAC patients.

Discussion

Considering the grim mean survival rate among pancreatic cancer patients as well as the limited improvement of clinicians' arsenal over the last twenty years, it has become urgent to explore new therapeutic avenues that target PDAC evolution as well as PDAC-associated phenotypes. Among the latter, neuropathic pain

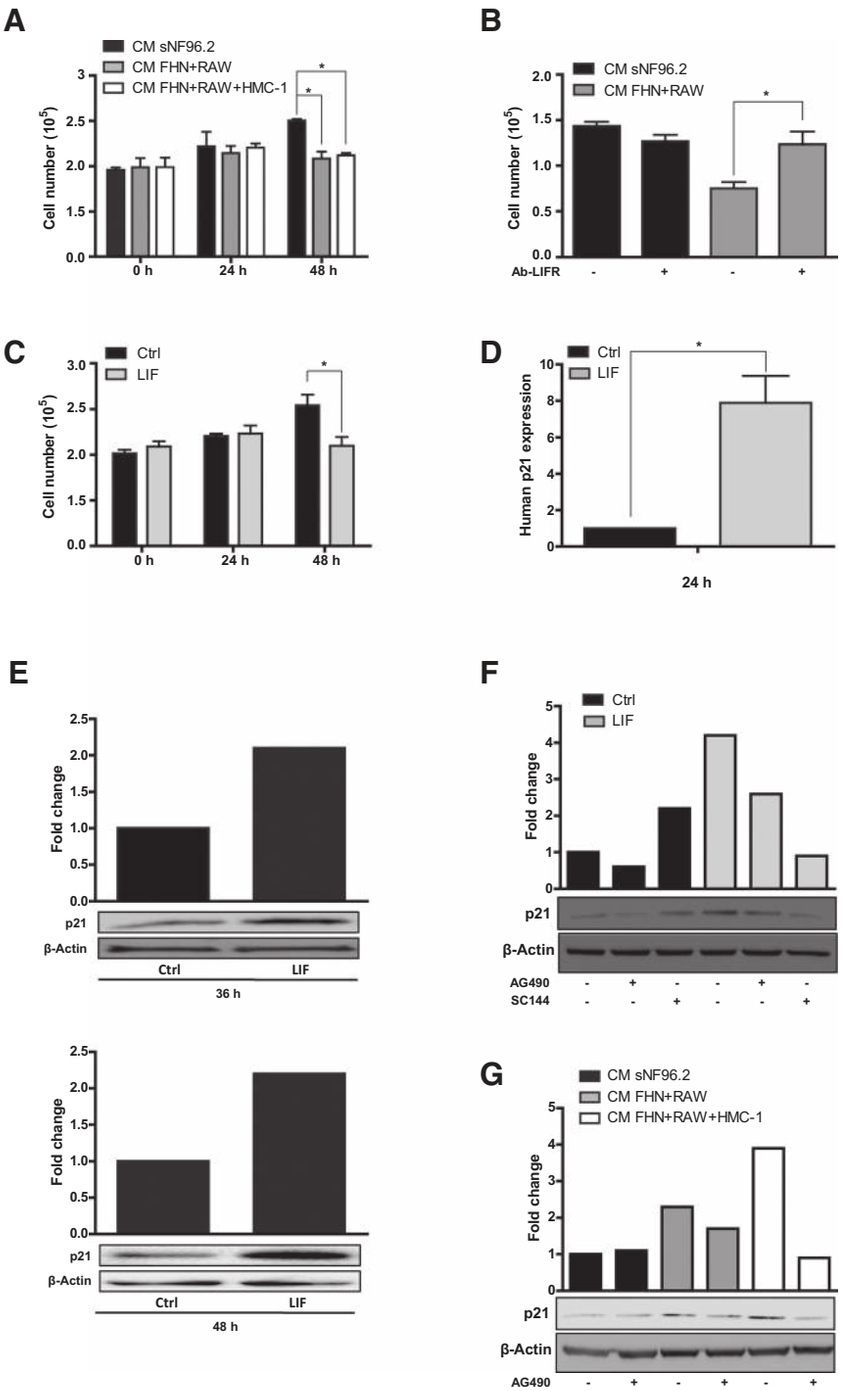


Figure 4.

