Transforming Growth Factor- β 1/Activin Receptor-like Kinase 5-Mediated Cell Migration is Dependent on the Protein Proteinase-Activated Receptor 2 but not on Proteinase-Activated Receptor 2-Stimulated G_q -Calcium Signaling[§]

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ABSTRACT

Transforming growth factor- β (TGF- β), serine proteinases such as trypsin, and proteinase-activated receptor 2 (PAR2) promote tumor development by stimulating invasion and metastasis. Previously, we found that in cancer cells derived from pancreatic ductal adenocarcinoma (PDAC) PAR2 protein is necessary for TGF- β 1-dependent cell motility. Here, we show in the same cells that, conversely, the type I TGF- β receptor activin receptor-like kinase 5 is dispensable for trypsin and PAR2 activating peptide (PAR2-AP)-induced migration. To reveal whether G_q -calcium signaling is a prerequisite for PAR2 to enhance TGF- β signaling, we investigated the effects of PAR2-APs, PAR2 mutation and PAR2 inhibitors on TGF- β 1-induced migration, reporter gene activity, and Smad activation. Stimulation of cells with PAR2-AP alone failed to enhance basal or TGF- β 1-induced

C-terminal phosphorylation of Smad3, Smad-dependent activity of a luciferase reporter gene, and cell migration. Consistently, in complementary loss of function studies, abrogation of the PAR2- G_q -calcium signaling arm failed to suppress TGF- β 1-induced cell migration, reporter gene activity, and Smad3 activation. Together, our findings suggest that the calcium-regulating motif is not required for PAR2 to synergize with TGF- β 1 to promote cell motility. Additional experiments in PDAC cells revealed that PAR2 and TGF- β 1 synergy may involve TGF- β 1 induction of enzymes that cause autocrine cleavage/activation of PAR2, possibly through a biased signaling function. Our results suggest that although reducing PAR2 protein expression may potentially block TGF- β 's prooncogenic function, inhibiting PAR2- G_q -calcium signaling alone would not be sufficient to achieve this effect.

Introduction

Transforming growth factor- β 1 (TGF- β 1) controls a plethora of cellular functions under physiological conditions and in disease states such as cancer development. In cancer, TGF- β resides in the tumor microenvironment in an inactive (latent) form and upon activation and release from its large latency

complex TGF- β binds to its cognate receptor(s) on target cells to control proliferation, cell motility, and morphologic plasticity. TGF- β signals by assembling the ALK5 and TGF- β type II ($T\beta$ RII) transmembrane serine/threonine kinase receptors into an active ternary complex. Following its phosphorylation by $T\beta$ RII on specific serine/threonine residues, ALK5 activates the Smad pathway and may also trigger non-Smad pathways, e.g., p38 and extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) signaling (Neuzillet et al., 2014). Phosphorylation of Smad2 and Smad3 on their C terminus by the ALK5 kinase represents a critical step in the initiation of TGF- β signaling. Smad2 and Smad3

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ABBREVIATIONS: ALK5, activin receptor-like kinase 5; AP, activating protein; CIM, cell-electrode impedance; CRISPR, clustered regularly interspaced short palindromic repeats; CTGF, connective tissue growth factor; ERK, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; PAR2, proteinase-activated receptor 2; PDAC, pancreatic ductal adenocarcinoma; RFP, red-fluorescent protein; TGF- β , transforming growth factor- β ; $T\beta$ RII, TGF- β type II.

phosphorylated in response to TGF- β stimulation bind to Smad4, which is translocated to the nucleus to regulate TGF- β target gene expression (Derynck and Zhang, 2003). Smad signaling is essential for inducing the activity of specific genes and, consequently, TGF- β -mediated responses including growth inhibition (Kretschmer et al., 2003), epithelial-to-mesenchymal transition (Miyazono et al., 2012), angiogenesis (Petersen et al., 2010), migration, invasion, and metastasis (Schniewind et al., 2007; Drabsch and ten Dijke, 2012; Miyazono et al., 2012; Neuzillet et al., 2014).

The family of proteinase-activated receptors (PARs), comprising PARs 1–4, is a subgroup of the G-protein-coupled receptor superfamily (Soh et al., 2010; Adams et al., 2011). The mechanism of proteolytic activation exhibited by PARs is unique; serine proteinases cleave the PARs at specific sites located in the extracellular N terminus, resulting in the exposure of a “tethered ligand.” The tethered ligand sequences, remaining attached to the receptor, bind to domains in the extracellular part of the receptor to induce conformational changes and various signaling events including activation of G-proteins and the β -arrestin pathway and transactivation of a variety of other receptors (Ramachandran et al., 2012; Gieseler et al., 2013). The prototype enzyme activator for PAR2 is trypsin, which cleaves PAR2 at its “canonical” R/S tethered ligand-generating activation site, whereas other serine proteinases, such as neutrophil elastase, utilize non-canonical sites within the extracellular domain of PAR2 (Ramachandran et al., 2011). Of note, although trypsin activation of PAR2 generates signaling via multiple G-proteins ($G_q/G_{12/13}$) and β -arrestin to signal via elevated intracellular calcium and mitogen-activated protein kinase (MAPK), “non-canonical” cleavage in the N-terminal domain of PAR2, e.g., by neutrophil elastase, can result in “biased signaling” to activate MAPK by a process that is $G_{12/13}$ -Rho kinase-dependent but both calcium- and β -arrestin-independent (Ramachandran et al., 2011; Hollenberg et al., 2014).

Both TGF- β and PAR2 are involved in the induction of fibrosis in pancreatic cancer (Ikeda et al., 2003), in part through their ability to upregulate TGF- β 1 and other profibrogenic genes (Ikeda et al., 2003; Knight et al., 2012). Like TGF- β , PAR2 promotes cell motility and invasion in many cancer types (Ge et al., 2004; Shi et al., 2004; Morris et al., 2006; Kaufmann et al., 2009; Su et al., 2009) including pancreatic ductal adenocarcinoma (PDAC) (Ikeda et al., 2003). The overlapping patterns of tissue expression and spectra of cellular responses as well as mutual regulatory interactions suggested functional cooperativity between TGF- β receptor(s) and PAR2. However, although PAR2 is known to cooperate with members of other receptor classes (Gieseler et al., 2013), until recently it was not known that PAR2 can also interact with the TGF- β receptor(s). The first evidence for this interaction came from the observation that PAR2 can transactivate ALK5 (and the epidermal growth factor receptor, EGFR) with relevance to renal fibrosis (Chung et al., 2013). More recently, we discovered another aspect of signaling crosstalk between TGF- β and PAR2: in PDAC-derived cells and immortalized keratinocytes, PAR2 protein was required for maintaining the expression of ALK5 and ALK5-mediated pro-oncogenic effects such as migration and invasion (Zeeh et al., 2016). The mutual functional interactions between these two receptors are also reflected in the sharing of common signaling pathways such as protein kinase

C (PKC)/IP $_3$ -calcium (Soh et al., 2010; Ramachandran et al., 2012), ERK1/2 (Lee et al., 2007; Guo et al., 2011), and, in some cells, canonical Smad signaling (Chung et al., 2013). In the present study, we ask whether PAR2 activation/ G_q -calcium signaling is required for PAR2 to stimulate TGF- β -dependent cell motility in PDAC-derived cells.

