Cell. Mol. Life Sci. (2017) 74:4231–4243
DOI 10.1007/s00018-017-2579-9

Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

PAP/REG3A favors perineural invasion in pancreatic adenocarcinoma and serves as a prognostic marker

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Received: 8 March 2017 / Revised: 8 June 2017 / Accepted: 20 June 2017 / Published online: 27 June 2017

Abstract Pancreatic ductal adenocarcinoma (PDA) is a fatal and insidious malignant disease for which clinicians' tools are restricted by the current limits in knowledge of how tumor and stromal cells act during the disease. Among PDA hallmarks, neural remodeling (NR) and perineural invasion (PNI) drastically influence quality of life and patient survival. Indeed, NR and PNI are associated with neuropathic pain and metastasis, respectively, both of which impact clinicians' decisions and therapeutic options. The aim of this study was to determine the impact and clinical relevance of the peritumoral microenvironment, through pancreatitis-associated protein (PAP/REG3A) expression, on PNI in pancreatic cancer. First, we demonstrated that, in PDA, PAP/REG3A is produced by inflamed acinar cells from the peritumoral microenvironment and then enhances the migratory and invasive abilities of cancer cells. More specifically, using perineural ex vivo assays we revealed that PAP/REG3A favors PNI through activation of the JAK/STAT signaling pathway in cancer cells. Finally, we analyzed the level of PAP/REG3A in blood from healthy donors or patients with PDA from three independent cohorts. Patients with high levels of PAP/REG3A had overall shorter survival as well as poor surgical outcomes with reduced disease-free survival. Our study provides a rationale for using the PAP/REG3A level as a biomarker to improve pancreatic cancer prognosis. It also suggests that therapeutic targeting of PAP/REG3A activity in PDA could limit tumor cell aggressiveness and PNI.

Keywords Pancreatic cancer · Perineural invasion · Peritumoral microenvironment

Electronic supplementary material The online version of this article (doi:10.1007/s00018-017-2579-9) contains supplementary material, which is available to authorized users.

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Introduction

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer death and is expected to become second in rank by 2030 [1]. It is among the most lethal of all cancers, with a 5-year survival rate of only 5% [1]. Because of its aggressiveness and the absence of symptoms, most patients are diagnosed at an advanced stage, often metastatic, limiting their access to surgery. Palliative treatments have a reduced efficacy, even for recent combinatory treatments such as Folfirinox or gemcitabine plus nab-paclitaxel [2, 3]. These combinations improve global survival by 3–5 months but are restricted to patients meeting several global health criteria, to ensure that they are likely to withstand important secondary effects. At present, numerous studies have suggested new therapeutic options integrating the impact of non-cancerous cells (stroma or intratumoral microenvironment) [4, 5] or targeting PDA-associated hallmarks [6, 7]. Among those, deciphering the drastic modulations of the nervous system compartment in PDA constitutes a potent source of biomarkers and therapeutic targets that could improve survival and quality of life.

The alterations of the nervous system in pancreatic cancer, which include neural remodeling (NR) and perineural invasion (PNI) [8], have been reported for decades. While NR is characterized by an increased nerve size and density due to peripheral nerve fiber infiltration and axonogenesis [8], PNI refers to the presence of cancer cells within the perineurium or the endoneurium of nerve fibers. NR and PNI are associated with a worse prognosis, shortened survival, recurrence, and are also linked with local or distant dissemination as well as neuropathic pain [9]. Such clinical associations suggest that interesting therapeutic options could be explored if the molecular basis of such profound and affecting alterations, at present unknown, could be uncovered. Interestingly, the recent confirmation of the importance of the nervous system in the initiation and progression of PDA suggests a link between inflammation and Kras-induced neoplasia [10]. Indeed, the consistent presence of an inflammatory environment in premalignant lesions of PDA as well as in PDA itself fosters niches where inflammatory mediators impact both cancer cells and the nervous system.

One of the main PDA hallmarks is the massive presence of non-tumor cells [11], mainly immune cells and cancer-associated fibroblasts (CAFs) [12, 13]. Interestingly, CAFs are widely reported as "factories", producing tremendous amounts of secreted factors that can impact cancer [5, 14] and nerve cells [15]. Among these mediators, proinflammatory cytokines correlate with clinicopathologic parameters, chemoresistance, and survival [16, 17]. A recent report showing an inflammatory gene signature in CAFs following chemotherapy treatment suggests a drastic impact of CAFs in the proinflammatory response following treatment and a consequent impact on patient survival [18]. Interestingly, inflammatory mediators have also been depicted as drivers of PDA-associated neural plasticity [8], reinforcing the need to improve our knowledge of the link between inflammation, nervous system alterations, and PDA.

In this study, we evaluated the role of PAP/REG3A as a prognostic marker associated with clinicopathologic features of PDA. Human PAP/REG3A (mouse PAP/REG3β), or pancreatitis-associated protein, is a C-type lectin-like secreted protein discovered for its involvement in pancreatic diseases such as acute pancreatitis [19], diabetes [20], and cystic fibrosis [21]. More recently, the tumor promoter role of PAP/REG3A in PDA was assessed because of its implication in M1/M2 macrophage polarization [22] and tumor cell growth under IL6-associated inflammatory conditions [23]. A suspected role of PAP/REG3A as a biomarker for PDA has been reported but remains unclear [24].

Because of these reports, as well as data showing the involvement of PAP/REG3A in the nervous system [25, 26], we evaluated its role in PDA-associated nervous system alterations, and more specifically in PNI, as well as its potential as a prognostic marker. We further examined the impact of the peritumoral microenvironment, through PAP/REG3A secretion, and aimed to unravel its impact on the survival of patients with PDA. Using a new ex vivo assay, we determined its influence on PNI and correlated it with the prognostic value of PAP/REG3A as a circulating biomarker to better stratify patients with PDA.

