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Proteinase-activated receptors differentially modulate *in vitro* invasion of human pancreatic adenocarcinoma PANC-1 cells in correlation with changes in the expression of CDC42 protein

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Abstract

Objectives—Proteinase-activated receptors (PARs) -1 and -2 have been associated with increased invasiveness and metastasis in human malignancies. The role of PAR-3 has been less investigated. We examined the role of PARs in a human pancreatic adenocarcinoma PANC-1 cell line phenotype *in vitro*.

Methods—We knocked down PAR-1, -2, or -3, while empty vector-infected cells served as controls. Specific peptide PARs agonists were used to stimulate the receptors. *In vitro* assays of colony formation, migration and invasion were used to characterize the phenotypes and Western analysis to follow CDC42 expression.

Results—PAR-1 and PAR-2 KDs were markedly less, while PAR-3 KDs were robustly more migratory and invasive than controls. Stimulation of PAR-1 or -2 by their peptide agonists increased, while PAR-3 agonist reduced the invasion of control cells. All three PARs knockdowns exhibited changes in the expression of CDC42, which correlated with the changes in their invasion. Conversely, stimulation of vector-control cells with PAR-1 or PAR-2 agonists enhanced, while PAR-3 agonist reduced the expression of CDC42. In the respective knock-down cells, the effects of agonists were abrogated.

Conclusion—The expression and/or activation of PARs is linked to PANC-1 cells invasiveness in vitro, probably via modulation of the expression of CDC42.

Keywords

Pancreatic adenocarcinoma; PANC-1 cell line; Proteinase-activated receptors; Invasion; Migration; CDC42

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Introduction

Proteinase-activated receptors (PARs) form a small family of four G-protein-coupled receptors, characterized by irreversible activation following proteolytic scission of their N-termini[1, 2]. Thrombin activation of PAR-1 was initially identified in the coagulation cascade, where it activates platelets[3] Currently, PARs have been identified in most mammalian tissues. PAR-1, -2 and -4 were traditionally linked to the calcium mobilization pathway via Gq/11, although they have been reported also to couple to other G-proteins, as well as to G-proteins-independent pathways[2]. PAR-3, although rarely shown to mobilize calcium[4,5], has been also shown to act as a co-receptor and enhance PAR-1 activation by forming PAR-1/PAR-3 heterodimers[6].

Many reports linked PAR-1 and PAR-2 activation and/or expression to proliferation, migration, invasion and metastasis of human cancers[7, 8, 9, 10, 11, 12, 13,14]. Although pancreatic adenocarcinoma (PAC) is one of the most deadly and therapy-resistant human malignancies[15, 16], there are few reports on PARs expression in PAC and their possible involvement in invasion and metastasis[11, 17]. We have previously reported that human fetal pancreas, human islet-derived pancreatic precursor cells, and human PAC PANC-1 cells express PAR-1, -2 and -3 mRNAs [18]. In order to assess the role of PARs in PAC cells phenotype, we depleted their expression in PANC-1 cells by shRNA knockdown technology and tested the effect of these knockdowns on in vitro proliferation, migration, and invasion. Our results show that PAR-1 or -2 knockdown markedly inhibits, while PAR-3 knockdown greatly promotes in vitro migration and invasiveness. Our results also suggest that the altered migration of PARs KDs is correlated with changes in the expression of CDC42.

Materials and Methods

Cell culture—Cells were routinely cultured in DMEM, 10% fetal bovine serum (FBS), penicillin and streptomycin (50U/ml and 50μg/ml, respectively) at 37° and in 6/94% CO_2/air mixture. Cells were re-fed twice each week.

Knockdown cells—PANC-1 Cells were infected with a retroviral vector which contained the specific shRNA essentially as previously described for plasminogen activator inhibitor 1 knockdown [19]. Selection for stable knockdowns was performed by growing the cells in medium containing hygromycin (200ug/ml). Higher passages were periodically maintained for 2 weeks in 200ug/ml hygromycin. Cells infected with empty retroviral vector were treated identically and used as controls.