Materials and Methods

Antibodies and Reagents. For immunoblot analyses, the following antibodies were employed: TGF β RI/ALK5 (V-22, cat. no. sc-398) and HSP90 α/β (H-114, cat. no. sc-7947) both from Santa Cruz Biotechnology (Heidelberg, Germany), phospho-Smad2 [cat. no. 3101, recognizing Smad2 that is phosphorylated on serines 465 and 467 (Ser465/467); Cell Signaling Technology, Frankfurt, Germany], phospho-Smad3 (Ser423/425) (cat. no. AB3226; R&D Systems, Wiesbaden, Germany), Smad2 (cat. no. 1736-1; Epitomics, Burlingame, CA), Smad3 (cat. no. ab40854; Abcam, Cambridge, UK), β -actin (Sigma, Deisenhofen, Germany), HA (clone 12CA5, cat. no. 1 583 816; Pharmacia, Uppsala, Sweden). TGF- β 1 was purchased from R&D Systems (cat. no. 240-B) or ReliaTech (Wolfenbüttel, Germany, cat. no. 300-023), EGF from PeproTech (Hamburg, Germany). BAPTA/AM, U0126, and NSC23766 were purchased from Calbiochem/Merck, SB431542 (Inman et al., 2002) from Sigma and the selective PAR2 antagonist and calcium signaling inhibitor ENMD-1068 (Kelso et al., 2006) from Enzo Life Sciences (# BML-N110-0005). The novel small molecule PAR2 anti-inflammatory antagonist, GB88 (5-isoxazolyl-Cha-Ile-spiroindene-1,4-piperidine), which inhibits PAR2 calcium signaling was kindly provided by D. Fairlie (University of Adelaide, Australia) (Suen et al., 2012). Antagonists were added to cells 30 minute before addition of TGF- β 1. The concentration of TGF- β 1 was 5 ng/ml in all experiments.

Peptides. The PAR2-selective peptide agonist SLIGKV-NH $_2$ and the PAR1-specific agonist peptide TFLLRN-NH $_2$ (STRAP-1) were obtained from Bachem (Bubendorf, Switzerland). The PAR2-specific peptide 2-furoyl-LIGRLO-NH $_2$ (2f-LI, EC $_{50}$ = 2.5 μ M), and the negative control peptides LRGILS-NH $_2$ (inverse) and LSIQRL-NH $_2$ (inactive) were synthesized as described in detail earlier (Kaufmann et al., 2009). The N-palmitoylated pepducin PAR2 peptide antagonist P2pal-18S (Sevigny et al., 2011), that like GB88 blocks PAR2 calcium signaling, as well as its reverse sequence negative control RP-P2pal (palm-KIASKRKKESNEDMASSR-NH $_2$) were synthesized and confirmed as described above for other peptides.

Cell Culture and Generation of PAR2 N-Terminal Mutants with CRISPR/Cas9 Technology. The TGF- β sensitive Panc1 and Colo357 human PDAC cell lines were cultured as described (Chen et al., 2002). HEK293T and HaCaT cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin.

Panc1 cells with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated genomic mutations in *F2RL1* were designed as follows (see Supplemental Fig. 1). We applied three RNA guide sequences of CRISPR (shown as red arrows in the PAR2 cDNA sequence), the first one of which is located in exon 1, whereas the second and third ones are in exon 2. The distance between exon 1 and 2 is about 15 kb and short enough to remove and repair the gap after Cas9 cleavage. The tethered ligand, SLIGKV-NH $_2$, is located just downstream of the second CRISPR. Thus the deletion of this genomic region should be from signal peptide to middle of the PAR2 cDNA including the entire tethered ligand region. Successfully mutated cells, termed Panc1-PAR2 CRISPR cells, were selected with puromycin.

Transient Transfections and Luciferase Reporter Assays. For transient transfection, cells were incubated for 4 hours with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) and either one of the following mutants: PAR2-R362Q (Sevigny et al., 2011), PAR2Y-dAKN9 (Δ 355-363) (Seatter et al., 2004) (calcium signaling defective

but ERK activation competent, according to data with transfected HEK293-PAR2-CRISPR cells, see below), PAR2-R36A (resistant to trypsin cleavage/activation of PAR2) (Sevigny et al., 2011), or ALK5-K232R (kinase-dead). At 48 hours after transfection, cells were subjected to various assays.

For luciferase reporter gene assays, cells in 96-well format were transfected serum-free with either p3TP-Lux, p6SBE-Luc, or p(CAGA)₁₂-Luc, along with pRL-TK-luc, a Renilla luciferase encoding vector (Promega, Heidelberg, Germany). In some assays, empty vector and PAR2-Myc-DKK (Origene, Rockville, MD) or empty vector and the above listed PAR2 mutants were transfected in parallel with the reporter constructs. On the next morning, cells were treated with TGF- β 1 for 24 hours and reporter gene activities were measured with the Dual Luciferase Assay System or Dual Glo Luciferase System (Promega). The data were calculated from six parallel wells and normalized with Renilla luciferase activity.

Cell-Electrode Impedance Random Cell Migration Assay.

Employing the xCELLigence DP device (ACEA Biosciences, La Jolla, CA), we performed impedance-based measurements of cell motility using nontransfected or transfected Panc1 and Colo357 cells. The migration assay was carried out as detailed earlier (Limame et al., 2012; Mandel et al., 2013). In brief, 165 μ l of serum-reduced medium (1% fetal bovine serum) was added to the lower chamber of a two-chamber device separated by a porous membrane (8 μ m pores), the cell-electrode impedance (CIM)-Plate 16 (OLS, Bremen, Germany). The lower side of the membrane contains microelectrodes for impedance-based detection of cells that have migrated through the pores. Before assembling the CIM-Plate 16, the lower side of the membrane had been coated with 30 μ l of collagen I (Sigma) to promote adhesion of the cells. Following assembly of the two chambers, the CIM-Plate 16 was equilibrated in medium for 1 hour in the incubator followed by a measurement step to monitor background signal. To begin an experiment, cells (30,000–50,000 per well) after overnight serum-starvation were seeded in the wells of the upper chamber of the CIM-Plates 16 and left in the laminar flow hood for 0.5 hour to allow cells to settle. In all assays, each condition was performed with three or four parallel wells, and signals were recorded every 15 minutes using the RTCA software version 1.2.1. (ACEA Biosciences). Assays were run for 12–48 hours, depending on the cell line. In some experiments, TGF- β 1, PAR-APs, or inhibitors, alone or in combination, were added to the media in the lower and upper wells (chemokinesis). In experiments with transfected cells, these cells were assayed 24 hours after the second transfection.

Immunoblot Analysis. After treatment with TGF- β 1, PAR2-AP, or inhibitors, cells were lysed in RIPA buffer containing proteinase inhibitors. SDS-PAGE and protein transfer to PVDF membranes was performed as described previously (Chen et al., 2002). Chemoluminescent detection was performed with a ChemiDoc Touch apparatus (BioRad, Munchen, Germany) and Image Laboratory software, version 5.2.1 (BioRad). Signal intensities were quantified by densitometry and computed with either NIH image J or Image Laboratory (version 5.2.1; BioRad).

Detecting In Situ PAR2 Cleavage in Cells Treated with TGF- β . To assess the impact of TGF- β treatment on the integrity of PAR2 expressed in Panc1 cells, we used a strategy employing “dually tagged” receptor, as described previously for N-terminal-mCherry/C-terminal-YFP-tagged PAR1 (Ramachandran et al., 2011; Mihara et al., 2013). For PAR2, we used red-fluorescent protein (RFP) instead of mCherry to tag the N terminus, with an eYFP-tagged C terminus (Mihara et al., 2016). The cDNA sequence of mRFP was inserted at the N terminus of human PAR2 between the signal peptide and proteinase cleavage-activation site that un masks the “canonical” PAR2 tethered ligand. The C-terminal stop codon of PAR2 was removed and eYFP was inserted after the signal peptide-mRFP-PAR2 fusion sequence. All open reading frame cDNA sequences were verified (University of Calgary DNA core facility). The fusion sequence was inserted under the cytomegalovirus promoter of the pCDNA3.1 plasmid vector. The PAR2 probe was

transfected into recipient Panc1 cells 48 hours before to their use, using Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA), and the functional integrity of the tagged receptors was verified using a PAR2 agonist (trypsin or 2f-LI) calcium signaling assay. The transfected cells were cultured on thin glass bottom plates and visualized using confocal microscope FV1000 (Olympus, Tokyo, Japan). More detailed methods for the use of dually-tagged PAR2, in keeping with the work done with dually tagged PAR1 and PAR2 are described elsewhere (Ramachandran et al., 2011; Mihara et al., 2013, 2016).