LIF reduces Schwann cells proliferation. **A–C,** Cell count of sNF96.2 cells incubated with stromal-conditioned media (**A**) together with Ab-LIFR (**B**), or LIF recombinant protein (**C**; mean ± SD). **D,** Effect of LIF recombinant protein (50 ng/mL) on *P21* mRNA expression in sNF96.2 cells (mean ± SD). **E,** P21 immunoblots from sNF96.2 incubated for 36 (top) or 48 (bottom) hours with 50 ng/mL of LIF. Quantifications noted are expressed as fold changes compared with sNF96.2 cells not incubated with LIF recombinant protein. **F** and **G,** P21 immunoblots from sNF96.2 incubated for 36 hours with 50 ng/mL of LIF (**F**) or various conditioned media (**G**) together with AG490 (**F** and **G**) or SC144 (**F**) treatments. Each experiment was reproduced at least three times. *, $P < 0.05$.

and cachexia are major problems; management of these symptoms is fraught with difficulties, and globally, there exists no agreed upon standard care or treatment. Importantly, both symptoms are often the determining factors in deciding between patients' eligibility for chemotherapy or palliative care. Among fields to explore to improve drug accessibility and maintenance of treatment in PDAC patients, deciphering mechanisms underlying PDAC associated neural remodeling could yield promising results.

Although clinicians have for many years reported nervous system reorganization in cancers, and specifically in PDAC, fundamental researchers have only recently realized its possible implications in PDAC evolution and patient survival (8, 13). It is now well acknowledged that infiltration of the tumor microenvironment by nerves, termed neoneurogenesis or axonogenesis, which occurs early in PDAC development (11), plays an active role in cancer progression (39) and correlates with shortened survival, pain and local tumor recurrence (8). Although