Western analysis—For PARs Western analysis, cells were washed with PBS and removed by rubber policeman in 0.7 ml of PBS containing 1mM EDTA and 1mM PMSF. After centrifugation (4°C X 10 minutes X 10000 RPM), the supernatant was collected and the particulate fraction sedimented by ultracentrifugation (4°C X 100,000g rotor TLA-120.2 X1h). The pellet was re-suspended in lysis buffer (Cell Lysis Buffer #9803, Cell Signaling Technology with 1mM PMSF). Protein concentration was determined by BCA assay (BCA Protein Assay Kit#23227 Pierce, Rockford, IL, USA), using bovine serum albumin as standard.

For CDC42 analysis, cells grown close to confluence were washed with PBS and lysed in RIPA buffer with protease inhibitors cocktail (Roche, Indianapolis, IN).

The lysate samples containing equal amounts of protein were separated using 10% SDS-PAGE and then transferred to a nitrocellulose membrane for detection. The membrane was blocked overnight with 5% low fat milk. Primary antibodies in TTBS solution + 1% BSA against each PAR (1:600), or anti-CDC42 (1:1000 from Cell Signaling Technology,

Danvers, MA), or anti-GAPDH (1:2000, from Abcam, Cambridge, MA) were added to the membrane for 3h at 25°C. The membrane was washed with TTBS solution + 0.2% BSA. A secondary HRP-conjugated antibody (1:2000 in TTBS solution + 0.2% BSA) was then added for 1h at 25°C. The membrane was washed with TTBS solution and the protein bands detected by chemiluminescence (ECL).

Calcium imaging—Microscopic single cell Fura 2 imaging to assay Vector-control and PAR-1, -2, or -3 KDs cytosolic calcium responses to their respective agonists were performed essentially as described (Wei C, Geras-Raaka E, Marcus-Samuels B *et al.*, 2006). The acquisition was done using Nikon TMD inverted microscope with X40 large numerical aperture (1.3) objective and Photonics Science Isis intensified camera. Consecutive 340/380 nm frames were acquired at 3/sec and analyzed with Metamorph version 6.1 (Molecular Devices LLC, Sunnyvale, CA). Results are presented as 340/380 ratios.

Colony formation assay in Matrigel—Assay was performed in 48-well clusters. Each well was plated with 150μl of Matrigel (BD Biosciences, San Jose, CA). Following 30min of polymerization at 37°, 300 cells/well were mixed with 150μl of 5% Matrigel in serum-containing medium, overlayed in triplicate and cultured 7–10 days. The pattern of the cells' outgrowth in Matrigel matrix was examined and photographed using a phase-contrast microscope. To obtain better contrast and visualize the entire well, cultures were incubated with 140μl medium containing 1mg/ml MTT for 2h and photographed using binocular stereoscope at X0.63 magnification. The resulting micrographs were analyzed using the Metamorph 6.1 (Molecular Devices, LLC, Sunnyvale, CA) software for colonies number and mean area.

Migration and invasion analysis—Cells were grown overnight with 0.5% FBS and detached with 0.05% trypsin or, when indicated, with Ca^{2+}/Mg^{2+}-free PBS and 5 mM EDTA. For invasion analysis, the Transwell inserts (8μm apertures, Costar, Lowell, MA) were coated with 100μl of 1mg/ml Matrigel for 60min at room temperature and the excess liquid removed. For migration assays, 5,000 cells, and for invasion assays 20,000–40,000 cells were applied in 100μl serum-free medium and placed over 400μl of FBS (10%) medium in the lower chambers. Following 24h incubation, the inserts were removed and the cells in the lower chamber were counted. The inserts were washed with calcium-free PBS and further incubated for 10 min in 400μl of 0.05% trypsin solution. 40μl of FBS were added to stop trypsin activity and the cells detached from the bottom of the insert were counted. Each experiment was run in triplicate wells.

PCR—Total RNA was extracted from two combined wells of 6 wells plate or from 25cm flask using either RnEasy or EZ-RNA-II kit according to the manufacturers' protocols. Reverse transcription was performed using random primers with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, Ca, USA) according to the manufacturer's protocol kit.

Quantitative real-time PCR was performed in 25ul reaction volume in 96-well plates using cDNA prepared from 1μg of total RNA, Universal PCR Master Mix (Applied Biosystems) and Taqman sequence-specific primers. Primers and probes were Assay-on-Demand (Applied Biosystems). Quantitative RT-PCR results were normalized to GAPDH.

