Blood coagulation-(in)dependent protease activated receptor signaling in pancreatic cancer

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Chapter 2

Protease activated receptor-2 induces migration of pancreatic cancer cells in an extracellular ATP dependent manner.

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Abstract

*Background:* Protease-activated receptor 2 (PAR-2) is a G protein-coupled receptor suggested to play an important role in the proliferation and migration of tumor cells of epithelial origin. However, the role of PAR-2 in the setting of pancreatic cancer remains largely unexplored. *Objectives:* To understand the importance of PAR-2 in pancreatic cancer cell migration. *Methods and results:* The present study shows that PAR-2 does not affect pancreatic cancer cell proliferation but significantly induces the migration of pancreatic cancer cells in scratch assays. Interestingly, PAR-2 does not affect migration in a trans-well setting. This apparent discrepancy depends on extracellular ATP release in the scratch assays and the addition of exogenous (ATP)-induced PAR-2-dependent migration in trans-well assays, whereas a specific P2Y11 receptor antagonist prevents PAR-2-driven migration in scratch assays. In the scratch assays, inhibitors of Src, Rac, protein kinase C, mitogen-activated protein kinase kinase, p38, and epidermal growth factor (EGF) receptor blocked PAR-2-driven migration, whereas they did not affect fetal calf serum-driven wound closure. *Conclusion:* Taken together, PAR-2 activation drives pancreatic cancer cell migration via an EGF-Src-Rac-p38/mitogen-activated protein kinase kinase/EGF1/2 signaling pathway, which is facilitated by extracellular ATP. Targeting the PAR-2/ATP-driven signaling pathway may therefore limit cell migration, which could inhibit pancreatic cancer metastasis.
Chapter 2

Introduction

Pancreatic ductal adenocarcinoma is a devastating disease due to its highly malignant phenotype associated with rapid tumor progression and metastasis [1, 2]. Despite improvement in the treatment of cancer in general, the 5-year survival rate of pancreatic cancer remains < 5% and overall mortality approaches 99% [3]. The high mortality results from the majority of patients presenting with locally advanced and/or metastatic disease, which is rapidly progressive and inevitably fatal. Less than 20% of patients undergo surgical resection, which is associated with improved 5-year survival rates of around 15% to 20%. However, the majority of this selected group of patients eventually also die from metastatic disease [4]. Protease-activated receptor (PAR)-2 is a seven-transmembrane domain receptor belonging to the family of G protein-coupled receptors [5]. The activation of PARs is unique, as it requires proteolytic cleavage instead of ligand binding. Proteolytic removal of the N-terminal extracellular region unmasks a novel tethered ligand that interacts with the body of the receptor to induce transmembrane signaling to G proteins. The activated G proteins subsequently trigger a variety of signaling pathways affecting diverse pathophysiological responses [6].

Traditionally, PARs are activated by blood coagulation factors. It is well known that PAR-2 is activated by the tissue factor–factor VIIa (FVIIa) complex and by FXa [7]. Interestingly, pancreatic cancer is among the most common malignancies associated with thromboembolic events, and thromboembolic events confer a significantly worse prognosis [8]. In addition to coagulation factors however, some other proteases have been shown to be PAR-2 agonists in vitro in different cell systems [9, 10].

Several lines of experimental evidence suggest that PAR-2 may play an important role in tumors from epithelial origin. PAR-2 is overexpressed in breast tumor specimens compared with normal breast tissue [11], whereas spontaneous breast cancer development and metastasis are reduced in PAR-2-deficient mice [12]. In line with these experimental animal data, PAR-2
induces migration of breast cancer cells in vitro [13, 14]. Moreover, PAR-2 stimulates both proliferation and migration of colon cancer cells [15, 16] and proliferation of cervical and pancreatic cancer cells [17].

As already touched on, metastasis is a major cause of morbidity and mortality in pancreatic cancer patients. To reduce the mortality from pancreatic cancer, it is essential to understand how pancreatic cancer cells escape the primary tumor and spread to secondary sites. Because increased cell migration is a key event in the metastatic cascade, we addressed the importance of PAR-2 in pancreatic cancer cell migration.