Results

PAP/REG3A shows a restricted pattern of expression in PDA

Deciphering the role of PAP/REG3A in PDA-associated nervous system alterations first requires identification of which cell type/compartment is the source of PAP/REG3A secretion. As reported previously, the cellular origin of PAP/REG3A levels in PDA could be the pancreatic acinar cells adjacent to the infiltrating adenocarcinoma [24], in a peritumoral zone that histologically resembles chronic pancreatitis. We assessed this cellular localization through PAP/REG3A staining on human PDA slides and observed a clear staining of the peritumoral areas, while no PAP/REG3A expression was observed in healthy and intratumoral areas (Fig. 1a). PDA from Pdx1-cre/$Kras^{G12D}$/$Ink4a^{fl/fl}$ mice also showed a similar peritumoral restricted

staining of mouse PAP/REG3β (the mouse homologue of human PAP/REG3A) (Fig. 1b). Using several specific cell markers, we investigated further to determine the cells from peritumoral areas of human PDA that express PAP/REG3A. Co-staining of PAP/REG3A with α-amylase (an inflamed acinar cell marker) occurred and confirmed the acinar cellularity of PAP/REG3A, while no co-staining occurred using CAF (αSMA, alpha-smooth muscle actin), tumor cell (PKRT, pan-cytokeratin), macrophage (CD68), or nerve cell (neurofilament) markers (Fig. 1c). PDAs from Pdx1-cre/$Kras^{G12D}$/$Ink4a^{fl/fl}$ mice displayed similar staining patterns (Supplementary Fig. 1). These data suggest that inflamed acinar cells from peritumoral areas are the main mediators of PAP/REG3A secretion in PDA. To assess whether PAP/REG3A can efficiently activate downstream signaling in recipient cells, we assessed gp130 expression (a co-receptor transducing PAP/REG3A signaling) and found it expressed in tumor cells and the nerve compartment in human PDA (Fig. 1d), suggesting that both are able to respond to PAP/REG3A. These data are supported by the interesting observation that nerves present in or around PDA are in close contact with peritumoral areas expressing PAP/REG3A (Fig. 1e). Altogether, our data reveal that PAP/REG3A is expressed by the peritumoral compartment of PDA, while tumor cells and nerve cells, through their expression of the PAP/REG3A co-receptor gp130, could respond to PAP/REG3A-induced signaling, reinforcing the possible role of PAP/REG3A in PDA-associated nervous system alterations.

High PAP/REG3A levels are associated with shortened survival and higher tumor grade in PDA

As a secreted factor, we first measured serum or plasma levels of PAP/REG3A in 85 healthy donors and 166 pancreatic cancer patients from three independent cohorts. Cohorts 1, 2, and 3 included 79, 33, and 54 patients, respectively. It should be noted that all PAP/REG3A measurements in biological fluids were carried out using an ELISA assay (Dynabio SA, France) that is currently used by several European countries in the newborn screening for cystic fibrosis. Statistical analysis revealed a significant increase in PAP/REG3A levels in all PDA patient cohorts (Fig. 2a, $P < 0.001$ or $P < 0.0001$). An optimal cut-off for PAP/REG3A levels of 17.5 µg/L was identified; patients with a PAP/REG3A level of 17.5 µg/L or above had shorter overall survival than patients with a PAP/REG3A level of less than 17.5 µg/L (Fig. 2b, c, $P = 0.0203$ and $P = 0.0363$ for cohort 1 and 2, respectively). In agreement with this, patients with PAP/REG3A level of 17.5 µg/L or above showed a higher grade of PDA at diagnosis; 42.3% of patients with PAP/REG3A levels of <17.5 µg/L had stage 3 PDA at diagnosis, which increased to 66.7% in patients with levels ≥17.5 µg/L (Fig. 2d). The percentage of patients with resectable tumors at diagnosis was also consistent with these data, as 72% of patients with PAP/REG3A levels of 17.5 µg/L or above were non-resectable vs. 40% of patients with low PAP/REG3A levels (<17.5 µg/L) (Fig. 2e). These results indicate that high circulating PAP/REG3A levels in patients with pancreatic cancer predict shortened survival and a worse PDA stage at diagnosis.

PAP/REG3A enhances PDA cancer cell aggressiveness

To determine the influence of circulating PAP/REG3A on the aggressiveness of PDA and patient survival, and regarding our hypothesis on the role of PAP/REG3A in nervous system alterations and PNI, we analyzed its impact on tumor cell motility using specific mouse PAP/REG3β (Fig. 3a) or human PAP/REG3β (Fig. 3b) recombinant proteins. As suspected, both proteins enhanced, respectively, mouse (Pk4A, Fig. 3a, $P < 0.05$ and $P < 0.01$) and human (Panc-1, Fig. 3b, $P < 0.01$ and $P < 0.001$) pancreatic cancer cell migration in a dose-dependent manner. We then wished to confirm whether the induction of the migratory ability of cancer cells was dependent on intracellular signaling activated specifically by PAP/REG3A. While little is known about specific PAP/REG3A receptors, the required activation of STAT3/AKT following PAP/REG3A stimulation has been reported [27, 28]. Using AG490, a STAT3-specific inhibitor, we could impair the PAP/REG3A effect on Panc-1 migration (Fig. 3c, $P < 0.05$). In addition, pancreatic cancer cells, in the presence of PAP/REG3β, exhibited increased invasive abilities (Fig. 3d, $P < 0.01$), which were also inhibited by AG490 treatment (Fig. 3d, $P < 0.05$). As we previously demonstrated that PAP/REG3A is mainly produced by pancreatic acinar cells (Fig. 1a, c), we confirmed the above data using acinar cell conditioned media (ACm). We extracted and cultured acinar cells from healthy mice and then retrieved their conditioned media, which we depleted in PAP/REG3β (ACm/PAP^{-}) or not (ACm/PAP^{+}). As depicted in Fig. 3e, ACm/PAP^{+} media enhanced Pk4A migratory abilities by tenfold ($P < 0.05$). Such an increase is significantly reduced either by AG490 treatment ($P < 0.05$) or following incubation with ACm/PAP^{-}, the PAP/REG3β-depleted media ($P < 0.05$) (Fig. 3e). These data demonstrate a significant role for PAP/REG3A/REG3β in the migratory and invasive abilities of pancreatic cancer cells, through JAK2/STAT3 signaling activation.