PANC-1, cells were purchased from the ATTC (VA, USA). DMEM, F12, Hank's salt solution, PBS, antibiotics and trypsin solution were purchased from Biological Industries, Beth HaEmek, Israel. Matrigel was from BD-Bioscience (Bedford, MA, USA). Thrombin was from MP Biomedicals, CA, USA. Rabbit anti-human PAR-1, -2, and -3 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Horseradish peroxidase-linked goat anti-rabbit IgG was from KPL (Gaithersburg, MD, USA). Fura 2AM was from Molecular Probes (Life Technologies, Grand Island, NY). PAR-1 (SFLLRN-NH2), PAR-2 (SLIGRL-NH2), and PAR-3 (TFRGAP-NH2) agonist peptides, respectively, were custom-synthesized by SBS Genetech, Beijing, China.

Solutions—TTBS solution: 0.15 NaCl, 0.1% Tween20, 0.1M Tris PH 7.5. ECL solution: 0.1M Tris pH 8.8, 90mM p-cumaric acid, 250mM luminol (A8511, Sigma-Aldrich, Israel), 6.2μL H_2O_2.

Statistics—All experiments were performed several times in triplicates or quadruplicates. Student's t-test was used and differences were considered significant when $p \leq 0.05$.

Results

PARs knock-down

We previously reported that PANC-1 cells, as well as human fetal pancreas, adult islets of Langerhans, and human islet-derived pancreatic precursor cells express PAR-1, -2 and -3 mRNAs and exhibit PAR-1 and -2-mediated calcium responses. PAR-4 mRNA was undetectable (Wei C, Geras-Raaka E, Marcus-Samuels B *et al.*, 2006). In order to study whether expression of PARs in PANC-1 cells affects their phenotype, we stably knocked down their expression by shRNA retroviral infection. PANC-1 cells stably infected with an empty vector were used as controls. The knock-downs were verified by Western analysis, which revealed a major band for each receptor, co-migrating approximately with a 50KD molecular mass marker. The stable knock-downs exhibited a large decrease in the major band, while the minor bands almost completely disappeared (see Fig. 1 insets).

It has been demonstrated that stimulation of PAR-1 or -2 activates the G-protein-coupled calcium mobilization cascade[20]. PAR-1 or -2 KDs exhibited inhibition of either the calcium transient amplitudes, and/or increases in the response delay, while knockdown of PAR-3 had no effect on Ca^{2+} responses (Supplementary data, Fig. 1S, Table 1S). These results proved that PAR-1 or -2 knock-down was sufficient to affect the acute physiological responses mediated by these receptors.

The effects of PARs knock-down on proliferation, culture morphology and gene expression

PAR-1 and -3 KDs exhibited the same proliferation rate as that of vector-controls. PAR-2 KD proliferated approximately 40% faster than vector controls (not shown).

The gross morphology of Vector-controls or PARs KDs was similar to that of parental PANC-1 cells. The cultures exhibited considerable shape and size variability, including numerous filopodia and lamellipodia, as well as areas where a second layer of cells grew over the monolayer (Fig. 1). PAR-2 knockdowns, however, exhibited a greater proportion of large cells and more compact colony morphology.

The effects of PARs knock-down on colony formation in Matrigel

Colony formation in three-dimensional semi-liquid Matrigel medium more closely resembles the *in vivo* conditions. The number of colonies reflects the survival of the unattached cells, while the size of the colony characterizes the ability to proliferate in the ECM-derived matrix. PAR-1 and -2 KDs exhibited modest, not statistically significant changes in both number of colonies and their mean area. PAR-3 KDs formed 87±12% more and 59±10% larger colonies than Vector-controls (Fig. 2, $p<0.01$ for each).

The effects of PARs knock-down on migration and invasion

The results of the colony formation experiments suggest that PARs may play a role in PAC invasiveness. Colony formation assay relies on experiments lasting between 7 and 10 days in semi-liquid matrix and, therefore, cannot be tested with specific PARs peptide agonists. To test the rapid effect of PARs, we examined the migration and invasion properties of PARs knockdowns. PAR-1 and PAR-2 KDs exhibited pronounced inhibition (by more than 70 and 90%, respectively) of both migration and invasion when compared to vector-controls. By comparison, PAR-3 KDs were more migratory (3.5-fold, $p<0.001$) in the Transwells assay and more invasive in Transwells coated with Matrigel (Fig. 3).