Materials and methods

Cell culture

Human pancreatic cancer cell lines BxPC-3 (ATCC; Manassas, VA, USA) and Capan-2 (Cell-Line-Service, Eppelheim, Germany) were maintained at 5% CO₂ and 37°C in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (BxPC-3) or 15% (Capan-2) fetal calf serum (FCS).

Reagents

PAR-2 agonist peptide (PAR2-AP; H-Ser-Leu-Ile-Gly-Lys-Val-NH₂; HPLC purity > 99%, mass spectrometry verified) was from GL Biochem (Shanghai, China). The reverse-sequence receptor-inactive PAR-2 peptide (H-Leu-Ser-Ile-Gly-Lys-Val-NH₂) was from Peptides International (Louisville, KY, USA). Sirolimus, U0126, and SB203580 were from LC Laboratories (Woburn, MA, USA). Chelerythrine, oligomycin, and apyrase were from Sigma (Zwijndrecht, the Netherlands). ATP receptor inhibitors NF157 and MRS2578 were from Santa Cruz Biotechnology (San Diego, CA, USA), MRS2179 and MRS2211 were from Abcam (Cambridge, UK), and PPADS and suramin were from Sigma. PP1 was from BioMol (Exeter, UK), and
NSC23766 was from Tocris Biosciences (Bristol, UK). The concentrations of all inhibitors are based on literature as detailed in the supplementary information. Antibodies against pERK and pSrc were from Cell Signalling (Boston, MA, USA). Antibodies against b-actin, a-tubulin, and GAPDH were from Santa Cruz Biotechnology.

**PAR-2 Expression analysis**

Publicly available Affymetrix expression data from pancreatic cancer cell lines were derived from the Cancer Cell Line Encyclopedia project database (http://www.broadinstitute.org/ccle [18]) using the R2 microarray analysis and visualization platform (http://r2.amc.nl). Expression levels in pancreatic cancer patients were derived by the R2 program from Illumina microarray data of 90 primary pancreatic ductal adenocarcinoma samples [19], which are available from the International Cancer Genome Consortium Data Portal (http://dcc/icgc.org) under the project code: Pancreatic Cancer (QCMG, AU).

**Wound Scratch assay.**

Cells were seeded onto six-well plates and maintained incomplete growth medium. After the cells formed a confluent monolayer, a scratch was created in the center with a p200 pipette tip. Next, cells were washed and incubated for 24 or 48 h with serum-free medium in the presence of the indicated agonists/inhibitors. Saline and 10% FCS were used as negative and positive controls, respectively. For inhibitor studies, cells were pre-incubated with the inhibitor 1 h before the scratch was made. The ability of cells to migrate was assessed by comparing the 0 and 24 or 48 h phase-contrast micrographs of six marked points along the wounded area at each well. The percentage of non-recovered wound area was calculated by dividing the non-recovered area after 24 or 48 h by the initial wound area at 0 h as previously described [20].
**Cell proliferation assay.**

Cells were seeded at a density of 5000 cells/well onto black 96-well plates in complete growth medium. After overnight incubation in serum-free medium, cells were cultured for 72 h in the absence/presence of PAR2-AP, after which cell proliferation was measured using the cell proliferation ELISA 5-bromo-2’-deoxyuridine (BrdU) assay (Roche, Almere, the Netherlands) as recommended by the manufacturer.

**Cell viability Glo-assay.**

Cells were seeded at a density of 5000 cells/well onto 96-well plates in complete growth medium. After overnight incubation in serum-free medium, cells were cultured for 24–72 h in the absence/presence of PAR2-AP. Cell viability was measured using the cell viability Glo-assay (Promega, Leiden, the Netherlands) as recommended by the manufacturer.

**Trans-well migration assay.**

Cell migration was performed as described previously [21, 22] with minor modifications. Briefly, $1 \times 10^5$ BxPC-3 cells were labelled with Cell Trace CFSE (Invitrogen) and transferred to 8- µm pore-size Cell Culture inserts coated with 0.1% (w/v) collagen. The cells were incubated in serum-free medium with or without PAR2-AP and/or ATP, and the inserts were incubated at 37 °C in growth medium supplemented with 10% FCS as chemoattractant. Subsequently, fluorescence values representing the number of cells on the bottom side of the insert were read on a BioTek plate reader at 485/528 nm (BioTek®, Bad Friedrichshall, Germany).
Live cell microscopy.