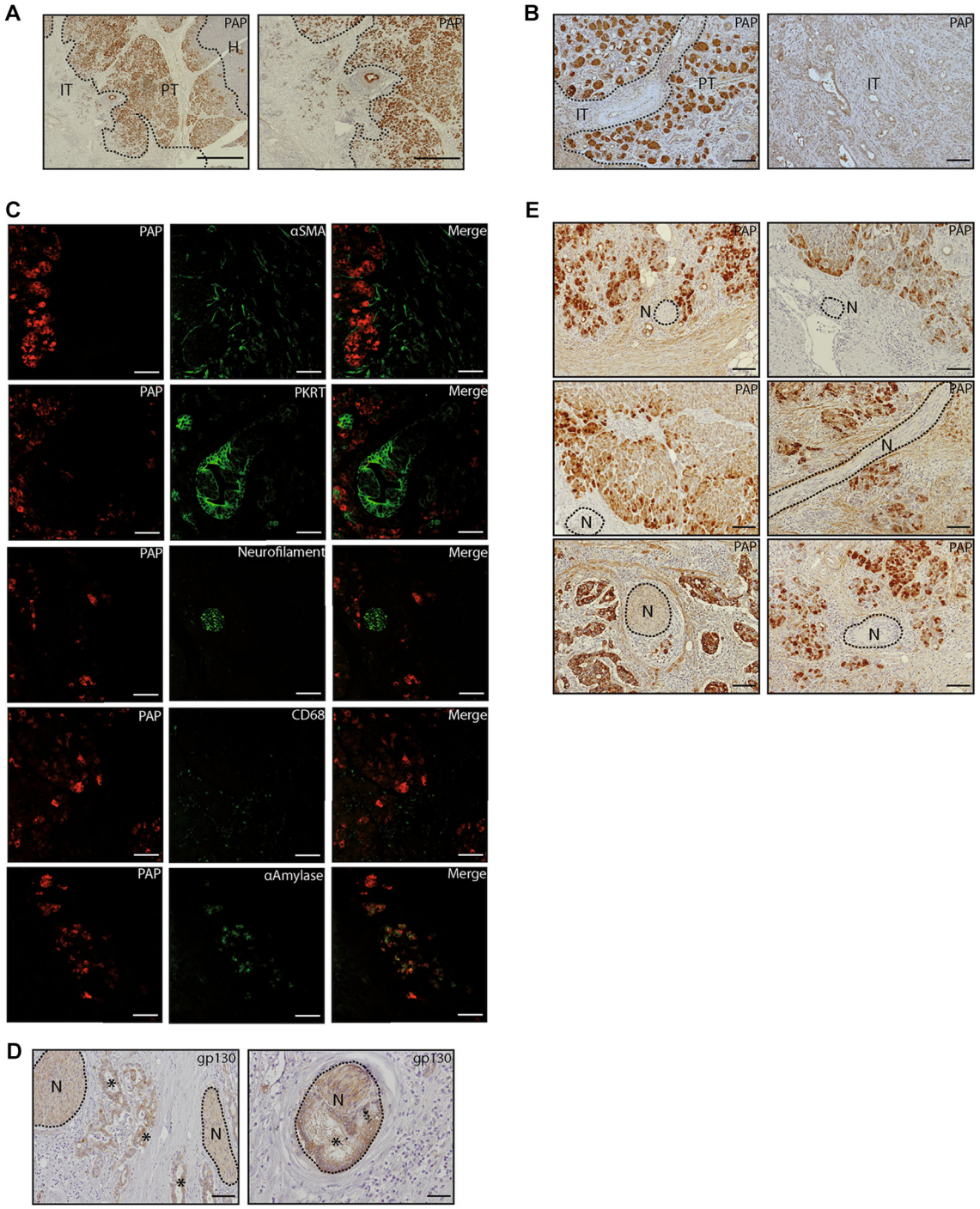

Fig. 1 PAP/REG3A/β expression is restricted to the peritumoral compartment in PDA. **a** Representative images of PAP/REG3A expression in human PDA tissues as determined by immunohistochemistry. *IT* intratumoral, *PT* peritumoral, *H* healthy. Magnification ×4. *Scale bar* 100 μm. **b** Representative images of PAP/REG3β expression in mouse PDA tissues as determined by immunohistochemistry. *IT* intratumoral, *PT* peritumoral. Magnification ×10. *Scale bar* 100 μm. **c** Immunofluorescence staining for PAP/REG3A (*left column*), αSMA, PKRT, Neurofilament, CD68, or αAmylase (*middle column*) and merged staining (*right column*) in human PDA tissues. Magnification ×20. *Scale bar* 100 μm. **d** Representative images of gp130 expression in human PDA tissues as determined by immunohistochemistry. *N* nerve section; *asterisk* tumor cells. *Dotted lines* highlight nerve fiber perimeters. Magnification ×20. *Scale bar* 100 μm. **e** Representative images of PAP/REG3A expression in human PDA tissues as determined by immunohistochemistry associated with nerve sections. *Dotted lines* highlight nerve fiber perimeters. *N* nerve section. Magnification ×10. *Scale bar* 100 μm