These results confirmed our hypothesis that PARs may be involved in the acute modulation of PANC-1 cells invasion. To test this possibility, we have assayed the invasion of vector-control and PARs KDs cells in the absence or presence of specific peptide agonists of PARs. PAR-1 peptide agonist weakly enhanced invasion, PAR-2 agonist markedly potentiated and PAR-3 agonist moderately inhibited invasion (Fig. 4).

The effect of PARs knock-down on the expression CDC42

Our results indicated that the knockdown of PARs affected their migration and invasion to the same extent, suggesting that the differences in invasion reflected their different migration properties. Cellular migration has been extensively linked to increased expression and/or activation of Rho family small GTPases[21, 22, 23]. In order to investigate the mechanism(s) of the altered behavior of PARs KDs, we performed Western analysis of CDC42, a member of the Rho small GTPases family involved in directed migation. The expression of CDC42 correlated with the migration and invasion characteristics of the three knockdowns. The expression of CDC42 was decreased in PAR-1 and markedly in PAR-2 KDs, whereas it was increased in PAR-3 KD. (Fig. 5A).

When the bends of CDC42 were normalized to the corresponding bands of GAPDH, the amounts of CDC42 were decreased by 87 and 95% in PAR-1 and PAR-2 KDs, respectively, and increased more than four-fold in PAR-3 KD cells (Fig. 5B).

24h stimulation of vector-control cells with the respective agonist peptides yielded the expected increases in CDC42 expression for PAR-1 or -2 activation (2.2±0.2- and 2.1±0.4-fold of control, Fig. 6). The PAR-3 agonist peptide (TFRGAP) modestly (0.75±0.05-fold) decreased CDC42 expression. Also, the respective PARs KDs did not respond to the peptide agonists. Exposure to thrombin, activating both PAR-1 and PAR-3, resulted in a decreased expression of CDC42.

Discussion

PARs are a unique class of G-protein-coupled receptors, characterized by their irreversible activation following proteolytic scission of the N-terminal sequence. There is ample indirect evidence that PAR-1 (physiological agonist – thrombin) and PAR-2 (putatively activated by trypsin or tryptase) are involved in cancer progression and metastasis [24, 25, 9, 10,11,12,13,14]. PAR-4 appears to be less ubiquitous and, consequently, the data concerning this receptor's involvement in cancer are less extensive. PAR-3, also activated by thrombin, although relatively well expressed in numerous tissues, is a special case. Firstly, there is conflicting evidence regarding its mechanism of activation. While several laboratories reported that activation of PAR-3 follows the common Gq/11-PIP2-InsP3-Ca mobilization pathway[4,5], others reported no independent activity and suggested that PAR-3 is a co-receptor, potentiating the activity of PAR-1[6]. As a result, a limited amount of information regarding this receptor exists and only two recent publications suggest its possible involvement in cancer. Liu et al. reported that biopsies of rectal tumors exhibited a decrease in PAR-3

expression in the tumor and the surrounding tissue, when compared to healthy tissue obtained from more remote sites [26]. Wysoczynski et al, using PAR-3 knockdown of human rhabdomyosarcoma cell line, recently reported that PAR-3 appeared to negatively affect PAR-1-mediated stimulation of proliferation, had no effect on chemotaxis, and had a negative growth effect on implanted tumor cells *in vivo*[27].

To delineate the role(s) of PARs in PANC-1 cells biology we knocked down each of the extant receptors. In the present report we present evidence that PAR-1 and -2, and importantly PAR-3, may play a role in cancer metastasis via their effects on the migration and invasion of human pancreatic adenocarcinoma cells in vitro. PAR-1 or PAR-2 knockdowns exhibited marked (70 and 90%, respectively) inhibition of both migration and invasion. PAR-3 knock-downs, however, exhibited robustly higher rates of three-dimensional migration and of invasion through a Matrigel barrier. Peptide agonists of PAR-1, (weakly) and PAR-2 (robustly) enhanced invasion, whereas the PAR-3 specific peptide agonist, TFRGAP, caused a 40% decrease of invasion in empty vector-infected cells.