Cells were seeded at a density of 5000 cells/well onto six-well plates. After overnight incubation in serum-free medium, cells were incubated with PAR2-AP, ATP, or PAR2-AP/ATP. Cells were imaged using a Leica IR-BE inverted wide field microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 37 °C in an atmosphere containing 5% CO2. Phase contrast images were acquired at 10-min intervals for 80 h using a x10 objective. Images were processed and analyzed using custom-made software and Image Pro Plus (Media cybernetics, Carlsbad, CA, USA).

Western blot assay.

Cells were seeded at 1x10^5 cells/well onto six-well plates and grown to 70% confluence. After overnight incubation in serum-free medium, cells were incubated with the indicated compounds for the indicated time, after which the cells were lysed in Laemmli lysis buffer and the lysates were incubated for 5 min at 95 °C. Cell extracts were resolved by SDS-PAGE (10%) and transferred to polyvinylidene difluoride membranes (PVDF). Membranes were blocked in 5% fat-free dried milk in TBS with 0.05% Tween (TBST) and incubated overnight at 4 °C with primary antibodies at 1:1000 dilutions in 1% BSA/ TBS-T. After washing in TBS-T, membranes were incubated with secondary antibodies (horseradish peroxidase conjugated) at 1:1000 dilutions in TBS-T for 1 h. Detection was performed by enhanced chemiluminescence on an LAS4000 imager (GE Healthcare Europe GmbH, Diegem, Belgium).

Statistics.

Differences between multiple groups were examined by one-way ANOVA and/or two-tailed ANOVA with Tukey t-tests using GraphPad Prism.
(GraphPad Software, Inc., La Jolla, CA, USA), with P < 0.05 considered significant.

**Results**

**PAR-2 is widely expressed in pancreatic cancer cell lines.**
To determine the potential importance of PAR-2 in pancreatic cancer cells, we assessed PAR-2 expression levels in a panel of 41 human pancreatic cancer cell lines [18]. All cell lines expressed PAR-2 in high amounts (Figure 1A). Panc-1 cells showed highest PAR-2 expression, whereas PAR-2 levels were low in pa-tu-8988t cells. BxPC-3 and Capan-2 cells, which were selected for subsequent experiments, showed intermediate PAR-2 expression levels. In line, PAR-2 was highly expressed in all 90 patient-derived pancreatic tumors examined [19]. For comparison, the expression levels of PAR-1 and tissue factor were analyzed, also showing high expression levels (Figure S1).

Next, we determined whether the expression of PAR-2 was associated with responses. As readout, we examined Erk1/2 phosphorylation, which is widely used as a surrogate marker for PAR-2 activation [10]. Indeed, Erk1/2 phosphorylation was observed 5 min after stimulation by PAR-2-AP and peaked after 10 min (Figure 1B, C). At 60 min post stimulation, Erk1/2 phosphorylation returned to basal levels. Hence, BxPC-3 and Capan-2 cells express functionally active PAR-2.
PAR-2 activation induces pancreatic cancer cell migration in scratch assays.

To evaluate the potential importance of PAR-2 on pancreatic cancer cells, the effect of PAR-2 stimulation was assayed in wound scratch assays. Both PAR2-AP-treated BxPC-3 cells as well as 10% FCS-treated cells (positive control) reduced the wound size by approximately 50% within 24 h compared with no significant reduction in wound size for control-treated cells (Figure. 2A). After 48 h PAR2-AP reduced the wound size by around 70%, whereas the wound was completely closed with 10% FCS treatment (Figure. 2A, C). Capan-2 cells were slightly less responsive to PAR-2 stimulation, but PAR2-AP also significantly induced wound closure in these cells after 48 h (Figure. 2B). Similar effects were seen after PAR-2 activation with trypsin (Figure. 2), FXa, and FVIIa (Figure. S2A), whereas a reverse-sequence receptor-inactive peptide (PAR2-AP-Neg) did not induce migration (Figure. 2). Pretreatment of cells with P2pal18 significantly inhibited PAR-2-AP-induced migration (Figure. S2B). Interestingly, PAR-1...
activation by thrombin did not induce migration of both cell lines (Figure. S2C).