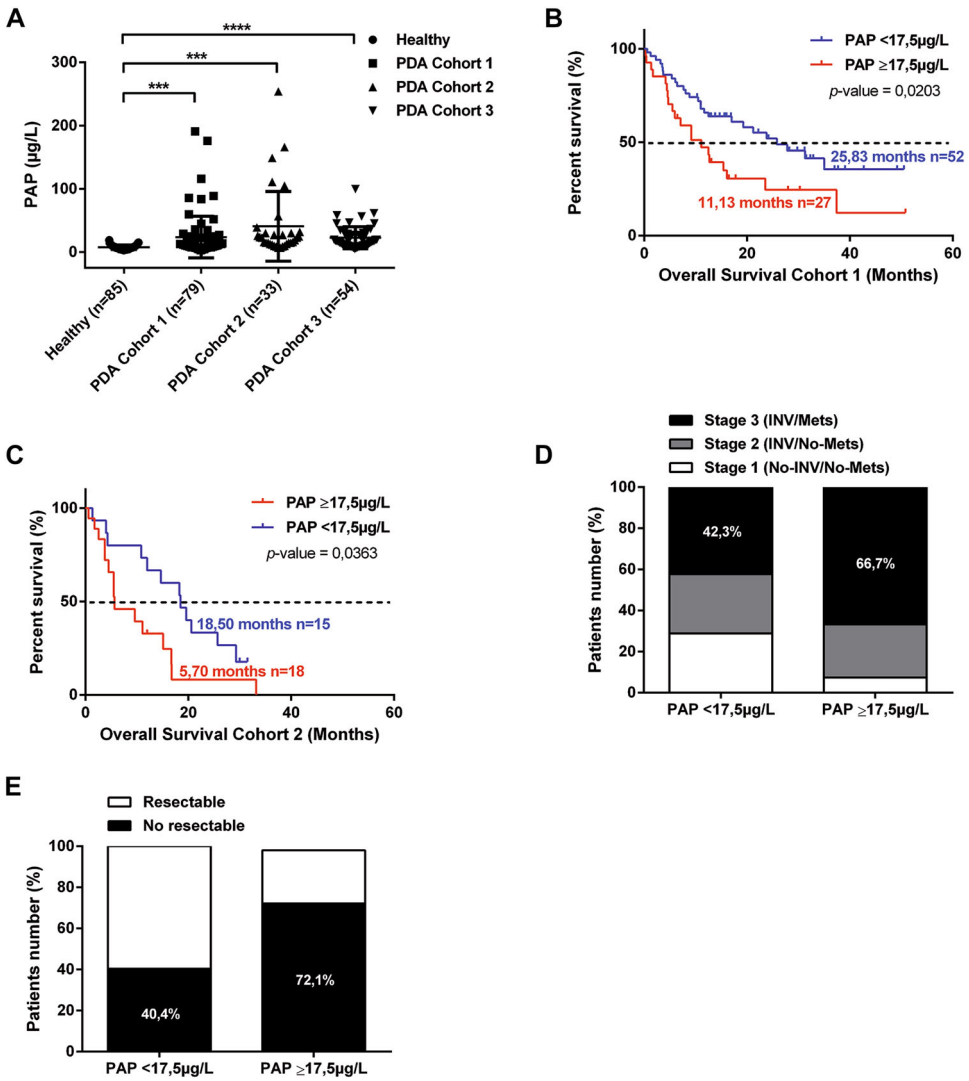

Fig. 2 High PAP/REG3A levels are associated with shortened survival and higher tumor grade in PDA. **a** Quantification of PAP/REG3A (µg/L) levels in blood obtained from healthy donors ($n = 85$) and patients with PDA from three different cohorts (1, $n = 79$; 2, $n = 33$; 3, $n = 54$) (median ± interquartile range). **b** Kaplan–Meier overall survival curve using PAP/REG3A measured in serum from patients with PDA from cohort 1, divided into high (≥17.5 µg/L) and low (<17.5 µg/L) groups ($n = 27$ and 51, respectively). **c** Kaplan–Meier overall survival curve using PAP/REG3A measured in serum from patients with PDA from cohort 2, divided into high (≥17.5 µg/L) and low (<17.5 µg/L) groups ($n = 18$ and 15, respectively). **d** Quantification of PAP/REG3A measured in serum from patients with stage 1, 2 and 3 PDA from cohort 1, divided into high (≥17.5 µg/L) and low (<17.5 µg/L) PAP/REG3A groups ($n = 27$ and 51, respectively). **e** Quantification of resectable and non-resectable PDA in high (≥17.5 µg/L) and low (<17.5 µg/L) PAP/REG3A groups from cohort 1 ($n = 27$ and 51, respectively)

PAP/REG3A increases perineural invasion

Immunohistochemical analysis of human PDA tissue revealed that PAP/REG3A staining is associated with nerve density, as shown in Fig. 1e. Indeed, nerve fibers are mainly present in the tissue areas where PAP/REG3A is strongly expressed. Moreover, immunostaining of human PDA slides showed that tumor cells present within PNI events express gp130 and, therefore, could respond to PAP/REG3A-induced signaling (Fig. 1e). To further investigate the suspected role of PAP/REG3A in favoring PNI, we designed a specific ex vivo experiment. Mouse sciatic nerve fibers were incubated with Pk4A cells ± PAP/REG3β mouse recombinant protein (Supplementary Fig. 2). After 72 h of co-culture, nerve fibers were fixed and paraffin-embedded sections were prepared to determine, using immunohistological staining, the presence of Pk4A cells that had invaded and migrated within nerve fibers. The presence of PAP/REG3β in the co-culture with nerve fibers/Pk4A cells led to a 2.9-fold increase in the number of tumor cells within nerve sections ($P < 0.05$, Fig. 4a). As for previous experiments (Fig. 3c–e), the use

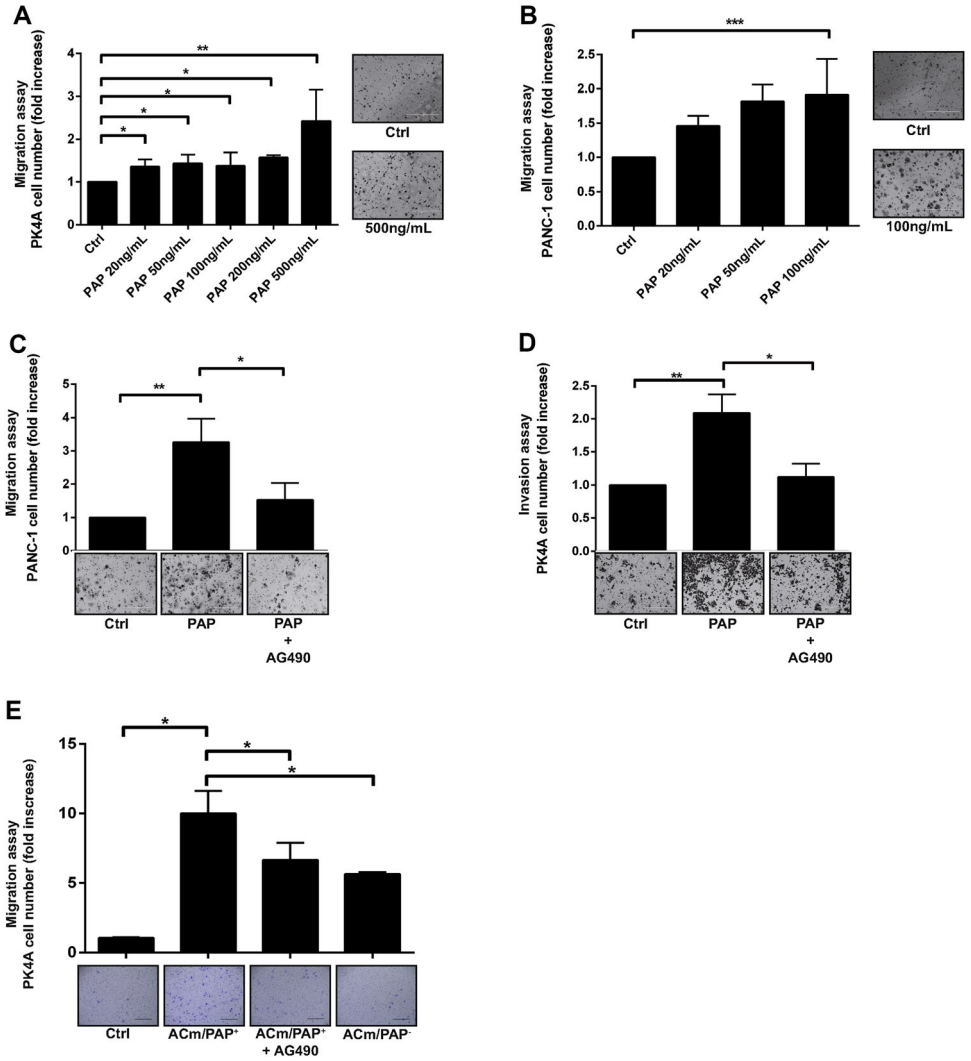

Fig. 3 PAP/REG3A enhances PDA cancer cell aggressiveness. **a** Migratory ability of mouse pancreatic cancer cells, Pk4A, in the presence of mouse PAP/REG3β recombinant protein concentrations ranging from 20 to 500 ng/mL (median ± interquartile range, $n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$. **b** Migratory ability of human pancreatic cancer cells, Panc-1, in the presence of human PAP/REG3A recombinant protein concentrations ranging from 20 to 500 ng/mL (median ± interquartile range, $n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$. **c** Migratory ability of human pancreatic cancer cells, Panc-1, ±100 ng/mL of mouse PAP/REG3A recombinant protein ± AG490 treatment (median ± interquartile range, $n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$. **d** Invasive ability of mouse pancreatic cancer cells, Pk4A, ±500 ng/mL of human PAP/REG3β recombinant protein ± AG490 treatment (median ± interquartile range, $n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$. **e** Migratory ability of mouse pancreatic cancer cells, Pk4A, ±control or PAP/REG3β-depleted acinar cell media (ACm/PAP^{+} or ACm/PAP^{-}, respectively) ± AG490 treatment (median ± interquartile range, $n = 3$). $^{*}P < 0.05$. Representative images in all panels were at magnification ×10. *Scale bar* 400 μm