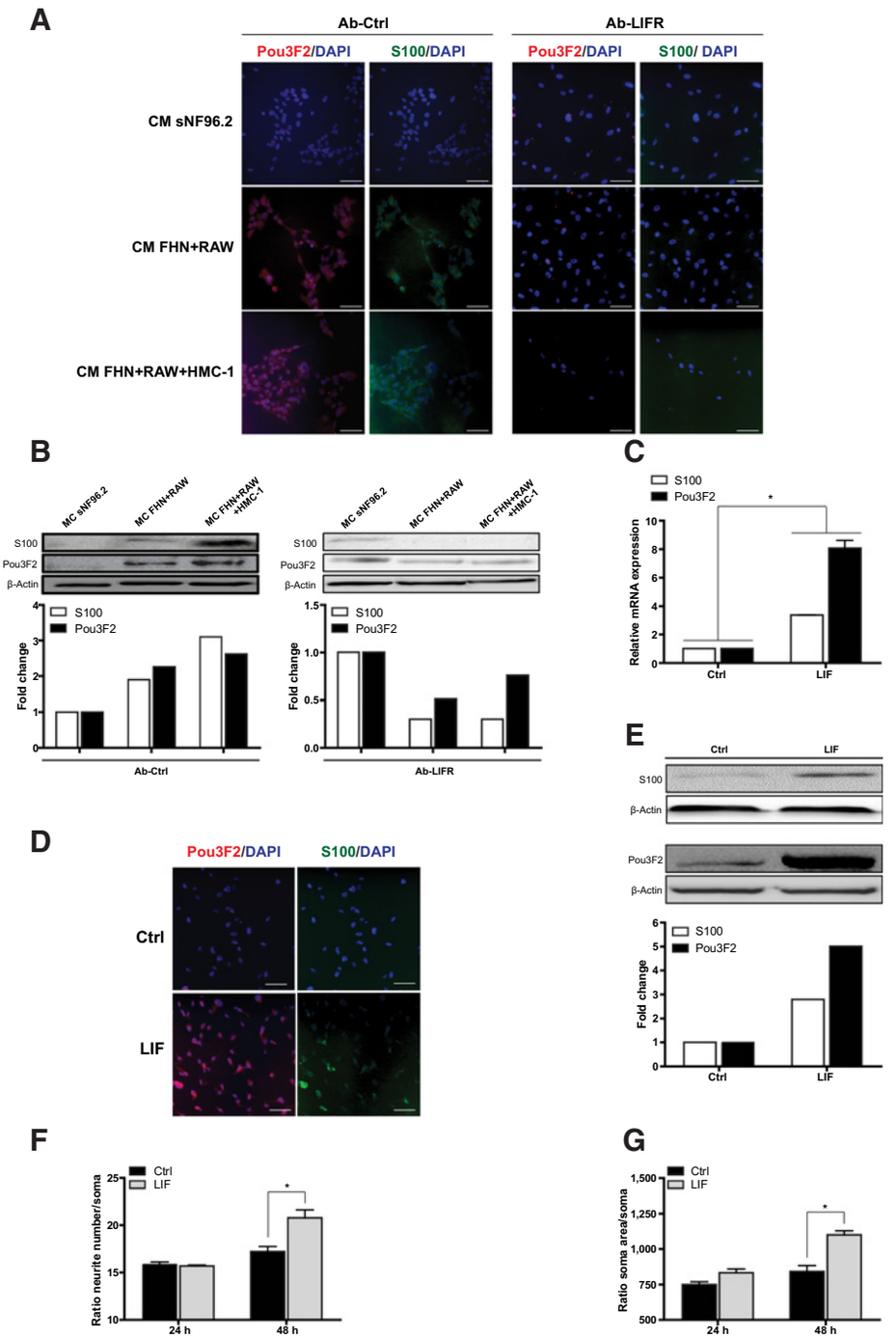


Figure 5.
LIF induces Schwann cell differentiation and neural plasticity. **A,** POU3F2 and S100 dual-staining in sNF96.2 cells incubated with control (sNF96.2) or stromal conditioned media, in presence of Ab-Ctrl (left) or Ab-LIFR (right; scale bar, 100 μm). **B,** S100 and Pou3F2 immunoblots in sNF96.2 cultured as in **A**. **C,** *S100* and *POU3F2* mRNA expression levels in sNF96.2 cells incubated with 50 ng/mL of LIF recombinant protein (mean ± SD). **D,** Representative images of POU3F2 and S100 dual-staining on sNF96.2 cells incubated with 50 ng/mL of LIF for 48 hours (scale bars, 100 μm). **E,** S100 and POU3F2 immunoblots from sNF96.2 cells incubated for 48 hours with LIF (50 ng/mL). **F** and **G,** Effects of 50 ng/mL of LIF (24 and 48 hours) on neuronal plasticity (**F,** neurite number; **G,** soma area) of neurons from DRG (mean ± SD). Each experiment was reproduced at least three times. *, $P < 0.05$.

several studies have reported the ability of cancer cells to attract nerve fibers (40, 41), very few have reported the impact of stromal cells in this process (12), especially in PDAC where stromal cells compose the vast majority of the tumor cell mass. Therefore, our goal was to identify molecular targets from the PDAC microenvironment that are involved in PANR, which may lead to the discovery of potent future adjuvant therapies that could prolong survival and reduce morbidity by blocking PANR. Here, we demonstrated for the first time that LIF, secreted by the PDAC microenvironment, induced nerve cell migration and differentiation and thereby is positively correlated with PANR and axonogenesis (Fig. 6F). Concomitantly, we have revealed that LIF is a potent biomarker for PDAC and helps in determining PANR in PDAC.

In this study, we considered knowledge associating tumor inflammation both with pancreatic cancer (42) and with the modulation of the nerve compartment (43, 44) to reach our hypothesis that stromal-driven inflammatory genes/pathways