Statistical Analysis. Depending on the assay type, mean and S.D. were computed with SPSS from at least three independent experiments. Statistical significance ($P < 0.05$) was calculated using either the Mann-Whitney U test or two-way analysis of variance with Bonferroni correction in case of multiple comparisons between data sets. For the sake of clarity, P values were provided at different significance levels, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

Characterization of PAR2 and TGF- β 1-dependent Cell Migration in PDAC Cells. To determine the role of PAR2-induced G_q-calcium signaling in TGF- β -enhanced cell migration, we first characterized the effect of stimulation of PAR2 pathways alone on the migration of PDAC-derived cells. To activate PAR2, we employed two distinct short receptor-selective synthetic peptides (PAR-activating peptides, PAR-APs), namely SLIGKV-NH₂ and 2f-LI that are capable of PAR2-specific activation without the requirement for receptor proteolytic cleavage (Scarborough et al., 1992; Ramachandran et al., 2012). We monitored the migratory activity of Colo357 cells after stimulation with each of these two PAR2-APs (Fig. 1, A and B). Stimulation of Colo357 cells for 24 hours with either SLIGKV-NH₂ (Fig. 1A, tracings C and D) or 2f-LI (Fig. 1B, tracing B) enhanced random cell migration compared with the vehicle negative control (Fig. 1A: $P < 0.001$ and Fig. 1B: $P < 0.001$; Supplemental Fig. 2). In contrast to SLIGKV-NH₂ and 2f-LI, the reverse-sequence PAR-inactive, negative control peptides LRGLS-NH₂ (inverse) and LSLIGRL-NH₂ (partial-reverse/inactive) had no effect (Fig. 1B, tracings C and D, respectively), demonstrating the specificity of the PAR2-AP effects on cell migration. We then monitored the effect of TGF- β 1 on Colo357 and Panc1 cell migration. TGF- β 1 treatment induced migration of these two cell lines with some different kinetics compared with the PAR2-AP SLIGKV-NH₂ (Fig. 1A; Supplemental Fig. 2, tracing B vs. A: $P < 0.01$ at 24:00 and Fig. 1C; Supplemental Fig. 2, tracing B vs. A: $P < 0.01$ at 16:00). In Colo357 cells, there was a time lag for TGF- β 1 relative to the PAR2-AP in promoting migration (Fig. 1A), whereas in Panc1 cells the kinetics of PAR2-AP and TGF- β 1-dependent migration were similar (Fig. 1C). Interestingly, a 20-hour treatment with a combination of TGF- β 1 and PAR2-AP enhanced migration over that of TGF- β 1 alone in both Colo357 (Fig. 1D, left-hand graph; Supplemental Fig. 2: tracing C vs. B, $P < 0.01$ at 20:00) and Panc1 cells (Fig. 1D, right-hand graph; Supplemental Fig. 2: tracing C vs. B, $P < 0.05$ at 20:00). These data show that PDAC-derived cells respond to both TGF- β 1 and PAR2-AP with enhanced migration and that both agonists use, at least in part, different signaling pathways.

Fig. 1. Effect of stimulation of PDAC cells with TGF- β 1 or PAR2 agonists on random cell migration measured by impedance-based real-time cell migration assay. The colored curves represent the capacity for cell migration, with the slope of the curve being proportional to the migration velocity of cells. Data (mean $\pm$ S.D.) were derived from triplicate wells of the various cell populations. Capitals on each graph's right-hand side allow for color-independent identification of the curves. In each panel a representative assay is shown. (A) Colo357 cells were treated with vehicle (phosphate-buffered saline, control) and either TGF- β 1 (5 ng/ml) or the PAR2-AP SLIGKV-NH₂ (100 or 200 μ M). (B) As in (A), except that cells were treated with the PAR2-AP 2f-LI (15 μ M), LRGILS-NH₂ (inactive, 100 μ M), or LSGRL-NH₂ (inactive, 100 μ M). (C) Panc1 cells were subjected to random cell migration assays in the presence of TGF- β 1 (5 ng/ml) or the PAR2-AP SLIGKV-NH₂ (100 or 200 μ M). Data are significantly different between the control and TGF- β 1 (red tracing A vs. blue tracing B curve) at 4 hours and all later time points, control and PAR2-AP 100 μ M (red tracing A vs. green tracing C curve at 16 hours, and control and PAR2-AP 200 μ M (red tracing A vs. magenta tracing D) at 12 hours and all later time points. (D) As in (A), except that Colo357 and Panc1 cells were treated with either TGF- β 1 (5 ng/ml) or a combination of TGF- β 1 (5 ng/ml) and 2f-LI (15 μ M). Data are significantly different between TGF- β 1 (blue tracing B vs. black tracing C curve) and TGF- β 1 + 2f-LI at 17:00 and all later time points for Colo357 cells and at 19:00 and all later time points for Panc1 cells, respectively. For measures of interassay variation see Supplemental Fig. 2.

TGF- β 1-Mediated Migration is ALK5- and PAR2-dependent while PAR2-AP and Trypsin-Mediated Migration is PAR2-dependent but ALK5-independent.

The biochemical and cellular responses to TGF- β 1 have been shown to require the expression of intact PAR2 (Zeeh et al., 2016). To analyze the requirement of PAR2-AP and TGF- β 1-dependent chemokinesis for the presence of expressed intact PAR2, we silenced the PAR2 encoding *F2RL1* gene by RNAi and monitored the cellular response to TGF- β 1 and PAR2-AP stimulation either alone or in combination. As expected, Panc1 cells in which PAR2 had been downregulated failed to respond to PAR2-AP activation with enhanced migratory activity (Fig. 2A, left-hand graph; Supplemental Fig. 2, tracing D vs. B: $P < 0.01$ at 10:00). In agreement with earlier data (Zeeh et al., 2016), PAR2 depletion also abolished the promigratory function of TGF- β 1 (Fig. 2A, right-hand graph; Supplemental Fig. 2, tracing D vs. B: $P < 0.001$ at 10:00).

PAR2-AP-induced connective tissue growth factor (CTGF) expression in kidney tubular epithelial cells has been shown to be inhibited by SB431542 (Chung et al., 2013), indicating a role of ALK5 transactivation in PAR2-induced gene expression. A question raised by these findings is whether TGF- β /activin signaling is involved in PAR2-dependent cell motility of PDAC-derived cells. As expected, SB431542 abolished TGF- β 1-mediated cell migration in Colo357 and Panc1 cells (Fig. 2B, left-hand graph; Supplementary Fig. 2, tracing D vs. B: $P < 0.05$ at 24:00, and Fig. 2C, right-hand graph; Supplemental Fig. 2: tracing E vs. B: $P < 0.01$ at 12:00). Interestingly, the ALK5 inhibitor inhibited the ability of the PAR2-AP and trypsin to induce migratory activity in Colo357 (Fig. 2B, right-hand graph; Supplemental Fig. 2, tracings D

and F vs. B for PAR2-AP: $P < 0.05$ (1 μ M SB431542) and $P < 0.01$ (5 μ M SB431542) at 20:00, and Fig. 2C, left-hand graph; Supplemental Fig. 2, tracing D vs. B for trypsin: $P < 0.01$ at 14:00). Depletion of endogenous ALK5 in Panc1 cells by RNAi suppressed the ability of TGF- β 1 to stimulate migration (Fig. 2D, left-hand graph; Supplemental Fig. 2, tracing D vs. B: $P < 0.01$ at 8:00), while a control siRNA did not affect it (Fig. 2D, left-hand graph; Supplemental Fig. 2, tracing B vs. A: $P < 0.01$ at 8:00). Together, these data suggest that endogenous ALK5 receptors mediate TGF- β 1-induced cell migration in PDAC-derived cells. In contrast with its effect on TGF- β 1-induced migration, the ALK5 siRNA did not abrogate PAR2-AP-stimulated cell migration compared with PAR2-AP treatment of cells exposed to the control siRNA (Fig. 2D, right-hand graph; Supplemental Fig. 2, tracing D vs. B: $P > 0.05$ at 8:00). These data show that activation of PAR2 by either PAR2-AP or trypsin promotes random cell migration in PDAC cells and that ALK5 does not appear to be required for this PAR2-stimulated process. We interpret the data to mean that while TGF- β 1-mediated cell migration is dependent on both ALK5 and PAR2, migration triggered by activation of PAR2 with either a PAR2-AP or trypsin is ALK5 independent. However, given the inhibitory effect of SB431542 on PAR2-AP and trypsin-induced migratory activity, a participation of the related activin/nodal receptors ALK4 and ALK7 both of which are sensitive to SB431542 inhibition cannot be entirely excluded.