of the STAT3 inhibitor AG490 significantly impaired the effect of PAP/REG3β on nerve fiber invasion by pancreatic cancer cells ($P > 0.05$, Fig. 4a). We confirmed the role of PAP/REG3β in PNI using acinar cell media containing or depleted in PAP/REG3β. The PNI that occurred following Pk4A incubation with nerve fibers and acinar cell media containing PAP/REG3β (ACm/PAP^{+}) was drastically reduced when using PAP/REG3β-depleted acinar cell media (ACm/PAP^{-}) ($P < 0.05$, Fig. 4b) or following treatment of Pk4A cells with SC144 (a gp130 inhibitor [29]), impairing PAP/REG3β stimulation ($P < 0.05$, Fig. 4b). These results confirm the direct impact of PAP/REG3β on PNI, in a JAK2/STAT3-dependent manner.

High PAP/REG3A levels are associated with a worse prognosis for resected patients

PNI is at present considered one of the major causes of tumor relapse for patients following PDA resection [30], and so impacts their overall survival. As our data revealed that PAP/REG3β enhances PNI, and also showed that PAP/REG3A detection in patients' sera is associated with worse prognosis,

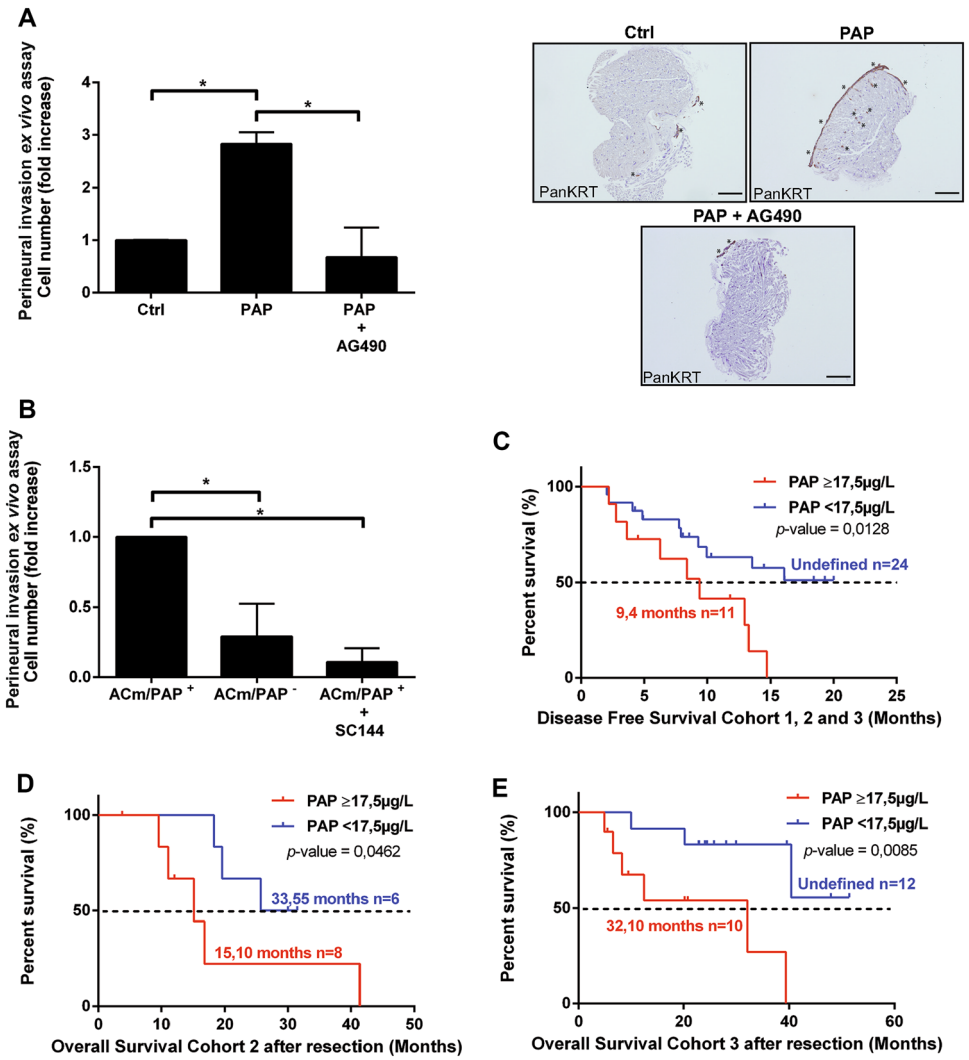

Fig. 4 PAP/REG3A increases perineural invasion (PNI) and is associated with a worse prognosis for resected patients. **a** Measurement of the PNI ability of human pancreatic cancer cells, Panc-1, using an ex vivo PNI assay in the presence or not of human PAP/REG3A recombinant protein (500 ng/mL) and AG490 treatment (median ± interquartile range, $n = 3$). $^*P < 0.05$. Representative images of Pancytokeratin (PanKRT) staining, revealing mouse pancreatic cancer cells, Pk4A, in paraffin-embedded nerve sections. *Asterisk* cells positive for PanKRT staining. Magnification ×4. *Scale bar* 100 µm. **b** Measurement of the PNI ability of human pancreatic cancer cells, Panc-1, using an ex vivo PNI assay in the presence of control or PAP/REG3A-depleted acinar cell media (ACm/PAP$^+$ or ACm/PAP$^-$, respectively) and SC144 treatment (median ± interquartile range, $n = 3$). $^*P < 0.05$. **c** Kaplan–Meier disease-free survival curve using PAP/REG3A serum levels of patients with resected PDA from cohort 1, 2, and 3, divided into high (≥17.5 µg/L) and low (<17.5 µg/L) groups ($n = 11$ and 24, respectively). **d** Kaplan–Meier overall survival curve following resection using PAP/REG3A serum levels of patients with resected PDA from cohort 2, divided into high (≥17.5 µg/L) and low (<17.5 µg/L) groups ($n = 8$ and 6, respectively). **e** Kaplan–Meier overall survival curve following resection using PAP/REG3A serum levels of patients with resected PDA from cohort 3, divided into high (≥17.5 µg/L) and low (<17.5 µg/L) groups ($n = 10$ and 12, respectively)