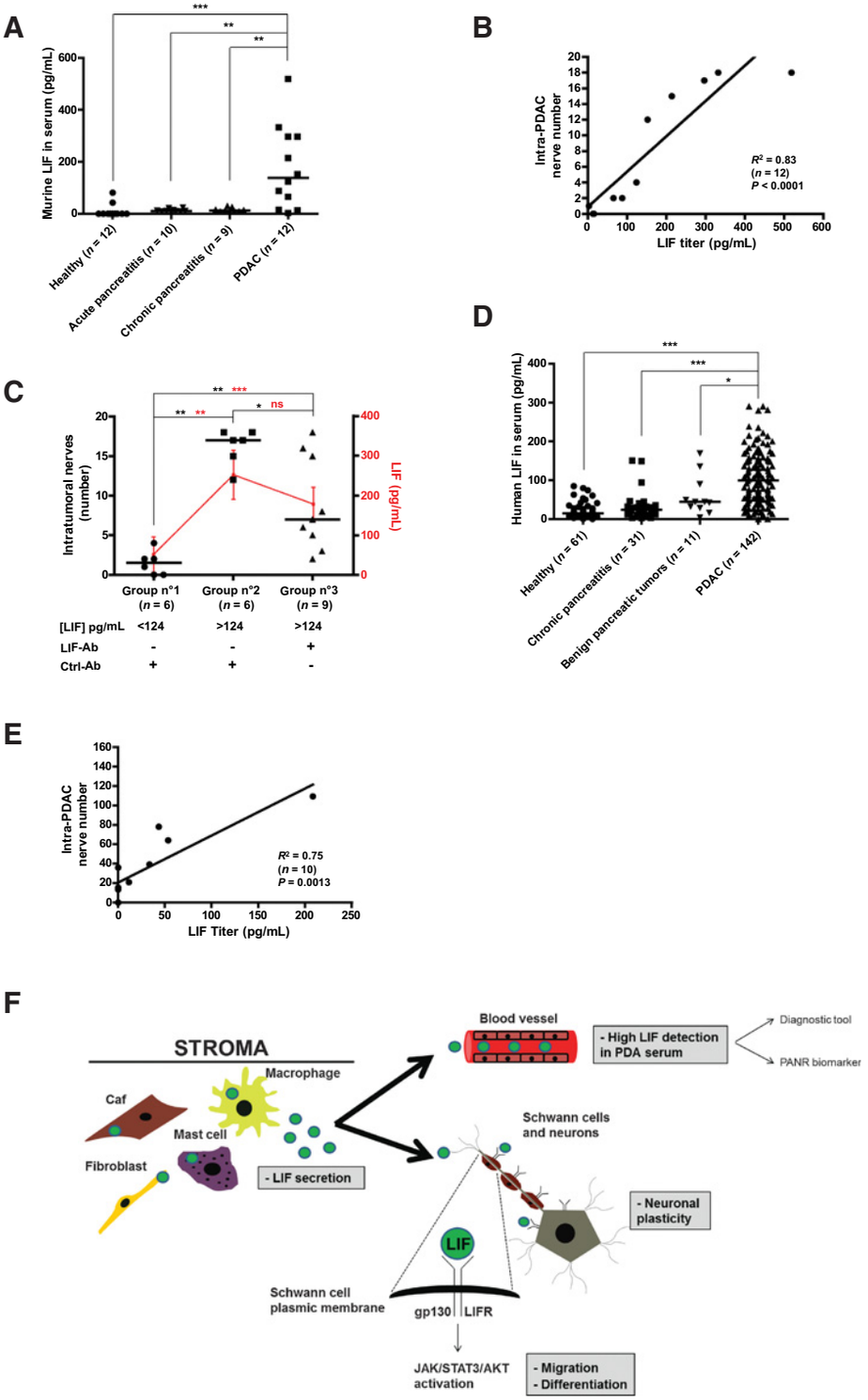


Figure 6.

LIF is a potent diagnostic and predictive biomarker for PDAC. **A,** Measurement of LIF level in serum from healthy ($n = 12$), acute ($n = 10$), or chronic ($n = 9$) pancreatitis as well as PDAC bearing ($n = 12$) mice. **B,** Linear regression of intra-PDAC nerve number versus LIF titer in PDAC-bearing mice sera ($n = 12$). **C,** Measurement of serum LIF level and intratumoral nerve number in PDAC-bearing mice treated with control antibody [$n = 6$, (LIF) < 124 pg/mL and $n = 6$, (LIF) > 124 pg/mL] or LIF-neutralizing antibody [$n = 9$, (LIF) > 124 pg/mL). **D,** Measurement of LIF level in human serum from healthy donors ($n = 61$), chronic pancreatitis ($n = 31$), benign pancreatic tumor ($n = 11$), and PDAC ($n = 142$) patients. **E,** Linear regression of intratumoral nerve number versus LIF titer in serum from PDAC patients ($n = 10$). **F,** Graphical representation summarizing the impact of stromal-secreted LIF on PANR and its potent use as a biomarker.

could, additionally to their effects on tumor cells, impact the nerve compartment and in particular PANR. Thus, we revisited previous transcriptomic analysis [Gene Expression Omnibus (GEO): GSE50570 for human PDAC, (12), and GSE61412 for mouse PDAC, (27)] and highlighted numerous genes that code for molecules involved in gp130 signaling and were overexpressed in the PDAC stromal compartment compared with PDAC tumor cells.

Among, the identified *GP130*-related genes, we focused on LIF due to its major role in regulating the nervous system (45).

Indeed, very little is known of its potent role in this context except a study suggesting that increased levels of LIF in PDAC could impact the STAT3 pathway in cancer cells (31). In addition to confirming these results in both human and mouse PDAC, our study has extended our knowledge on LIF from its expression pattern to its mode of secretion within PDAC, revealing that although both tumor and stromal cells (CAFs, mast cells, and macrophages) were able to express LIF, only stromal cells could secrete it. This striking and somewhat unexpected result reinforces the potent role of the stromal compartment in PANR but also raises questions about the role of this nonsecreted LIF within PDAC cancer cells. In our study, we were interested in the effect of the stromal cell–secreted LIF in human PDAC and found that infiltrating nerve fibers expressed LIFR and gp130, indicating a possible triggering of LIF signaling within these nerve cells.