To identify the molecular target(s) that are affected by PARs knockdowns, we assayed the expression of small GTPases of the Rho family, which have been demonstrated to be instrumental in cellular migration[21, 22, 23]. Only CDC42 exhibited changes that were compatible with the observed changes in the invasion of the three knockdowns. While the expression of CDC42 was largely inhibited in PAR-1, and even more so in PAR-2 knockdown, it was up-regulated in PAR-3 knockdown, suggesting that this small GTPase may be responsible for the anti-invasion effect in PAR-1 and PAR-2 KDs, as well as the pro-invasion effect in PAR-3 KD cells. CDC42 expression also responded to the stimulation of each PAR in a manner compatible with the above presented hypothesis. PAR-1 or -2 peptide agonists increased the expression of CDC42 in vector-control cells and had no effect on the respective PARs knockdowns. PAR-3 agonist caused a modest decrease in CDC42 expression and no effect on the PAR-3 KD cells. These results suggest that the effects of PARs activation are rapidly translated into changes of CDC42 expression.

It is generally accepted that all Rho family GTPases are involved in migration, albeit affecting different specific functions, such as actin dynamics at the front of migrating cell (Rac) and the necessary contraction and retraction of the cell body and rear to allow forward motion (Rho)[21]. It has been suggested that CDC42 specifies the direction of migration[21] compatible with our results of directed invasion. The identification of CDC42 as the GTPase regulating cancer cell migration and invasion is amply documented in the literature for many cell lines, including sarcomas and melanoma [28, 29, 30, 31, 32, 33, 8], as well as pancreatic adenocarcinoma cells lines, including PANC-1[34]

Our results are compatible with the proposed enhancement of cancer progression by PAR-1 and -2 and suggest that the expression of PAR-3 maybe playing a cancer-limiting role (Fig. 7). This hypothesis is supported by a recent report on PAR-3-mediated decreased proliferation of human rhabdomyosarcoma cells [27]. Hence, to the best of our knowledge, ours is the second report pointing to PAR-3-mediated attenuation of cancer growth and metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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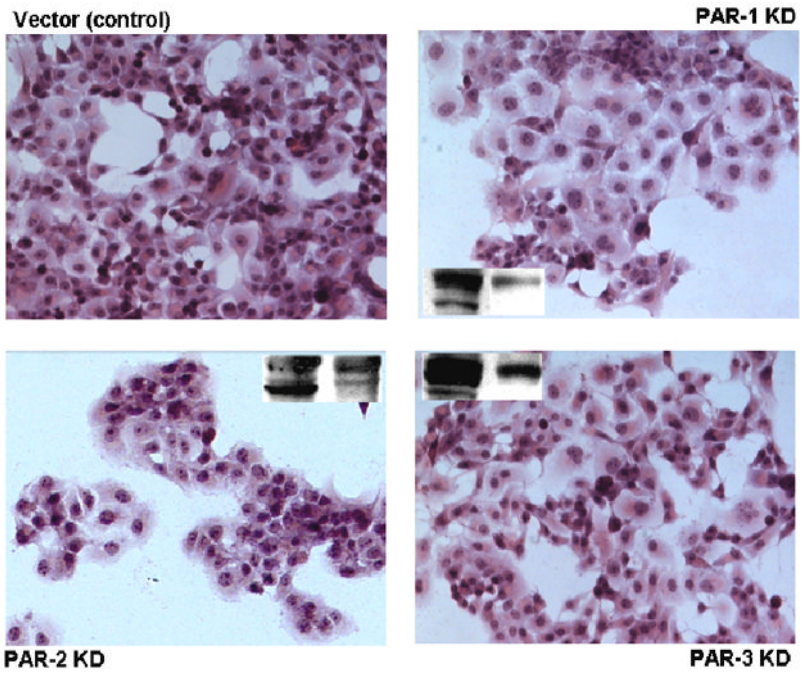

Fig. 1.
Gross morphology of sub-confluent control (empty vector-infected) and PARs KDs. Hematoxylline-eosin stain. Objective magnification X4. Insets-Western analysis of PARs bands.