**Figure 2.** Protease-activated receptor 2 (PAR-2) activation induces pancreatic cancer cell migration in scratch assays. Quantification of the wound size of (A) BxPC-3 and (B) Capan-2 monolayers after treatment with saline (Neg Ctrl), PAR2-AP (100 μmol/L), 10% FCS (Pos Ctrl), trypsin (10 nmol/L), or PAR2-AP-Neg (100 μmol/L) for 24 and 48 h. (C) Representative pictures of BxPC-3 cells before and 48 h after the scratch was made (mean SD; five experiments with n =3). ***P < 0.0001 and **P < 0.001 compared with the negative control.
**PAR-2 activation does not affect pancreatic cancer cell proliferation**

Wound closure in scratch assays may depend on both cell migration and proliferation. To discriminate between migration versus proliferation as being important for the observed effect in the scratch assays, we determined PAR-2-dependent proliferation. Importantly, stimulation of both BxPC-3 and Capan-2 cells with PAR2-AP for 24–72 h did not induce proliferation (Figure 3A shows proliferation after 72 h), whereas treatment with 10% FCS induced proliferation by ~ 3-fold. In line, PAR-2 stimulation also did not affect the number of viable cells as assessed by the cell viability-Glo assay (Figure. 3B). Both BxPC-3 and Capan-2 cells showed a small increase in luminescence over time, but this increase was independent of PAR-2 stimulation. Cells incubated in 10% FCS did show a large increase in viability/proliferation as evident from an almost 4-fold increase in luminescence. To confirm that the observed effect of PAR-2 stimulation in the scratch assays is not dependent on proliferation, we performed scratch assays in the absence and presence of a well-known proliferation inhibitor (rapamycin/Sirolimus). As shown in Figure. 3C, PAR2-AP induced wound closure in both BxPC-3 and Capan-2 cells is independent of rapamycin, showing that PAR-2-driven wound closure is solely dependent on cell migration.
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**Figure 3.** Activation of protease-activated receptor 2 (PAR-2) has no effect on proliferation of pancreatic cancer cells. (A) Proliferation of BxPC-3 (left) or Capan-2 (right) cells in the absence/presence of PAR2-AP. Saline served as negative control and 10% FCS as positive control. Shown is mean SD (three experiments with n = 6) at t = 72 h. (B) Cell viability in the presence/absence of PAR2-AP (mean SD; three experiments with n = 4). (C, D) PAR-2-driven migration is not affected by rapamycin (20 nmol/L) in (C) BxPC-3 or (D) Capan2 cells. Shown is the wound size (mean SD) at 48 h (three experiments with n = 3). **P < 0.001 and ***P < 0.0001 compared with the negative control.

**ATP potentiates PAR-2-driven pancreatic cancer cell migration**

To substantiate that PAR-2 activation drives pancreatic cancer cell migration, we evaluated migration of BxPC-3 cells in trans-well assays. Surprisingly, PAR2-AP stimulation did not affect migration compared with control-treated cells (Figure. 4A) in these assays. As it is well known that cells in proximity of a scratch release high amounts of ATP [23, 24], we hypothesized that PAR-2-induced migration may depend on ATP release. To prove or refute this hypothesis, we performed transwell migration assays in
the absence and presence of exogenous ATP. As shown in Figure 4A, the addition of ATP or PAR2-AP alone did not induce migration of BxPC-3 cells. However, the combination of ATP and PAR2-AP did significantly induce migration of BxPC-3 cells.

To confirm that ATP is required for PAR-2-driven migration, we analyzed the effect of oligomycin and apyrase on PAR-2-induced migration in scratch assays. Importantly, the addition of oligomycin and/or apyrase significantly blocked PAR-2-driven cell migration in both BxPC-3 (Figure 4) and Capan-2 cells (data not shown). Interestingly, oligomycin and apyrase did not affect FCS-driven migration (data not shown).

ATP may potentiate PAR-2-dependent migration by serving as an energy reservoir or ATP may act on the P2Y receptor thereby enhancing the effect of PAR-2. To discriminate between these hypotheses, we treated cells with suramin (P2Y receptor blocker). As shown in Figure 4B and C, Suramin treatment completely blocked PAR-2-driven cell migration in BxPC-3 and Capan-2 cells. Again, FCS-driven migration was not affected by the ATP receptor blocker (data not shown).

