Activation of FAK/PI3K/Rac1 Signaling Controls Actin Reorganization and Inhibits Cell Motility in Human Cancer Cells

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Key Words

Actin • Migration • FAK • PI-3K • Rac1 • Melanoma cells • Breast cancer cells

Abstract

We have recently identified a specific signaling pathway that regulates actin reorganization in malignant human breast and prostate epithelial cells associated with FAK, PI-3K and Rac1 activation. Here we report that this pathway operates in MCF7 cells upon activation of membrane androgen receptors (mAR). Stimulation of mAR by the non-permeable testosterone-BSA conjugate resulted in early actin reorganization documented by quantitative measurements of actin dynamics and morphological analysis of microfilament organization. This effect was regulated by early phosphorylation of FAK and subsequent PI-3K and Rac1 activation. The functional role of this pathway was further shown in A375 melanoma cells. Treatment with the opioid antagonist α_51 casomorphin resulted in rapid and potent actin remodeling in A375 cells, regulated by rapid activation of the FAK/PI-3K/Rac1 signaling. Pretreatment of both cell lines with the specific PI-3K inhibitor wortmannin blocked actin reorganization. Interestingly, wound

healing assays revealed that testosterone-BSA and α_51 casomorphin significantly inhibited MCF7 and A375 cell motility respectively. These effects were abrogated through blockade of PI-3K signaling by wortmannin. The results presented here indicate that actin reorganization through FAK/PI-3K/Rac1 activation operates in various human cancer cell systems supporting a functional role for FAK/PI-3K/Rac1/actin signaling in controlling cell motility.

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Introduction

Aberrant cell migration and invasion typically characterize cancer cells and actin remodeling is one of the crucial elements of the cell-motility process suggesting the involvement of a deregulated actin system in malignant transformation and progression. Indeed, the dynamic state of actin cytoskeleton is actively modulated by various extracellular signals [1-3] and microfilament organization and stability is impaired in malignant compared to normal cells [4, 5]. Initial signals controlling actin cytoskeleton rearrangements may include modification of FAK, a non-

receptor tyrosine kinase that is localized in focal adhesions [6]. Subsequently, FAK serves as a scaffold or adaptor to recruit a number of structural and signaling molecules, including PI-3K, to promote cell adhesion turnover and the actin cytoskeleton remodeling [7]. FAK signaling controls cell migration [8] mainly by mediating the initial assembly [9] or the disassembly of focal adhesions [10]. FAK has been also shown to promote invasion and metastasis by influencing actin cytoskeleton dynamics that in turn modify cell-substratum adhesion [11].

Recently we have reported that exposure of malignant breast MCF7 cells to the opioid agonist α_{s1} casomorphin [1] and malignant prostate LNCaP cells to the non-permeable testosterone-BSA conjugate (testosterone-BSA) [12] induced potent reorganization of the actin cytoskeleton and regulation of cell migration, proliferation and apoptosis [13]. A specific signaling pathway involving FAK phosphorylation and subsequent phosphatidylinositol-3 kinase (PI-3K) and Rac1 activation was shown to regulate these effects in both cellular systems [1, 14], indicating a functional role of this signaling cascade in cellular responses. It remains however unclear, whether activation of this cascade may be a common mechanism linking actin cytoskeleton rearrangements and migration potential in various tumor cell systems.

The aim of the present study was to investigate whether actin reorganization, initiated by the phosphorylation of FAK and mediated by downstream PI-3K and Rac1 activation is functional in different human cancer cell systems. To this end we have used MCF7 breast epithelial cancer cells and A375 melanoma cells. We demonstrate here that MCF7 express functional membrane androgen receptors (mAR) and that activation of these receptors by testosterone-BSA induces rapid actin reorganization regulated by non-genomic FAK/PI-3K signaling. In addition, we report that opioid treatment of A375 melanoma cells induces both, activation of the specific signaling cascade and actin reorganization. Finally we provide evidence that activation of the FAK/PI-3K/Rac1/actin signaling inhibits cell motility, supporting its broader role in cancer progression.

Materials and Methods

Materials

DMEM culture media, trypsin, EDTA, fetal bovine serum (FBS) were all from GIBCO-BRL (Co, MD, USA). Wortmannin was purchased from Sigma (St. Louis, MO, USA). ECL western blotting Kit and protein-A Sepharose beads were obtained from Amersham (Arlington Heights, IL, USA). Rhodamine-phalloidin

and FITC-IgG antibody were from Molecular probes Inc (Eugene OR USA). α_{s1} casomorphin was synthesized and testosterone-BSA was prepared as previously described [1, 12]. Rac1/Cdc42 activation assay kit and anti-PI-3K antibody were purchased from Upstate Biotechnology (Inc, USA). Antiphosphotyrosine monoclonal antibody (PY20) as well as anti-FAK and anti-PI-3K (p85) polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other chemicals were obtained from usual commercial sources at the purest grade available.

Cell cultures and Wound healing assay

A375 melanoma cells obtained from ATCC (American Type Culture Collection) were cultured in (v/v) 1:1 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 mg/ml penicillin/streptomycin. MCF7 mammary adenocarcinoma cells, provided from ATCC were cultured in 1:1 Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 30 mM NaHCO_3 , 16 ng/ml insulin and 50mg/ml penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 in 25cm² or 75 cm² flasks. Subcultivation was performed with Ca^{+2} and Mg^{+2} free phosphate buffer saline (PBS) containing 0.25% trypsin and 5mM EDTA. Cells were studied between passages 2 and 10. All experiments were performed during the logarithmic growth phase. Prior to the experiments all cell lines were transferred to DMEM containing penicilline/streptomycin.