Pharmacologic Inhibition of PAR2 Activation/Signaling Blocks PAR2-AP-dependent but not TGF- β 1-dependent Cell Migration. Both PAR2-dependent (Kaufmann et al., 2011; Kaufmann and Hollenberg, 2012; Wu et al., 2014)

Fig. 2. TGF- β 1-mediated cell migration is ALK5- and PAR2-dependent while PAR2-AP and trypsin-mediated cell migration is PAR2-dependent but ALK5-independent. (A) Panc1 cells were transiently transfected with control siRNA (siCo) or PAR2 siRNA (siPAR2) and subjected to chemokinesis assay in the presence of either PAR2-AP (left-hand graph) or TGF- β 1 (right-hand graph). (B) Cell migration assay of Colo357 cells stimulated with TGF- β 1 (5 ng/ml, left-hand graph) or PAR2-AP (2f-LI, 15 μ M, right-hand graph) or (C, left-hand graph) trypsin in the presence or absence of vehicle (Dulbecco's modified Eagle's medium) or SB431542 (1 and 5 μ M). In the left-hand graph, differences are significant between vehicle (red curve, tracing A) and SB431542 (green curve, tracing C) and between vehicle + trypsin (blue curve, tracing B) and SB431542 + trypsin (magenta curve, tracing D) at the 24-hour time point. (C, right-hand graph) Migration assay of Panc1 cells treated with TGF- β 1 or PAR2-AP and either vehicle or SB431542. (D) Panc1 cells were transfected with siCo or siALK5 and subjected to migration assay in the presence of TGF- β 1 (left-hand graph) or PAR2-AP (right-hand graph). For measures of interassay variation, see Supplemental Fig. 2.

and TGF- β -dependent (Chow et al., 2008) migration have been reported to require intracellular calcium signaling. In BxPc3 pancreatic carcinoma cells, TGF- β rapidly induces an increase in cytoplasmic free calcium from intracellular stores, leading to subsequent PKC- α activation. Moreover, knockdown of PKC- α prevents the TGF- β -induced increase in cell migration (Chow et al., 2008). To analyze whether other PDAC-derived cells respond in a similar way, we performed inhibition experiments with the cell-permeant calcium chelator BAPTA/AM. By sequestering intracellular calcium, this inhibitor strongly suppressed both basal (Fig. 3A, tracing C vs. A) and TGF- β 1-dependent (Fig. 3A; Supplemental Fig. 2, tracing D vs. B; $P < 0.001$ at 8:00) random migratory activity of Panc1 cells.

We (Hollenberg et al., 2014) and others (Suen et al., 2012, 2014) previously verified that GB88 and the pepducin P2pal-18S, a small molecule and a peptide inhibitor of

PAR2, respectively, effectively and selectively block PAR2 G_{q/11}, calcium, and PKC signaling in diverse cell types, including HEK and CHO-hPAR2 cells. Consistently, we found that GB88 strongly inhibited PAR2-AP-dependent random migration of Colo357 cells (Fig. 3B, left-hand graph; Supplemental Fig. 2, tracing D vs. B; $P < 0.01$ at 24:00). To determine whether G_q-calcium signaling induced by PAR2 contributes to TGF- β 1 signaling-induced cell migration, we compared the effects of GB88 and SB431542 on the ability of TGF- β 1 to enhance random migration of Colo357 cells. Remarkably, whereas the ALK5 inhibitor SB431542 completely blocked TGF- β 1 promotion of cell migration (Fig. 3B, right-hand graph; Supplemental Fig. 2, tracing F vs. B; $P < 0.001$ at 24:00), GB88 at 10 μ M enhanced rather than inhibited TGF- β 1-induced migratory activities as monitored in the cell-electrode impedance assays (Fig. 3B, right-hand graph;

Fig. 3. Pharmacologic inhibition of PAR2 activation/signaling blocks PAR2-AP-dependent but not TGF- β 1-dependent cell migration. The colored curves indicate the migratory/invasive activities of the various Colo357 cell populations (numbers on each graph's right-hand side allow for color-independent identification of the curves). Data were derived from three to four parallel wells and represent the mean $\pm$ S.D. of a representative assay. (A) Real-time cell migration assay with Panc1 cells treated or not with TGF- β 1 (5 ng/ml) and either vehicle or the calcium chelator BAPTA/AM (25 μ M). (B) Cells remained untreated or were treated with TGF- β 1 in the presence of various concentrations, as indicated, of GB88 or the ALK4/5/7 inhibitor SB431542 (right-hand graph). As control for the functionality of GB88, Colo357 cells were treated with PAR2-AP in the presence of 10 μ M GB88 (left-hand graph). Differences are significant between vehicle + TGF- β 1 (right-hand graph, blue curve, tracing B) and between SB431542 + TGF- β 1 (right-hand graph, magenta curve, tracing F) on the one hand and GB88 [10 μ M] + TGF- β 1 (right-hand graph, black curve, tracing E) on the other hand at 24 and 16 hours, respectively, and all later time points, and between vehicle + PAR2-AP (left-hand graph, blue curve, tracing B) and GB88 + PAR2-AP (left-hand graph, magenta curve, tracing D) at 16 hours and all later time points. For measures of interassay variation, see Supplemental Fig. 2.

Supplemental Fig. 2, tracing E vs. B: $P < 0.05$ at 24:00). ENMD-1068, another PAR2 antagonist and calcium signaling inhibitor, was also found not to affect TGF- β 1-stimulated migration (Supplemental Fig. 3A, tracing D vs. B: $P > 0.05$ at 12:00). In addition, a failure to inhibit TGF- β 1-induced migration was observed when cells were cotreated with the selective PAR2 inhibitor, P2pal-18S (Supplemental Fig. 3B, tracing D vs. B, tracing F vs. B, and tracing H vs. B, all $P > 0.05$ at 24:00), which like GB88 blocks PAR2-AP (SLIGRL-NH₂)-stimulated calcium signaling in pancreatic acinar cells (Michael et al., 2013). Thus, our data suggest the possibility that TGF- β 1-ALK5-signaling-induced migration requires the presence of PAR2 protein but not PAR2-mediated calcium signaling independent of ERK signaling (see *Discussion*).