Suramin inhibits P2Y1, P2Y11, P2Y12, and P2Y13 in the used concentrations, of which P2Y12 can be excluded as a potential involved receptor because it is only expressed on platelets and microglia [25]. To further characterize the specific P2Y receptor involved in ATP-PAR-2-dependent migration, we treated cells with PPADS (P2Y1 inhibitor), MRS2211 (P2Y1 and P2Y13 inhibitors), and MRS2179 (P2Y1 inhibitor), and none of these inhibitors inhibited PAR-2-dependent migration (Figure 4D, E). Next, we treated cells with NF157, and this specific P2Y11 inhibitor [26] did inhibit PAR-2-dependent migration.
Figure 4. ATP potentiates Protease-activated receptor 2 (PAR-2)-driven pancreatic cancer cell migration. (A) PAR-2 activation does not induce migration of BxPC-3 cells in trans-well assays (left). Migration of BxPC-3 cells stimulated with PAR2-AP (100 µmol/L), ATP (100 µmol/L), or both PAR2-AP and ATP after 10 h (right). Shown is mean SD (three experiments with n = 2). (B) Inhibition of ATP synthesis by oligomycin (+, 100 nmol/L; ++, 500 nmol/L) or apyrase (10 units/ml) inhibits PAR-2-dependent migration of BxPC-3 cells in
scratch assays. Shown is mean SD (three experiments with n = 3). (C) Representative pictures of BxPC-3 cells before and 48 h after the scratch was made. (D) PAR-2–dependent migration of BxPC-3 cells in the presence of specific ATP receptor inhibitors: PPADS (10 µmol/L), MRS2179 (30 µmol/L), MRS 2211 (30 µmol/L), MRS 2578 (0.5 µmol/L), suramin (+, 10 µmol/L; ++, 30 µmol/L), or NF157 (0.5 µmol/L). Shown is mean SD (three experiments with n = 3). (E) Representative pictures of BxPC-3 cells before and 48 h after the scratch was made. ***P < 0.0001 compared with negative control.

Finally, we assessed cell migration using live cell microscopy. As shown in Figure 5, cells cultured in the presence of PAR2-AP or ATP alone did not seem to migrate in the time span of the experiment. In contrast, cells cultured in the presence of both PAR2-AP and ATP did move around in all directions with remarkable cell shape changes (evident after 40–80 h).
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Figure 5. Life cell microscopy of BxPC-3 cells. Images of BxPC-3 cells in the presence of protease-activated receptor 2 agonist peptide (PAR2-AP (100 µmol/L), ATP (100 µmol/L), or a combination of PAR2-AP and ATP. Shown are representative pictures from different time points (two experiments with n = 5).

PAR-2 drives pancreatic cancer cell migration in an epidermal growth factor receptor/Src/Rac/p38/mitogen activated protein kinase kinase-dependent manner.

To analyze the underlying signaling pathways by which PAR-2 drives pancreatic cancer cell migration, we determined phosphorylation of the classic PAR-2 downstream targets Src and ERK1/2 in BxPC-3 cells. PAR2-AP and ATP alone or in combination did induce Erk1/2 phosphorylation (Figure 6A). Erk1/2 activation was observed after 5 min, peaked at 10 min, and returned to basal levels after 60 min in all conditions. PAR2-AP did not induce phosphorylation of Src, whereas ATP did induce Src phosphorylation at 5 min after stimulation. These ATP-induced pSrc levels returned to baseline 10 min after stimulation. Interestingly, the combination of PAR2-AP and ATP induced phosphorylation of Src within 5 min and pSrc levels remained induced for up to 30 min. Notably, the inhibition of Src does not affect PAR-2-induced ERK phosphorylation, whereas (as expected) inhibition of the P2Y receptor by suramin also does not affect PAR-2-induced ERK phosphorylation, indicating that these kinases are independently activated in these conditions (Figure 6B).