we wondered whether measurement of PAP/REG3A in serum from pancreatic cancer-resected patients might also be associated with PNI-related parameters. Supporting our hypothesis, there was a significant reduction in disease-free survival for patients with PAP/REG3A levels of 17.5 µg/L or above compared to patients with PAP/REG3A levels lower than 17.5 µg/L ($P = 0.0128$, Fig. 4c). This result correlated to overall survival of patients with PDA following surgical resection of their pancreatic tumor. Using the same optimal PAP/REG3A cut-off value of 17.5 µg/L, we revealed, in two different cohorts, that patients with PAP/REG3A levels of 17.5 µg/L or above had a shorter survival following PDA resection than patients whose levels were below 17.5 µg/L ($P = 0.0462$, Fig. 4d and $P = 0.0085$, Fig. 4e). Altogether, our data confirm the clinical association between PAP/REG3A levels in serum and a worse prognosis, even for PDA-resected patients, suggesting a role for PAP/REG3A in PDA relapse.

Discussion

The management of PDA is limited by the absence of effective therapies. Besides which, clinicians urgently require accurate disease biomarkers to allow stratification of patients based on how they are likely to respond to treatment and on their likelihood of developing clinico-pathological features or PDA-associated phenotypes, both of which impact clinical care and survival. In the present study, we demonstrated that the peritumoral microenvironment, through the secretion of PAP/REG3A by inflamed acinar cells, increases cancer cell aggressiveness and favors perineural dissemination (Fig. 5).

First, we revealed that inflamed acinar cells from the peritumoral compartment are the original source of PAP/REG3A expression in PDA. Regarding our starting hypothesis on the influence of PAP/REG3A on PNI in PDA, it is important to note that the PAP/REG3A-expressing areas are those rich in nerve fibers. This correlation supports our original concept, as both tumor cells and nerve fibers, which are found in those areas, can respond to PAP/REG3A-driven signaling, as both express gp130. The association of PAP/REG3A staining and nerve fibers in similar areas could also lead to a new hypothesis that PAP/REG3A favors NR. Further evaluation of this hypothesis could bring new insights into the mechanisms associated with NR in PDA, from the timepoint of early lesions.

Looking at PNI, numerous factors from several cell types within the PDA tumor mass have been determined to impact PNI. Indeed, cancer cells, macrophages, and CAFs have previously been shown to foster PNI [31–33] or NR [15]. Interestingly, our study revealed the concrete influence of peritumoral areas on PNI. Our data, by showing an important role of the peritumoral areas, suggest that further studies should be conducted to investigate the various ways in which inflamed acinar cells impact PNI or NR in more detail. Beyond the numerous secreted proteins already associated with PNI, extracellular vesicles, reported as giving important CAFs-mediated support to tumor cells [5], and free circulating miRNA [34] are two major intercellular communication modes that should be explored in terms of the connection between inflamed acinar cells and PNI. Indeed, while CAFs are the PDA official "factories" for secreted proteins that impact neighboring and more distant stromal or tumoral cells [35], our data shed some light on an underestimated compartment combining both inflamed acinar cells and infiltrating immune cells. Determining the impact of inflamed acinar cells on neighboring tumor cells and their relative activity within PDA carcinogenesis, and associated phenotype, is important in the development of new clinical tools. Towards this, the evaluation of PAP/REG3A serum levels in patients with PDA constitutes a proof of concept, as well as holding promise for patient stratification.

We also highlighted that PAP/REG3A is able to enhance pancreatic tumor cell aggressiveness through the activation of STAT3 proteins, which are known to govern several key oncogenic signaling pathways [36]. Recently, Wormänn and colleagues reported that persistent activation of STAT3 is involved in the progression of PDA and is associated with p53 mutation in tumor cells [37]. Consistent with data obtained from Wormänn's study, most pancreatic cancer cells express constitutively activated STAT3 [38] as well as mutated p53 [39]. However, while constitutive/persistent STAT3 activation can be present in the absence of active p53 [37], p53-mutated pancreatic cancer cells are still able to respond and then enhance STAT3 signaling [40]. Interestingly, our study shows that PAP/REG3A is able to enhance the migratory and invasive ability of p53-mutated pancreatic tumor cells, through a STAT3-dependent mechanism. These data confirm that even if STAT3 is constitutively/persistently activated in PDA tumor cells, STAT3 is still able to respond then drive PAP/REG3A stimulation. A therapeutic strategy using STAT3/JAK2 inhibitors reduced tumor growth and chemoresistance [41], as well as impacting the desmoplastic stromal reaction [42]. These data reinforce the need to thoroughly understand the various processes that activate STAT3 signaling or STAT3 downstream effectors in PDA. Indeed, determining a STAT3 bypass, which renders STAT3/JAK2 inhibitors ineffective, could highlight potent STAT3-associated targets for the treatment of PDA.

In our study, we revealed that PAP/REG3A favors PNI, a well-known associated factor of pancreatic tumor cell dissemination and tumor recurrence [9]. Recent studies have revealed some of the molecules and mechanisms associated with PNI [43, 44]. However, the importance of such phenomenon on patients with PDA, and any possible use as therapeutic targets, remains unclear. In our model, PAP/REG3A-favored PNI is dependent on STAT3 activation, which strengthens the interest in STAT3-targeting therapy for PDA as well as for pancreatic cancer-associated PNI [45]. Indeed, STAT3 inhibitors in combination with PAP/REG3A-blocking antibody treatments should be investigated for patients who have undergone PDA surgical resection, to determine the impact of these treatments on tumor recurrence and disease-free survival.

In our study, blood PAP/REG3A levels were not significantly associated with patients' age, gender, or TNM (tumor, node, and metastasis) staging. Interestingly, when we evaluated patients with PDA who had undergone surgery, we found that patients with resected PDA and a high serum level of PAP/REG3A had a significantly shorter survival than patients with a low level of PAP/REG3A.