Genetic Inhibition of PAR2 Activation/Signaling Blocks PAR2-AP-dependent but not TGF- β 1-dependent Cell Migration. To further test the idea that G_q-calcium signaling is not required for the PAR2-dependent increase in cell migration stimulated by TGF- β 1, we next evaluated the effect of expression of three PAR2 receptor mutants [R362Q, dAKN9 (Δ 355-363), R36A] with distinct signaling properties due to alterations in the C- or N-terminal sequences (Seatter et al., 2004) on TGF- β -induced signaling and cell migration. Initially, we employed the calcium signaling-defective mutant (PAR2-R362Q) described by Sevigny et al. (2011). This mutant has an intact protease cleavage site and tethered ligand but

cannot signal to G_q, although β -arrestin interactions may still be possible (Sevigny et al., 2011). It is thus expected that the G_q signaling-compromised R362Q mutant may on its own and, via heterodimerization, diminish calcium signaling by endogenous PAR2 receptor in a dominant-negative fashion. Accordingly, expression of PAR2-R362Q blocked PAR2-AP stimulated migration in these cells (Fig. 4A, left-hand graph; Supplemental Fig. 2, tracing D vs. B: $P < 0.001$ at 24:00). However, the PAR2-R362Q mutant did not suppress TGF- β 1-induced cell migratory activity (Fig. 4A, right-hand graph; Supplemental Fig. 2, tracing D vs. B: $P > 0.05$ at 16:00), which was blocked by the expression of the kinase inactive ALK5-KR (Fig. 4A, right-hand graph; Supplemental Fig. 2, tracing F vs. B: $P < 0.001$ at 16:00).

Next, we wanted to test the effect of expression of PAR2-dAKN9 on TGF- β 1-induced migration to determine if additional C-terminal sequences in PAR2 are required for enhancing this TGF- β -dependent response. We first confirmed that in contrast to transfected (wild type) PAR2, transfection of PAR2-dAKN9 did not restore a calcium signal in response to 2f-LI stimulation in HEK293-PAR2 CRISPR cells (Supplemental Fig. 4A). Consistently, the PAR2-dAKN9 mutant abolished the migratory response to PAR2-AP (Fig. 4B, left-hand graph; Supplemental Fig. 2, tracing D vs. B: $P < 0.05$ at 12:00). In contrast, expression of PAR2-dAKN9 stimulated rather than suppressed TGF- β 1-dependent cell migration (Fig. 4B, right-hand graph; Supplemental Fig. 2, tracing D vs. B: $P < 0.05$ at 24:00).

Fig. 4. Genetic inhibition of PAR2 activation/signaling blocks PAR2-AP-dependent but not TGF- β -dependent cell responses. (A) Colo357 cells were transiently transfected with PAR2-RQ or ALK5-KR as control and subjected to cell migration assay in the presence or absence of PAR2-AP (left-hand graph) or TGF- β 1 (right-hand graph). Differences are significant between vector + TGF- β 1 (right-hand graph, blue curve, tracing B) and ALK5-KR + TGF- β 1 (right-hand graph, cyan curve, tracing F), and between vector + PAR2-AP (left-hand graph, blue curve, tracing B) and PAR2-RQ + PAR2-AP (left-hand graph, magenta curve, tracing D) at the 16-hour and all later time points. (B) As in (A), except that Panc1 cells were transfected with PAR2-dAKN9 instead of PAR2-RQ and stimulated with PAR2-AP (left-hand graph) or TGF- β 1 (right-hand graph). Differences are significant between vector + TGF- β 1 (right-hand graph, blue curve, tracing B) and PAR2-dAKN9 + TGF- β 1 (right-hand graph, magenta curve, tracing D) at 16-hour and all later time points. (C) As in (A), except that Colo357 cells were transfected with PAR2-RA instead of PAR2-RQ. Cells were stimulated with either 10 nM trypsin (left-hand graph) or 5 ng/ml TGF- β 1 (right-hand graph). Data are significantly different between vector + TGF- β 1 (right-hand graph, blue curve, tracing B) and ALK5-KR + TGF- β 1 (right-hand graph, cyan curve, tracing F) and between vector + trypsin (left-hand graph, blue curve, tracing B) and PAR2-RA + trypsin (left-hand graph, magenta curve, tracing D) at the 16-hour and all later time points. (D–F) Analysis of Panc1 cells engineered by CRISPR/Cas9 technology to express endogenous PAR2 with a nonfunctional tethered ligand region (CRISPR) and wild-type (WT) cells as control. (D) Real-time migration assay. Data are the mean $\pm$ S.D. of four wells and are representative of three independent assays. Data are significantly different between WT cells + TGF- β 1 (right-hand graph, blue curve, tracing B) and CRISPR cells + TGF- β 1 (right-hand graph, magenta curve, tracing D) at the 4- and 16-hour time points. For measures of interassay variation, see Supplemental Fig. 2. (E) Luciferase assay with the TGF- β /Smad responsive reporter plasmid p3TP-Lux and pRL-TK-Luc. Data are the normalized mean $\pm$ S.D. of six wells and are representative of three independent assays. ** $P < 0.01$. (F) Immunoblot analysis of TGF- β 1-induced C-terminal phosphorylation of Smad3 (p-Smad3C) and Smad3 as loading control in cells treated with TGF- β 1 for 1 hour. The graph below the immunoblot depicts the results from densitometric analysis of three independent experiments (mean $\pm$ S.D., $n = 3$). CR, CRISPR.

The observation that concomitant agonist-mediated PAR2-AP activation was unable to amplify the TGF- β 1 response, in theory, may have resulted from the fact that the pool of surface-associated PAR2 molecules had already been activated maximally before PAR2-AP addition. This activation in principle could result from the continuous activity of residual trypsin or another serine proteinase(s) present in the cell-conditioned medium. To evaluate this possibility, we used a “trypsin-resistant” PAR2 receptor (PAR2-R36A) wherein the R/S canonical cleavage site was changed to A/S. This substitution of alanine for arginine in the PAR2 sequence blocks the unmasking of the PAR2 canonical tethered ligand by tryptic activation. As a consequence, any endogenous G_q-calcium signaling by the mutant PAR2 receptor via activation of its “canonical” tethered ligand would have been suppressed. Although resistant to trypsin activation, this mutant is, nonetheless, susceptible to activation by PAR2-AP. Transient

expression of PAR2-RA in Colo357 cells abolished the migratory response to trypsin (Fig. 4C, left-hand graph; Supplemental Fig. 2, tracing D vs. B: $P < 0.01$ at 24:00), but unlike ALK5-KR (Fig. 4C, right-hand graph; Supplemental Fig. 2, tracing F vs. B: $P < 0.01$ at 20:00) was unable to alter the migratory response to TGF- β 1 (Fig. 4C, right-hand graph; Supplemental Fig. 2, tracing D vs. B: $P > 0.05$ at 20:00). Based on all these findings, we conclude that the PAR2-TGF- β synergy does not involve G_q-calcium signaling or activation of the PAR2 canonical tethered ligand.

PAR2 can be cleaved at non-canonical sites in the tethered ligand region by serine proteinases other than trypsin, eventually resulting in biased signaling (Hollenberg et al., 2014). To analyze whether one of these sites is involved in the TGF- β promoting effect, we generated mutant Panc1 cells by introducing a genomic deletion in *F2RL1* that spans exon 1 and part of exon 2 (see Supplemental Fig. 1 for choice of

Fig. 4. Continued.

guide sequences of CRISPR). Stimulating these cells with PAR2-AP (2f-LI) in a calcium flux assay confirmed that the PAR2-CRISPR cells were signaling-defective with respect to G_q -calcium signaling (Supplemental Fig. 4B). The Panc1 PAR2-CRISPR cells were then challenged with PAR2-AP or TGF- β 1 and compared with wild-type Panc1 cells for their chemokinetic response. The migration of PAR2-CRISPR cells was not enhanced by PAR2-AP (Fig. 4D, left-hand graph; Supplemental Fig. 2, tracing D vs. C: $P > 0.05$ at 8:00) compared with that of wild-type cells (Fig. 4D, left-hand graph; Supplemental Fig. 2, tracing B vs. A: $P < 0.05$ at 8:00). Thus, PAR2 agonist responsiveness was absent in the PAR2-CRISPR cells. In contrast, in those cells, TGF- β 1-induced migration was retained (Fig. 4D, right-hand graph; Supplemental Fig. 2, tracing D vs. C: $P < 0.05$ at 12:00) and both basal and TGF- β 1-dependent migration tended to be stronger in the PAR2-CRISPR cells compared with the wild-type cells (Fig. 4D, right-hand graph).