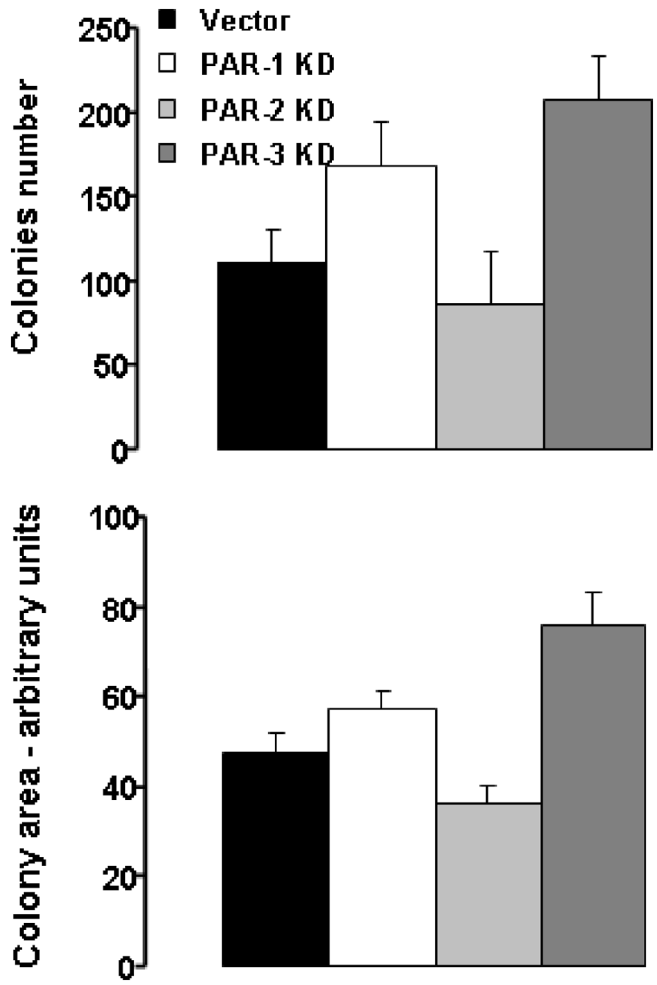

Fig. 2. Colony formation in Matrigel

Vector-control and each of the three PARs KD cells were plated in Matrigel (see Methods) at 500 cells/well. Following 7–10 d, the colonies were stained and analyzed for number of colonies and their mean area. Results of three independent experiments are shown.

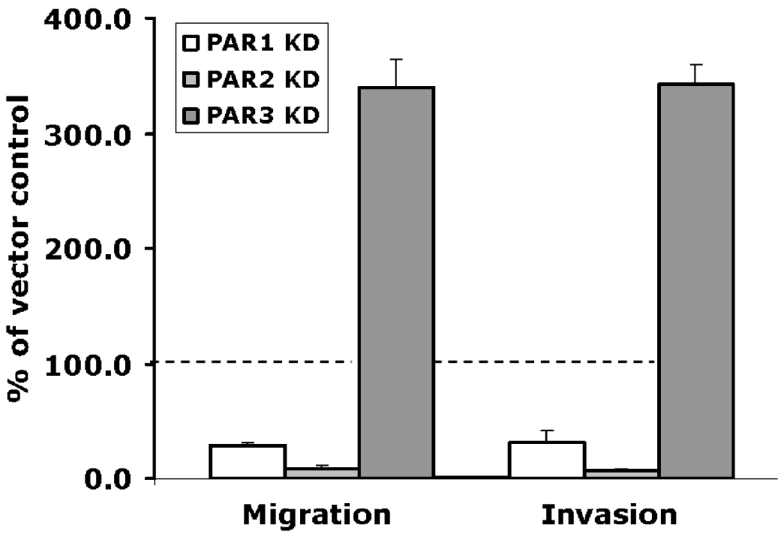

Fig. 3. Migration and invasion of PARs knockdown cells

The migration and invasion assays were performed as described in Methods. Results of five experiments in triplicates are presented as % change from values obtained for the vector-controls. The mean±SE values for vector-control cells were: migration-222±12 (n=15); invasion-151±15 (n=15) cells/well, respectively. p for all <0.001.