To further delineate the underlying mechanism by which PAR-2 induces pancreatic cancer cell migration, we performed scratch assays in the absence/presence of specific inhibitors for key signal transduction pathways involved in cell migration. As shown in Figure 6C, PAR-2-induced migration was slightly but significantly inhibited in the presence of chelerythrine (PKC inhibitor). Moreover, in both BxPC-3 and Capan-2 cells, PAR-2-driven cell migration was blocked in the presence of inhibitors for Rac (NSC23766), mitogen-activated protein kinase kinase (MEK) (U0126),
p38 (SB203580), and Src (PP1), suggesting all these kinases are involved in PAR-2-dependent pancreatic cancer cell migration. Interestingly, none of the inhibitors modified wound closure induced by FCS (data not shown). Finally, we assessed the potential transactivation of the epidermal growth factor receptor (EGFR) by analyzing cell migration in the presence of the EGFR inhibitor erlotinib. As shown in Figure 6C, blocking EGFR significantly inhibits PAR-2-induced migration. Overall, these data suggest that PAR-2-driven pancreatic cancer cell migration is dependent on EGFR transactivation with subsequent activation of a Src/Rac/MEK/ERK/p38-dependent pathway (Figure 7).

**Figure 6.** Protease-activated receptor 2 (PAR-2)-dependent pancreatic cancer cell migration is dependent on an Src/ERK signaling pathway. (A) Src and Erk1/2

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phosphorylation after addition of PAR2-AP (100 µmol/L), ATP (100 µmol/L), or PAR2-AP and ATP to BxPC-3 cells. Shown are representative blots of an experiment performed three times. (B) PAR2-AP induced Src and Erk1/2 phosphorylation in the absence/presence of PP1 (Src inhibitor) or suramin (ATP receptor blocker). (C) Quantification of the wound size of BxPC-3 (left) and Capan-2 (right) monolayers after treatment with PAR2-AP in the presence of the indicated inhibitors (mean SD; two experiments with n = 2). *P < 0.05 and ***P < 0.001 compared with PAR2-AP-stimulated cells in the absence of inhibitor.

Figure 7. Proposed model of protease-activated receptor 2 (PAR-2)/ATP-dependent pancreatic cancer cell migration. PAR-2 activation leads to phosphorylation/activation of the MEK-ERK pathway, in combination with PKC-dependent Src activation. However, only after ATP-dependent sustained Src phosphorylation is EGFR trans-activated, leading to downstream activation of PKC/Rac/MEK/p38 resulting in pancreatic cancer cell migration.
Discussion

Pancreatic cancer is a devastating disease with the worst survival outcome of all human cancers. Importantly, metastasis is a major cause of morbidity and mortality in pancreatic cancer patients, and it is thus essential to fully appreciated pancreatic cancer cell metastasis (as highlighted in several recent reviews [27, 28]). Interestingly, PAR-2 is highly expressed in pancreatic cancer cell lines (Figure. 1A) and patient-derived neoplasms (Figure. 1B), whereas PAR-2 expression is rarely expressed in the pancreatic duct in healthy individuals [29]. It is thus tempting to speculate that PAR-2 may be involved in pancreatic cancer development, and here we addressed this hypothesis by specifically analysing the importance of PAR-2 in pancreatic cancer cell migration and proliferation. We show that PAR-2 does not modify pancreatic cancer cell proliferation, whereas it potentiates migration of pancreatic cancer cells. Interestingly, PAR-2 only drives migration of pancreatic cancer cells in the presence of endogenous ATP.

We show that PAR-2 drives migration of pancreatic cancer cells in scratch assays but not in trans-well migration assays. An important difference between these two assays is that cells in close proximity of a scratch do release large amounts of ATP [30]. This is interesting as several recent reports implicate ATP in cell migration in various cell types [24, 31], and PAR-2-dependent wound closure may thus depend on ATP release. Indeed, we show that an inhibitor of ATP synthesis, an ATP diphosphohydrolase, and specific ATP receptor blockers prevent PAR-2-driven migration in scratch assays (Figure. 4). Moreover, we show that PAR-2 does induce migration of pancreatic cancer cells in trans-well migration assays in the presence of ATP, whereas both PAR-2 and ATP alone do not induce migration in these assays. Overall, our data thus show that PAR-2 and ATP contribute to drive pancreatic cancer cell migration.