To gain additional evidence that PAR2 activation/calcium signaling is dispensable for TGF- β signaling, we monitored general transcriptional activity of the Smad responsive reporter gene p3TP-Lux and Smad3 activation in the PAR2-CRISPR and wild-type cells in response to TGF- β 1 stimulation (Fig. 4, E and F). Again, the TGF- β 1-dependent transcriptional activity was not lost in the PAR2-CRISPR cells but was even higher than that of the wild-type control cells (Fig. 4E, $P < 0.01$). Finally, the proportion of C-terminally phosphorylated Smad3 (p-Smad3C) in Panc1-PAR2-CRISPR

cells after 1 hour of TGF- β 1 stimulation tended to exceed those in the wild-type cells (Fig. 4F). Together, these results are in agreement with earlier observations that promotion of cell migration by PAR2-AP or trypsin-cleaved PAR2 tethered ligand is mediated by G_q -calcium-dependent signaling, whereas in contrast, the dependence of TGF- β 1-induced migration on endogenous abundance of the protein PAR2 does not require PAR2-mediated G_q -calcium-mediated signaling but may require PAR2's ERK-activating function (see Discussion).

Agonists and Antagonists of PAR2 are Unable to Alter TGF- β 1-Induced Reporter Gene Activity. The data presented above indicated that PAR2-stimulated G_q -calcium signaling is not involved in the TGF- β promigratory effect, despite the dependence of this action of TGF- β 1 on PAR2 protein expression. To test whether this result is reflected at the level of TGF- β transcriptional regulation, we performed reporter gene assays with two different TGF- β /Smad reporter genes, p(CAGA)₁₂ MLP-Luc and p6SBE-Luc. In previous work, we have shown that siRNA-mediated depletion of PAR2 attenuates the induction of these reporter genes by TGF- β 1, whereas ectopic overexpression of PAR2 enhances their activity in the presence of TGF- β 1 (Zeeh et al., 2016). However, neither the treatment of cells with PAR2-AP alone nor the combined treatment with TGF- β 1 and PAR2-AP (2f-LI) together (Fig. 5A) drove the signal from the p(CAGA)₁₂ MLP-Luc higher than in the control cells or in the TGF- β 1-alone-treated cells, respectively. The data show

Fig. 5. Agonists and antagonists of PAR2 are unable to alter TGF- β 1-induced reporter gene activity. (A) Panc1 cells were transfected on day 1 with p(CAGA)₁₂ MLP-Luc and the *Renilla* luciferase-encoding vector pRL-TK-Luc using Lipofectamine 2000. Two days later, cells were treated with 5 ng/ml TGF- β 1 or 15 μ M PAR2-AP (2f-LI) alone or in combination for 24 hours, which was followed by lysis and dual luciferase assay. Data represent the mean $\pm$ S.D. of four assays, *** P < 0.001, two-way analysis of variance with Bonferroni correction. (B) As described in (A) except that TGF- β 1 stimulation was performed in the absence or presence of the indicated concentrations of GB88, P2pal-18S, or SB431542 as control. Data represent the mean $\pm$ S.D. of four assays, *** P < 0.001, Mann-Whitney U test. (C) Panc1 cells were transfected on day 1 with p6SBE-Luc and pRL-TK-Luc along with empty vector or vectors for the indicated PAR2/ALK5 mutants. Forty-eight hour after the start of the first transfection, cells were stimulated with TGF- β 1 for another 24-hour period followed by dual luciferase measurements. Data represent the mean $\pm$ S.D. of four assays, ** P < 0.01 vs. TGF- β 1-treated vector control, Mann-Whitney U test. (D) As in (C), except that cells were transfected with p(CAGA)₁₂ MLP-Luc along with the indicated PAR2 mutants, n.d., not determined. * P < 0.05, ** P < 0.01; *** P < 0.001 vs. empty vector control, Mann-Whitney U test. Each assay in (A–D) was performed with six parallel wells for each condition and represent firefly luciferase values normalized with those for *Renilla* luciferase.

that stimulation of cells with the receptor-selective PAR2-AP was without effect on the TGF- β reporter signal.

The use of GB88 and P2pal-18S that can selectively block PAR2 G_{q/11}, calcium, and PKC signaling but not other PAR2 signal pathways (Seigny et al., 2011; Suen et al., 2012, 2014; Hollenberg et al., 2014) allowed us to test whether the activation status of PAR2-mediated calcium signaling has an impact on TGF- β /Smad transcriptional activity. In contrast to the TGF- β /ALK5 inhibitor, SB431542, both GB88 and P2pal-18S did not block TGF- β 1-stimulated transcription in Panc1 cells (Fig. 5B).

Next, we tested the effect of ectopically overexpressing the PAR2 mutants R362Q, dAKN9 (Δ 355–363), and R36A, and the kinase-dead ALK5-KR mutant as control, on TGF- β /Smad-induced reporter gene activity in Panc1 cells. Expression of these calcium signaling-deficient PAR2 mutants, in contrast to ALK5-KR, failed to inhibit the TGF- β 1-dependent activity

of p6SBE-Luc (Fig. 5C) and p(CAGA)₁₂ MLP-Luc (Fig. 5D). Rather, cells expressing the RA mutant, which cannot trigger trypsin-mediated PAR2 calcium signaling led to a significant increase in TGF- β 1-dependent luciferase activity relative to empty vector transfected cells (Fig. 5, C and D).

PAR2-AP Stimulation and Pharmacologic and Genetic Inhibition of PAR2 Calcium Signaling are Unable to Alter TGF- β 1-Induced Smad Activation or ALK5 Expression. Previous data have shown that PAR2 depletion by RNAi blunts TGF- β 1-induced transcription and migration raising the key question of the mechanism(s) by which PAR2 regulates TGF- β signaling. Surprisingly, our data suggest that, as opposed to PAR2 translation inhibitors, agonists or antagonists of PAR2 signaling do not alter TGF- β -mediated signaling and migration. To confirm these results at the level of individual components of the TGF- β signaling pathway, we monitored the phosphorylation status of Smad3. To this end,

PAR2-AP did not induce C-terminal phosphorylation of Smad3 (Fig. 6A) and Smad2 (data not shown) in Panc1 cells. Colo357 cells showed high basal abundance of p-Smad3C (Fig. 6A) and p-Smad2C (data not shown), both of which were not further enhanced by PAR2-AP (Fig. 6A and data not shown, respectively). Moreover, PAR2-AP did not further increase TGF- β 1-induced p-Smad3C (Fig. 6B) or p-Smad2C (data not shown) levels in Colo357 and Panc1 cells when used in combination.

GB88, which selectively blocks PAR2 $G_{q/11}$, calcium, and PKC signaling but not other PAR2 signal pathways (Suen et al., 2012; Hollenberg et al., 2014) was used to test whether the activation status of PAR2-mediated calcium signaling impacts TGF- β 1-induced Smad activation. Likewise and in contrast to the ALK5 inhibitor SB431542, GB88 did not suppress TGF- β 1-induced p-Smad3C levels in Colo357 and Panc1 cells (Fig. 6C). Moreover, ectopic expression of the PAR2-RQ or the PAR2-dAKN9 mutant failed to alter TGF- β 1-dependent Smad3 activation in Panc1 cells (Fig. 6D) despite

their ability to block PAR2-AP-triggered calcium signaling (Seatter et al., 2004; Sevigny et al., 2011, and Supplemental Fig. 4A).

We recently showed that PAR2 protein promotes TGF- β signaling by sustaining expression of ALK5 (Zeeh et al., 2016), suggesting the possibility that PAR2 signaling targets ALK5 abundance either by increasing its synthesis or by inhibiting its degradation. However, in Panc1 cells, activating PAR2 by PAR2-AP neither stimulated an increase in ALK5 protein abundance on its own nor in combination with TGF- β 1 (Supplemental Fig. 5). Consistent with the inability to alter p-Smad3C levels in TGF- β 1 treated cells (see Fig. 6D), expression of PAR2-RQ or dAKN9 was unable to affect the abundance of ALK5 (data not shown).