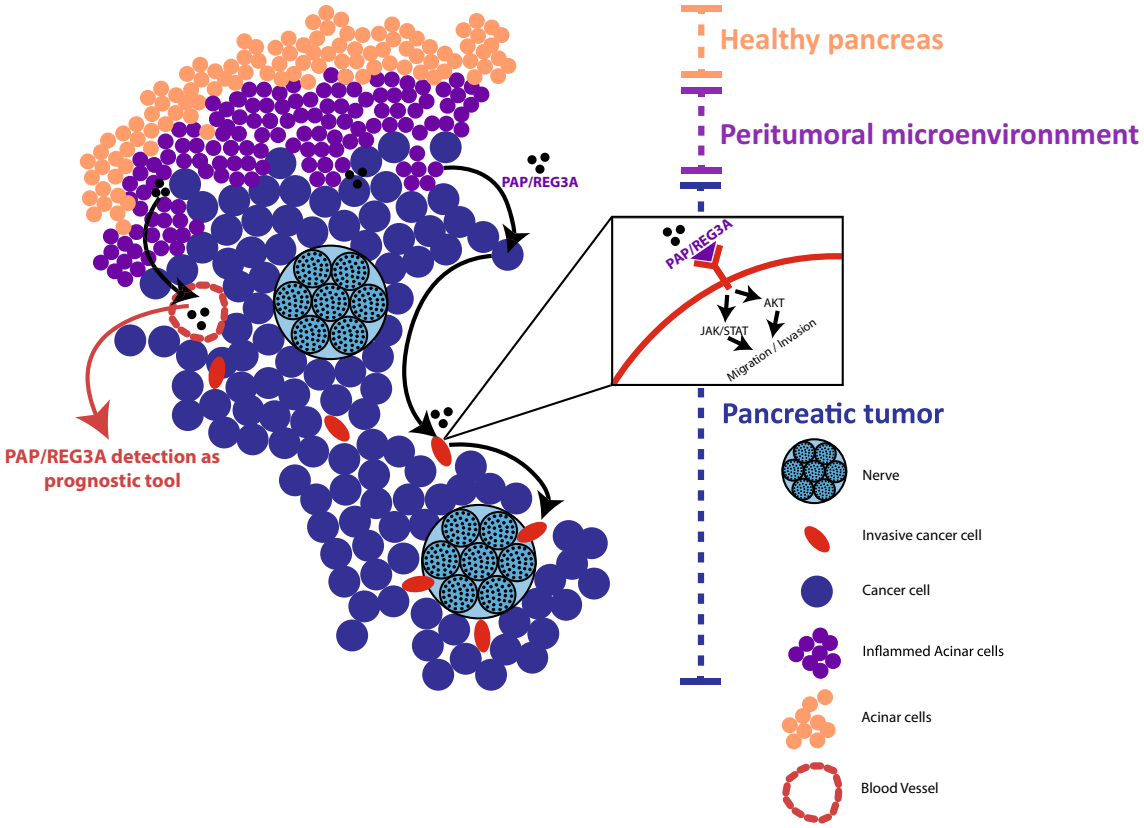

Fig. 5 Mode of action of the peritumoral microenvironment in pancreatic cancer evolution, through inflamed acinar cell production of PAP/REG3A. The peritumoral microenvironment, mainly composed of inflammatory cells and inflamed acinar cells, is at the interface of the healthy pancreas and PDA. This cellular compartment is able to drastically impact on PDA evolution. Indeed, inflamed acinar cells can secrete PAP/REG3A, which acts as a paracrine mediator. As a consequence of the PAP/REG3A-induced pathway, cancer cells enhance their aggressiveness and metastatic potential, favoring perineural invasion and tumor cell dissemination through nerve fibers. Moreover, detection of PAP/REG3A in sera meets stringent criteria as an effective biomarker, improving PDA prognosis and stratification

Moreover, the disease-free survival, reflecting the time before disease recurrence, is also shorter for patients with PDA with a high PAP/REG3A serum level compared to patients with a low PAP/REG3A level. These data, along with data showing that the level of PAP/REG3A found in the sera of patients with PDA originates from peritumoral acinar cells, it is not unreasonable to suppose that PAP/REG3A blood levels in PDA could reflect primary tumor aggressiveness or dimensions that are ultimately related to the clinical outcome of patients. As clinicians urgently require ways to accurately diagnose patients with PDA to provide effective therapeutic options, the discovery of new, effective biomarkers (enabling the prognosis and stratification of patients with PDA) is a meaningful goal to aim for. While immune biomarkers [46] and miRNAs [47] are important recent discoveries in the field [48], our data on PAP/REG3A highlights the peritumoral component as an underestimated, potent source of effective biomarkers.

In summary, our results demonstrate the influence of PAP/REG3A on PDA-associated PNI, strengthen our knowledge of the impact of the peritumoral compartment, as well as STAT3 signaling, in PDA carcinogenesis, and may open new therapeutic fields. It is also tempting to suggest that high levels of PAP/REG3A in the blood may serve as a promising tool for the stratification of patients with PDA, thereby favoring targeted and individualized therapy to prevent tumor relapse and local dissemination through NR or PNI.

Materials and methods

Mouse strains and tissue collection

Pdx1-Cre; Ink4a/Arf$^{fl/fl}$; LSL-Kras^{G12D} mice were obtained by crossing the following strains: Pdx1-Cre; Ink4a/Arf$^{fl/fl}$ and LSL-Kras^{G12D} mice kindly provided by Dr. D. Melton (Harvard Stem Cell Institute, Cambridge, MA, USA), Dr. R. Depinho (Dana-Farber Cancer Institute, Boston), and Dr. T Jacks (David H. Koch Institute for Integrative Cancer

Research, Cambridge, MA, USA), respectively. PDA-bearing 8- to 12-week-old male mice were killed with their mating control littermates. Sections of tumor or control pancreas were fixed in 4% formaldehyde for further immunostaining analysis. For isolation of acinar cells, control mice were killed between 8 and 12 weeks old. All animal care and experimental procedures were performed in agreement with the Animal Ethics Committee of Marseille number 13.

Human tissue samples

Human pancreatic adenocarcinomas, used for immunostaining, were collected from 36 patients with PDA with available clinical history during surgery at the Gastroenterology Department of the Hôpital Nord de Marseille, France. 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.