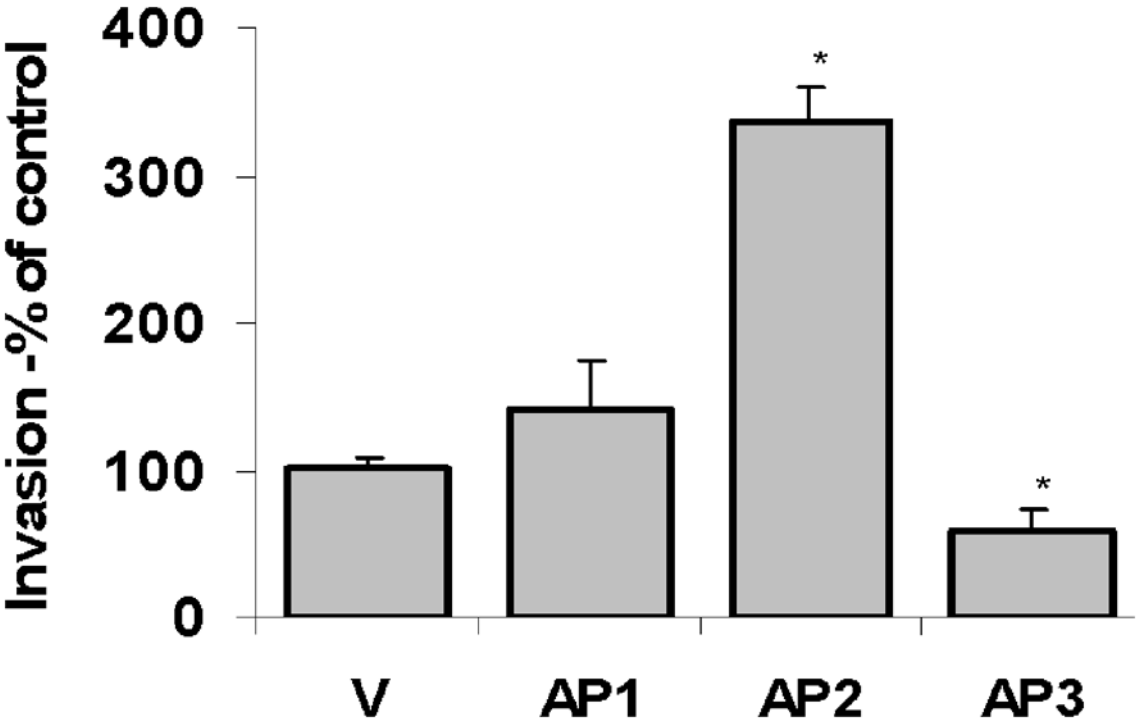

Fig. 4. PARs specific agonists change the invasion of vector-control cells

Vector-control cells were assayed for invasion as described in Methods. The PARs specific agonist peptide amides (AP1, AP2, and AP3, respectively, 100μM) were added to the upper chamber of the Transwells in the indicated wells. The results show mean±SE of 3–5 experiments, each run in triplicates. The vector-control value (159±14 cells/well) was set at 100% and the effects of agonists presented as mean±SE % of controls. Asterisk – $p<0.05$.

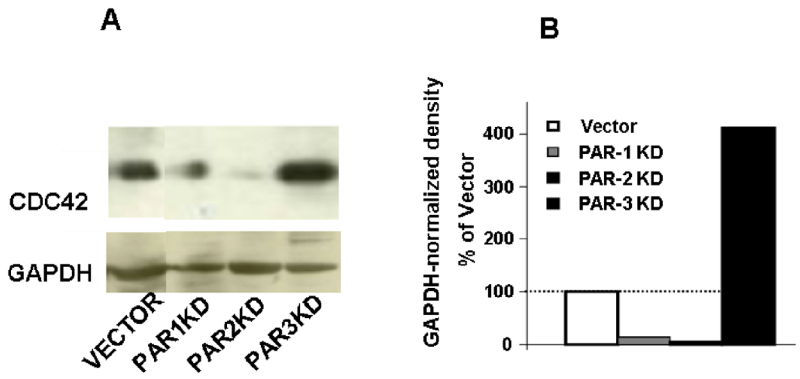

Fig. 5. PARs knockdowns change the expression of CDC42

Vector-control and each PAR knockdown cultures were grown to 70–80% confluency in 100mm dishes. The cells were washed free of medium with Hank's solution and solubilized in homogenization medium as described in Materials and Methods. The homogenates were separated on 10% PAGE-SDS gels, electrotransferred to nitrocellulose and assayed for the indicated proteins as described in Material and Methods. A –Results of a typical experiment. B – Densitometric quantitation of CDC42 bands normalized to the GAPDH band. The experiment was performed three times on two separate biological samples.