Transactivation of PAR2 via TGF- β Stimulation. The demonstration that the PAR2 inhibitors that selectively block PAR2 calcium signaling (Hollenberg et al., 2014) do not suppress the TGF- β effect on Smad activation and Smad-dependent responses, whereas our other data clearly implied a

Fig. 6. PAR2-AP stimulation and pharmacologic and genetic inhibition of PAR2 calcium signaling are unable to alter TGF- β 1-induced Smad3 activation. (A) Detection of p-Smad3C in Panc1 and Colo357 cells after single treatment with PAR2-AP. M, molecular weight marker (shown is the 60-kDa band). Detection of HSP90 was used here to verify equal loading. Band intensities from immunoblots (p-Smad3C/HSP90) of three independent experiments ($n = 3$) were quantified by densitometric analysis and displayed as mean $\pm$ S.D. below one representative blot, Mann-Whitney U test. (B) The same as (A), except that Panc1 (top panel) or Colo357 cells (bottom panel) were stimulated with TGF- β 1 alone (T) or with a combination of PAR2-AP + TGF- β 1 (P+T). The graphs below the blots show the signal intensity ratios (mean $\pm$ S.D.) from three experiments ($n = 3$), Mann-Whitney U test. (C) P-Smad3C immunoblot of Panc1 (top panel) or Colo357 cells (bottom panel) treated with TGF- β 1 (5 ng/ml) in the absence or presence of various concentrations (as indicated) of GB88 or SB431542 (SB) as control. The graphs below the blots show the signal intensity ratios (mean $\pm$ S.D.) from three experiments ($n = 3$). *** $P < 0.001$, Mann-Whitney U test. (D) Panc1 cells were transfected with empty vector, wild-type PAR2, or the indicated PAR2 mutants and immunoblotted for p-Smad3C and total Smad3. The graph below the blots shows mean $\pm$ S.D. from three experiments ($n = 3$), Mann-Whitney U test.

participation of PAR2 in these TGF- β -triggered events, suggested the possibility that PAR2 might cooperate with the TGF- β receptor via activation of biased signaling. Such calcium-independent PAR2 biased signaling is triggered by the proteolytic unmasking of a “noncanonical” PAR2 tethered ligand revealed by enzymes other than trypsin (Ramachandran et al., 2011; Hollenberg et al., 2014). Furthermore, since PAR2 activation can be accompanied by proteolytic transactivation of the TGF- β receptor (Little et al., 2011; Chung et al., 2013; Kamato et al., 2015), we wondered if concurrently, TGF- β receptor activation might in turn lead to the production of enzymes that can cleave transactivated PAR2. To test this possibility, we expressed dually labeled PAR2 in a Panc1 cell background, activated the cells with TGF- β , and evaluated the cleavage of Panc1 expressed PAR2 induced by TGF- β 1. Confocal imaging revealed that TGF- β 1 treatment resulted in the production of proteinases that released the N-terminal red fluorescent protein tag from PAR2, which was then visualized as a “green” receptor that remained predominantly at the cell surface (Fig. 7C). In contrast, trypsin treatment not only released the N-terminal mRFP tag from PAR2, turning it “green,” but also triggered receptor internalization (Fig. 7B). In contrast, in the untreated Panc1 cells, dually tagged PAR2 was visualized as an intact “yellow” receptor at the cell surface (Fig. 7A). Our data therefore indicate that TGF- β 1 is able to induce enzymes that cause autocrine cleavage/activation of PAR2 in PDAC cells. This autocrine cleavage resulting from TGF- β 1 action does not drive PAR2 internalization, in keeping with the action of elastase (Ramachandran et al., 2011), and may well stimulate a calcium-independent biased PAR2 signal.

Discussion

The main finding of our study is that the migratory response to TGF- β 1 in Colo357 and Panc1 cells depends on the kinase activity of ALK5 and on the presence of PAR2 protein, but not on canonical PAR2-induced calcium signals (Fig. 8). Furthermore, our data indicate that targeting PAR2 signaling with receptor antagonists will not affect the impact of ALK5 activation on cancer cell migration. Our data thus reveal an unexpected role for PAR2 in affecting TGF- β 1 action in tumor cells. These results are in accord with our recent study where we showed in PDAC-derived cells and HaCaT keratinocytes

that PAR2 is required for various TGF- β 1-mediated cellular effects including random cell migration and that PAR2 functions to sustain expression of ALK5 (Zeeh et al., 2016). We also observed earlier that PAR2-AP-activated PAR2 transactivates both ALK5 and the EGF receptor that leads to Smad2C phosphorylation and upregulation of CTGF expression in human proximal tubular epithelial cells (Chung et al., 2013). These results prompt the question whether PAR2-dependent cell motility is ALK5 dependent. However, in the PDAC-derived cells we show here that neither PAR2-AP nor trypsin-stimulated PAR2-induced cell migration requires ALK5 activation as determined by the selective RNA interference-based approach. Thus, in the PDAC cells, PAR2-dependent ALK5 transactivation is not required for PAR2-stimulated cell migration. Hence, the requirement of PAR2-dependent ALK5 transactivation for PAR2-mediated signaling and responses might be cell type dependent.

Since PAR2 is a cell surface receptor and its cellular actions are thought to be mediated by G_q/G_{12/13} and β -arrestin signaling (Soh et al., 2010; Ramachandran et al., 2011), an important issue therefore was to determine whether PAR2 cleavage-dependent signaling is needed to enhance TGF- β signaling. By using several approaches, our findings indicate that the PAR2-induced G_q-calcium signal is not required for the ability of PAR2 to support TGF- β /ALK5-stimulated cell migration. Thus, 1) PAR2-AP (2f-LI) treatment did not enhance TGF- β 1-induced reporter activity, either through endogenous (Fig. 5A) or overexpressed PAR2 (not shown), and 2) p-Smad3C levels in PAR2-AP + TGF- β 1 stimulated cells were indistinguishable from those in TGF- β 1-only treated cells (Fig. 6B). In this regard, PAR2-AP on its own had no (Panc1) or only a minor (Colo357) effect. The failure of the PAR2-AP to trigger phosphorylation of Smad2/3 on its own or to enhance TGF- β 1-induced Smad2/3C phosphorylation in PDAC-derived cells support the idea that the additional increase in TGF- β 1-dependent migration upon combined treatment with PAR2-AP (see Fig. 1D) is PAR2-dependent but ALK5 independent. In this respect, PDAC-derived Panc1 cells appear to differ from renal tubular epithelial cells, in which Smad2 activation and CTGF expression were synergistically enhanced by PAR2-AP and TGF- β 1 (Chung et al., 2013). Moreover, no block was observed in any of the above Smad phosphorylation responses by sequestering intracellular calcium with a chelator, with the use of the calcium

Fig. 7. TGF- β induces autocrine proteinase-mediated cleavage-activation of PAR2 in Panc1 cells. Panc1 cells growing adherently on cell culture plastic and transfected with dually tagged N-terminal-RFP/C-terminal YFP-PAR2 as described in *Materials and Methods* were exposed or not to trypsin (1 U/ml, 5 minutes, room temperature) or TGF- β 1 (10 ng/ml, 4 hours, 37°C). Fixed cells were then visualized using confocal microscopy, as outlined in the text. (A) Normal control: The dually tagged intact receptor appears predominantly yellow (left-hand panel, untreated cells), whereas (B) trypsin treatment results in the release of the mRFP tag and internalization as a “green” receptor (middle panel, green arrow). (C) In contrast, TGF- β 1 treatment results in the production of endogenous proteinase activity that cleaves the receptor at a “non-canonical” site, releasing the mRFP tag and leaving the “green” YFP-tagged receptor mostly at the cell surface (right-hand panel, green arrows). The Scale bar in the middle panel, 10 μ M.