Blood sample cohorts

For cohort 1, from January 2011 to April 2015, a translational research study of blood samples was proposed to all consecutive patients ($n = 79$) with histologically proven pancreatic adenocarcinoma and treated in "Hôpital Pitié-Salpétrière" (Paris, France). This translational research was approved by the local ethics committee ("Comité de Protection des Personnes Ile de France IV"). After patients accepted and signed informed consent was given, blood was collected directly from the vena cava and the central catheter on the day of the first chemotherapy cycle. For the patients with pancreatic adenocarcinoma who underwent curative surgical resection, blood samples were collected after surgery, on the day of the first cycle of adjuvant chemotherapy. For the patients with an advanced pancreatic adenocarcinoma (locally advanced or metastatic), blood samples were collected on the day of the first cycle of chemotherapy. Two EDTA tubes and two BD® P100 tubes were used. After the blood sampling, as soon as possible and always within 3 h of collection, the EDTA and BD® P100 tubes were centrifuged at 3500 rpm for 15 min at 4 °C. Plasma was collected and stored at −80 °C in several aliquots (2 ml Eppendorf tubes) at the biological resources center of the treatment center. Data from medical records were recorded in a database. The following information was collected prospectively: patient and tumor characteristics at inclusion (gender, age, medical history, date of diagnosis, location of the primary tumor, primary tumor diameter, tumor differentiation grade, stage of the disease), biological data before first chemotherapy cycle (ACE, Ca 19-9, albuminemia, bilirubinemia) and follow-up data (date of primary resection, date and type of relapse, date of diagnosis of metastasis, date and type of chemotherapy regimen, date and type of chemoradiotherapy, date of death or last follow-up).

For cohort 2, plasma samples of the Brussels cohort were obtained from patients with histologically proven PDA ($n = 33$) any treatment was given. Among patients diagnosed with PDA, 14 underwent PDA resection and 19 had metastatic disease. Plasma samples were prospectively collected in Erasme University Hospital at the time of diagnosis, and stored under and according to rigorous standard operating procedures. Clinical and pathological data were prospectively collected and regularly updated. All research samples were collected after obtaining written informed consent for participation, in accordance with the Declaration of Helsinki, and with ethical approval from the local institutional review boards (ref:B2011/005).

For cohort 3, plasma samples were obtained from patients diagnosed with PDA ($n = 54$) in Hospital Clínic of Barcelona before treatment was given. All specimens were obtained according to the Institutional Review Board-approved procedures for consent. Ethically approved informed consent was obtained from all subjects and all the experiments conformed to the principles set out in the Declaration of Helsinki. Data from medical records were recorded in a database. Blood samples were collected in tubes containing EDTA and plasma was separated by two consecutive centrifuges ($1600\times g$ for 10 min at 4 °C followed by further centrifugation at $16{,}000\times g$ for 10 min at 4 °C to completely remove cellular components). Plasma was then aliquoted and stored at −80 °C until use.

Blood samples of healthy persons were collected by EFS (Etablissement Français du Sang).

Measurement of PAP/REG3β concentrations in blood samples and culture medium

Measurement of the concentration of human PAP/REG3β in blood samples occurred using a commercially available ELISA kit PANCREPAP (Dynabio SA, Marseille, FR) according to the manufacturer's instructions. Results were expressed as μg of PAP/REG3β per L of plasma (μg/L). Plasma samples were diluted 1/200. All samples were run in triplicate and a standard curve was established for each assay. The absorbance was measured on the Thermo Scientific™ Multiskan™ Spectrum spectrophotometer.

Cell migration assay

Cancer cell migration was studied using PKA4 and PANC-1 cell lines under different media (medium with or without recombinant PAP/REG3A or conditioned medium from

acinar cells) on Boyden chambers. Culture inserts from BD Biosciences (353097, Le pont de Claix, France) with a porous membrane of 8 µm, were coated with a mix made of gelatin 0.1% from Sigma-Aldrich (G1890, St Quentin, FR) and fibronectin 10 µg/mL from Sigma-Aldrich (F0895, St Quentin, FR) then seeded with PK4A or PANC-1 cells (100,000 or 75,000 cells, respectively, per insert) and placed into wells containing culture media (with or without recombinant PAP/REG3A). PAP/REG3A or β were used at concentrations ranging from 0 to 500 ng/mL. Migration was performed for 4 h for medium with or without recombinant PAP/REG3A or β, and for 1 h 45 min for conditioned medium from acinar cells. After cleaning and briefly staining inserts with coomassie blue, migration was assessed by counting (Image J software) the number of colored cells in 8–16 high-power fields (magnification 10×).

Cell invasion assay

Cancer cell invasion was studied using PKA4 cells on Boyden chambers according to the manufacturer's protocol (354480, Corning Lifesciences, Corning, NY, USA). Mouse recombinant PAP/REG3β was used at 500 ng/mL and AG490 at 30 µmol/L. Culture inserts were pre-coated with matrigel matrix then seeded with PK4A (100,000 cells per insert) and placed into wells containing the medium. Cells were counted after 24 h of incubation. After cleaning and briefly staining inserts with coomassie blue, invasion was assessed by counting (Image J software) the number of colored cells in 8–16 high-power fields (magnification 10×).

Ex vivo perineural invasion assay

The day before the experiment, 125,000 PK4A cells were seeded into 24-well plates in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic. After 24 h, inhibitors (AG490 at 30 µmol/L or SC144 at 2 µmol/L) were added for 2 h. Culture medium was then replaced by DMEM supplemented with 2% FBS, 1% antibiotic/antimycotic with or without PAP/REG3β (500 ng/mL) and inhibitors. A mouse sciatic nerve section (5 mm) was placed in every well and cultured for 48 h. Nerve sections were then fixed for 24 h in 4% formaldehyde and embedded in paraffin for immunohistochemistry analysis. Nerve sections from each condition were cut to make 4 µm sections from two different depths spaced by 50 µm and fixed on slides. Slides were processed for cytokeratin immunostaining by immunohistochemistry as described above. Cells stained with cytokeratin inside or in contact with the nerve were recorded (Image J software).

Statistical analysis

The results shown are medians, and error bars in graphs represent standard deviations (SD). The Mann–Whitney test, recommended for the comparison of two independent groups, was performed when required. Overall and disease-free survival were estimated using the Kaplan–Meier method using GraphPad Prism software. 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 three times.

Acknowledgements We would like to thank Bruno Olivier from INSERM U1068 (Marseille, France) for his technical assistance, Laurence Borge from the U1068 Cell Culture Platform (Marseille, France), and Karim Sari and Régis Vitestelle of the animal colony facility "PSEA" (Marseille, France). This project was supported by SIRIC PACA-OUEST (Grant INCa-DGOS-Inserm 6038), the French National Institute of Cancer (INCa, PLBIO13-134), the European Research Council under the European Union's Seventh Framework Program (FP/2007–2013)/ERC Grant Agreement No. [282036], the Instituto de Salud Carlos III (for CIBEREHD support) and FIS Grant, PI13/01224 to EF-P and PI13/02192 to MG, co-funded by FEDER-European Union. J.N., C.B., and J.R. were supported by the European Research Council under the European Union's Seventh Framework Program (FP/2007-2013)/ERC Grant Agreement no. [282036]. S.L. was supported by the "Programme Investissements Avenir" from Aix-Marseille University and J.R. by "Cancéropole PACA".

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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