We extended our *in vivo* data with *in vitro* experiments performing heterotypic cocultures of stromal cells. We observed that cocultures with macrophages drastically enhanced LIF secretion by fibroblasts, which is consistent with recent findings concerning LIF expression by activated fibroblasts (46). Here, we demonstrated that LIF is a strong modulator of nerve cell status, in terms of motility, proliferation, and differentiation. Interestingly, this part of the study is highly similar with our previous study about the stromal-derived SLIT2 impact on PANR (12). Indeed, we mentioned in this work the ability of CAF-derived SLIT2 to induce PANR in PDAC. While the relevance of SLIT2 as an efficient biomarker was not reported, the connection between SLIT2 and LIF and their possible association to a further common signaling pathway should be investigate to determine whether SLIT2/LIF-impact on PANR are both due to JAK/STAT activation. In addition, we confirmed, with blocking antibodies or chemical inhibitors of gp130, LIFR or the JAK/STAT3 signaling pathway, that all modulations observed on cell behaviors were dependent on LIF signaling. These results are particularly relevant from a therapeutic point of view: targeting LIF signaling through the inhibition of either LIF binding to its receptors or LIF-triggered signaling could, in addition to the reported effect on targeting cancer cells, have an impact on tumor progression via the inhibition of nerve infiltration.

However, while we had demonstrated that LIF could modulate nerve cell status *in vitro* and backed this up by revealing a plausible cellular expression of LIF, LIFR, and gp130 in human PDAC, we needed a correlation between the presence of LIF in PDAC patients and PANR. Therefore, we measured LIF titer in serum from human or mouse PDAC and correlated it with the nerve density in the corresponding PDAC sample. In both models, we found a positive correlation between the amount of LIF in the serum and the intratumoral nerve density, supporting a link between LIF and PANR, as well as revealing LIF as a valuable biomarker to determine PANR level in PDAC. In addition to confirming our hypothesis, we observed that LIF titration in serum from PDAC patients could have other uses. Indeed, as already reported for IL6 and IL11, cytokine serum levels are valuable diagnostic and prognostic tools (47–49). The specificity given by LIF to distinguish PDAC from other benign pancreatic diseases suggests that the combined detection of LIF and CA19.9 could be greater than CA19.9 alone in the diagnosis of PDAC. Finally, LIF titration in serum of PDAC patients has a real potent value as a stratifying biomarker of PDAC to classify PDAC patients regarding their possible responsiveness to JAK/STAT targeting agent as ruxolitinib. Such ongoing investigation could improve PDAC patient management.

Altogether, our results have potential therapeutic implications by providing a rationale for the use of LIF inhibitors in PDAC, but also diagnostic implications by suggesting the usefulness of combining LIF and CA19.9 titration as a diagnostic and predictive marker. Indeed, our study is a proof-of-concept that the stroma impacts nervous system reorganization and thus PANR through the secretion of LIF. This secreted LIF (titrated in the serum), in addition to correlating with nerve density in PDAC, exhibited a strong specificity with PDAC tumors. While potentially useful in PDAC detection, LIF titration should also be explored in a large panel of human cancers, especially those developing axonogenesis or perineural invasion such as prostate, colon, and breast cancers. Further work will also be needed to determine the exact effect of LIF inhibitors in PDAC as well as the potent value of LIF tittering as a stratifying biomarker for JAK/STAT–targeting therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Bressy, S. Vasseur, R. Tomasini
Development of methodology: R. Tomasini
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Bressy, S. Lac, J. Nigri, J. Leca, J. Roques, V. Secq, T.-T. Bui, R. Tomasini
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Bressy, J. Nigri, J. Leca, J. Roques, S. Granjeaud, J.-B. Bachet, S. Vasseur, R. Tomasini
Writing, review, and/or revision of the manuscript: C. Bressy, J.-B. Bachet, S. Vasseur, R. Tomasini
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.-N. Lavaut, F. Guillaumond, D. Pietrasz, J.-B. Bachet, M. Ouaissi, J.L. Iovanna, S. Vasseur, R. Tomasini
Study supervision: R. Tomasini

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