Fig. 8. Concluding figure to illustrate the main finding of this study. PAR2 activated via its agonists, trypsin, or PAR2-AP stimulates cell migration in a G_q -calcium signaling-dependent fashion (G_q -calcium dep., green arrows). In contrast, TGF- β 1 acting through its type I receptor ALK5 also requires PAR2 protein expression to promote cell migration (black double-headed arrow). However, PAR2's G_q -calcium signaling function is not necessary to facilitate TGF- β 1 signaling (G_q -calcium indep., red arrows).

signaling-selective antagonists GB88 or P2pal-18S, or by eliminating the PAR2 calcium signaling-deficient PAR2 mutants, R362Q and dAKN9. Our data therefore exclude the possibility that elevation in intracellular calcium by PAR2 is responsible for PAR2 regulation of TGF- β actions. Given the ability of GB88 and P2pal-18S to block PAR2-induced inflammatory responses *in vivo* (Sevigny et al., 2011; Suen et al., 2012), these antagonists might have been expected to mimic the effect of downregulation of PAR2 by RNAi on TGF- β responses. The anti-inflammatory actions of GB88 and P2pal-18S can be linked to their ability to block PAR2 G_q -calcium signaling, because they do not inhibit other PAR2-driven responses like MAPK activation and PAR2/ β -arrestin-mediated responses (Hollenberg et al., 2014). Since neither of these two antagonists was able to inhibit the TGF- β responses in cells co-expressing PAR2 along with ALK5, one can conclude that the impact of PAR2 expression on TGF- β 1-mediated responses is independent of its ability to upregulate intracellular calcium concentrations. Of interest, GB88 at the highest concentration tested (10 μ M) did not diminish but rather appeared to *enhance* TGF- β 1-induced cell migration (see Fig. 3). Given the strong dependency of TGF- β 1-induced migration on MEK-ERK signaling, this phenomenon can possibly be explained by the biased agonism of GB88, which on its own has been shown to act as a PAR2 agonist to increase ERK1/2 phosphorylation (Hollenberg et al., 2014; Suen et al., 2014) and as a consequence TGF- β -dependent migration.

The above results have shown that the C-terminal region of PAR2 involved in calcium signaling is not required for PAR2 to facilitate TGF- β -mediated signaling and migration. Moreover,

proteolytic cleavage of PAR2 at its trypsin activation site appears to be dispensable for PAR2 effects on TGF- β responses, because expression of the trypsin-resistant PAR2 mutant R36A did not impede TGF- β -induced migration. As observed for the dAKN9 mutant, in cells expressing the RA mutant there was a small but significant increase in TGF- β -dependent luciferase activity (see Fig. 5). We conclude that if activation/signaling by PAR2 is required to enhance TGF- β signaling, proteolytic cleavage, if it occurs at all, would unmask a non-canonical PAR2-tethered ligand that might cause biased signaling (Hollenberg et al., 2014). Unfortunately, dominant negative mutants for non-canonical cleavage site(s) are not yet available, prohibiting a genetic approach to test this hypothesis as we did with the RA mutant that is resistant to canonical trypsin signaling.

Panc1 cells and, to a lesser extent, Colo357 cells are known to secrete large amounts of TGF- β (Geismann et al., 2009) through which they can enhance, in an autocrine fashion, several responses to this cytokine, such as growth inhibition and migration. In a similar way, the PAR2-TGF- β cosignaling event may possibly relate to the ability of TGF- β to induce PAR2-cleaving enzymes that can act in an autocrine way. Indeed, our data suggest the possibility that TGF- β may induce PAR2-cleaving enzyme activity that in an autocrine manner can remove the extracellular N-terminal part of the receptor, leaving PAR2 predominantly at the plasma membrane rather being internalized (Fig. 7). The location of cleaved PAR2 is associated with its ability to signal in a $G_{12/13}$ -dependent/ β -arrestin-independent manner. From the observations that PAR2 did not enhance TGF- β action either via an elevation in intracellular calcium (no block caused by

the calcium signaling-selective PAR2 antagonists GB88 or P2pal-18S or via use of the calcium signaling-defective RQ and dAKN9 mutants), we suggest that PAR2 may possibly promote TGF- β signaling via a biased PAR2 signal mechanism that remains to be elucidated. The TGF- β -induced proteinase(s) that resulted in the release of the PAR2 N terminal (Fig. 7) did not trigger receptor internalization and thus appears to cleave PAR2 at a non-canonical tethered ligand site within its extracellular domain. This non-canonical cleavage could result in biased PAR2-dependent calcium-independent signaling via an effector that remains to be determined (Hollenberg et al., 2014; Suen et al., 2014).

Other than the possibility of signaling through an as yet undetermined “biased” mechanism, our results prompt the question of whether PAR2 activation/signaling is required at all for promoting TGF- β signaling. If activation by cleavage (at both canonical and non-canonical sites) is not required for PAR2 to aid in TGF- β signaling, another attractive scenario is that PAR2 serves an intracellular chaperone function, e.g., in aiding the anterograde transport of TGF- β receptors from intracellular stores to the cell surface. In this case, silencing PAR2 would be expected to reduce surface expression of T β RRII and/or ALK5. Support for this hypothesis comes from work showing that PAR2 can act as a chaperone to facilitate anterograde transport, glycosylation, surface expression, and signaling of PAR4 (Cunningham et al., 2012). This action of PAR2 involves a direct physical interaction between PAR2 and PAR4. It is thus possible that there is a direct physical association between PAR2 and ALK5 as was previously shown for PAR2 and TLR4 in a similar HEK293T cell-based ectopic expression system (Rallabhandi et al., 2008). If indeed PAR2 is acting as a chaperone rather than a cell surface signaling receptor, this interaction would take place inside the cell where PAR2 likely has no access to exogenous ligands, and hence PAR2 activation/signaling may be dispensable for promoting TGF- β signaling. Moreover, when PAR2 acts as a surface receptor, it is believed to be capable of partnering with PAR1 (Jaber et al., 2014). Our observation that depletion of PAR1 did not mimic the effects of PAR2 depletion on several responses to TGF- β 1 (Zeeh et al., 2016) further suggests that both PARs act via different mechanisms and adds to our contention that PAR2 has an activation-independent function.

Taken together, the results of our study indicate that in PDAC cells various TGF- β responses require PAR2 protein expression but *not necessarily* activation of PAR2. Although our dual-tag PAR2 cleavage assay shows that TGF- β 1 can induce the production of autocrine PAR2-cleaving enzyme activity, it is not possible to determine if the cleaved receptor generates an intracellular signal or rather acts via non-covalent interactions with ALK5 itself or with domains on other nearby membrane proteins. The identity of the TGF- β 1-induced enzyme(s), including details of the possible signal pathways activated by the proteinase or the precise nature of the non-signaling mechanism, respectively, remain topics for continuing investigation. Moreover, by demonstrating a failure of all presently available specific inhibitors of PAR2 activation/signaling, in contrast to inhibitors of PAR2 protein translation, to block the TGF- β promoting effect, we show that a renewed effort to develop PAR2-targeted antagonists is warranted (Ramachandran et al., 2012). We suggest that blocking TGF- β receptor-PAR2 interactions in the tumor microenvironment rather than blocking PAR2 activation

may be a promising novel approach to interfere with Smad and non-Smad pathway activation and hence with TGF- β 's protumorigenic functions. Furthermore, this strategy may not be limited to anticancer treatments. It may also have pathophysiological relevance for the development of vascular thrombotic and fibroproliferative disorders/atherosclerosis. Targeting the PAR2-TGF- β crosstalk in these tissues may dramatically enhance the therapeutic strategies for these diseases.

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