One may argue that scratch-induced ATP release driving PAR-2-dependent migration is somewhat artificial. Importantly, however, ATP concentrations
are much higher in the interstitium of solid tumours compared with healthy tissues [32]. As expected, ATP is released from the cytosol of necrotic cells, which are omnipresent in the centre of fast-growing tumours [33]. Moreover, conventional anticancer therapies induce ATP release from dying tumour cells [34]. Taken together, extracellular ATP is highly relevant in the setting of cancer, and our data suggest that PAR-2 drives pancreatic cancer cell migration in a relevant setting of tumour necrosis and/or chemotherapy-induced autophagy.

Rather surprisingly, we did not observe any effect of PAR-2 on pancreatic cancer cell proliferation (Figure. 3). Inline, we did not observe any effect of mammalian target of rapamycin (mTOR) inhibition on PAR-2-induced migration in scratch assays. Importantly, mTOR is a key signalling element involved in the control of cell growth and proliferation, and mTOR has been shown to be critically involved in cancer cell survival and proliferation [35]. This is unexpected as previous reports showed that PAR-2 activation is involved in proliferation of (among others) pancreatic cancer cells [17, 29, 36]. At this moment, we do not have a proper explanation for the different PAR-2-induced responses with respect to pancreatic cancer cell proliferation.

G protein-coupled receptors are able to use EGFR as a signalling intermediate [37], and PAR-2 seems to not be an exception [38]. Here, we show that erlotinib inhibits PAR-2-induced migration pinpointing EGFR transactivation as an early event in PAR-2-driven migration. Moreover, recent studies showed that PAR-2 mediates proliferation, migration, and invasion of malignant glioma cells through the ERK1/2 pathway [39]. Here, we show that PAR-2-driven pancreatic cancer cell migration is also dependent on the ERK pathway. Indeed, PAR-2 leads to ERK phosphorylation, and inhibition of both ERK and MEK prevents PAR-2-induced migration in scratch assays. Next to MEK/ERK, p38 seems involved in PAR-2-dependent migration as evident from the fact that a specific p38 inhibitor inhibits PAR-2-dependent migration, whereas inhibition of Rac (which is upstream of p38) also completely blocks PAR-2-
driven migration of pancreatic cancer cells. Intriguingly, however, activation of the MEK/ERK and Rac/p38 pathways is not sufficient for pancreatic cancer cell migration, as evident from the trans-well assays. Actually, extracellular ATP is essential for PAR-2-driven migration as it leads to sustained Src phosphorylation, and the combination of sustained Src, MEK/ERK, and p38 signalling pathways ultimately leads to (PAR-2-dependent) migration of pancreatic cancer cells.

In conclusion, PAR-2 drives pancreatic cancer cell migration via an EGFR-Src-Rac-p38/MEK/ERK1/2 signalling pathway in an ATP-rich environment. Targeting the PAR-2/ATP-driven signalling pathway in such a setting (i.e., during tumour cell necrosis/autophagy) may therefore limit cell migration, thereby preventing pancreatic cancer metastasis. In vivo experiments should prove or refute this hypothesis however.
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References


Supplementary Figures.

**Figure S1.** PAR-1 and tissue factor are expressed in pancreatic cancer cells with different levels. (A) PAR-1 expression in a panel of 41 pancreatic cancer cell lines and (B) 90 patient samples was evaluated by expression microarray analysis. (C) Tissue factor expression in a panel of 41 pancreatic cancer cell lines and (D) 90 patient samples was evaluated by expression microarray analysis. Shown are the absolute expression levels on a 2-log scale.

**Figure S2.** (A) Quantification of the wound size of BxPC-3 monolayers after treatment with saline (Neg Ctrl), FXa (1 U ml⁻¹) or FVIIa (100 nmol/L) for 48 h. (B) Quantification of the wound size of BxPC-3 monolayers after treatment with PAR2-AP in the absence or presence of P2pal-18S for 48 h. (C) PAR-1 activation does not induce pancreatic cancer cell migration in scratch assays. Quantification of the wound size of BxPC-3 monolayers after treatment with either saline (Neg Ctrl), 10% FCS (Pos Ctrl), or thrombin (1 unit/ml) for 48 h. All data are expressed as mean ± SD (two experiments with n = 2–3).