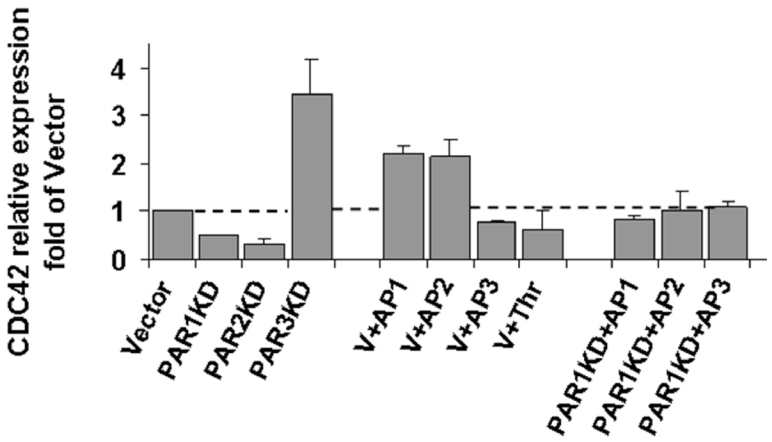

Fig. 6. PARs peptide agonists change the expression of CDC42

Vector-control and each PAR knockdown cultures were grown to 70–80% confluency in 100mm dishes. The cells were exposed to the desired peptide agonists (100μM) for 24 h. The cells were washed free of medium with Hank's solution and solubilized in homogenization medium as described in Materials and Methods and legend to Fig. 5. The experiment was performed four times on two separate biological samples. Abbreviations: PAR-1-KD (KD1), PAR-2-KD (KD2), PAR-3-KD (KD3), V – Vector-controls, PAR-1, -2 or -3 activating peptides – AP1, 2 or 3, Thr – thrombin (2U/ml).

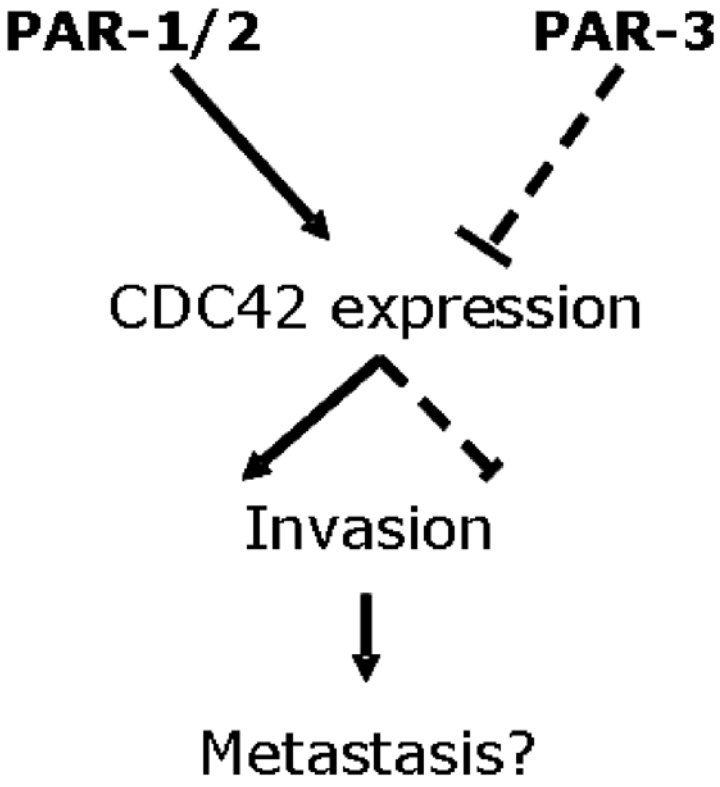

Fig. 7.
Schematics of PARs modulation of migration/invasion