For the wound healing assay, confluent cell cultures were scrapped with a pipette tip across a 60 mm dish. Following wounding, culture medium was replaced with fresh medium and cells were exposed to 10^{-8} M α_{s1} casomorphin or 10^{-7} M testosterone-BSA in the presence or absence of 10^{-7} M wortmannin for the indicated time points.

Immunoblot analysis of soluble and insoluble actin

Cells treated as indicated were incubated in 500 μ l of Triton extraction buffer (0.3 % Triton X-100, 5mM Tris HCl, 2mM EGTA, 300mM sucrose, 400 μ M PMSF, 10 μ M Leupeptin, 2 μ M phalloidin, pH 7.4) for 5 min on ice. The Triton soluble proteins were precipitated with equal volumes of 6% perchloric acid (PCA). The Triton insoluble fractions were precipitated with 1 ml 3% PCA. Equal volumes from each fraction were subjected to SDS electrophoresis and proteins were transferred to nitrocellulose membrane. Membranes blocked with 5% non-fat dry milk were then incubated with monoclonal anti-actin antibody, followed by incubation with the appropriate labelled secondary antibody. Blots were developed using the enhanced chemiluminescence (ECL) western blot kit. Nitrocellulose blots were exposed for variable lengths of time. Bands were quantified using PC-based image analysis (Image analysis Inc, Ontario, Canada).

Confocal Laser Scanning Microscopy

For testosterone-BSA-FITC staining cells cultured on coverslips were washed twice with PBS 1.5% FBS for 1.5min and incubated for 1h with 1%BSA in PBS at RT. After two washes with PBS 1.5% FBS cells are exposed to testosterone-

Fig. 1. Testosterone membrane receptors and actin reorganization in MCF7 cells: (A): Confocal laser scanning microscopic analysis of MCF7 cells stained with (a) BSA-FITC and (b-d) testosterone-BSA-FITC, showing specific FITC related fluorescence at the cell membrane. (b-d) represent consecutive scanning sections (0.5μ) from the upper cytoplasmic region to the attachment site of the cells. (B): Reorganization of actin structures in MCF7 cells treated for 10 and 30min with testosterone-BSA and stained with rhodamine-phalloidin. Magnification $\times 400$.

BSA-FITC 10^{-7} while control cells are incubated with BSA-FITC 4×10^{-7} M for 1h at RT. After two washes with PBS 1.5% FBS and fixation with 0.5% paraformaldehyde for 30min, cells were washed twice with PBS 1.5% FBS for 3min and mounted with slow anti-fade.

For rhodamine-phalloidin staining of actin microfilaments cells treated as indicated were fixed using 3.7% formaldehyd, followed by short incubation (4min) with acetone at -20°C . Cells were then incubated for 40 min with rhodamine-phalloidin to stain the filamentous actin. Samples were examined using a confocal laser-scanning microscope (Leica Lasertechnik, Heidelberg Germany) and images were analyzed with the respective software.

Immunoprecipitation and tyrosine phosphorylation of proteins.

Cells were incubated with α_{s1} casomorphin or testosterone-BSA for the indicated time points, washed twice with ice-cold PBS and suspended in 500 μ l ice-cold lysis buffer (50mM Tris/HCl, 1% TritonX-100 pH 7.4, 1% Sodium deoxycholate, 0.1% SDS, 0.15% NaCl , 1mM EDTA, 1mM sodium orthovanadate). Protein concentration was determined using the Bio-Rad protein kit. Equal amounts of protein (500 μ g) were subjected to immunoprecipitation with a monoclonal anti-phosphotyrosine antibody (7 μ g/500 μ g total protein). Antigen-antibody complexes were then bound to protein A-Sepharose beads. Samples were resuspended in SDS sample buffer, subjected to SDS electrophoresis and transferred to nitrocellulose membrane. Proteins were incubated with FAK, and PI-3K monoclonal antibodies followed by the appropriate secondary antibody. Detection of protein bands was succeeded with ECL kit. Protein bands were quantified using a PC based image analysis system (Image analysis Inc, Ontario, Canada).

Rac activation assay

Rac activation was analyzed by affinity precipitation using a GST fusion-protein (Rac/cdc42 assay reagent Upstate Biotechnology Inc, USA kit), corresponding to p21-binding domain (PBD 67-150) of human PAK-1 bound to glutathione agarose. This protein specifically binds to and precipitates Rac-GTP from cell lysate [15]. Cells were treated as indicated, washed twice with ice-cold PBS and scrapped off in 400 μ l MLB lysis buffer (25mM PH 7.5 Hepes, 150mM NaCl, 1%, Nididet P-40, 10% Glycerol, 25mM NaF, 10mM MgCl_2 , 1mM EDTA, 1mM sodium orthovanadate, 10 μ M Leupeptin, 10 μ M Aprotinin). 500 μ g from each sample were incubated with PAK-1 PBD agarose for 60 min at 4°C . Agarose beads were collected by centrifugation at 14000g for 5 seconds. After resuspension of agarose beads in SDS sample buffer, samples were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Blots were incubated with polyclonal anti-Rac antibody followed with the appropriate secondary IgG antibody, using the ECL Western blotting kit. All bands were quantified by a PC-based image analysis (Image analysis Inc, Ontario, Canada).

Results

MCF7 breast cancer cells express functional mAR that mediate rapid actin reorganization

Functional membrane androgen receptors (mAR) resulting in the activation of non-genomic signaling pathways upon stimulation with non-permeable steroid conjugates (such as BSA-bound steroids), have been recently described in prostate (LNCaP and DU145) and

Ts/Ts+Ti	Control	5min	10min	30 min
MCF7	0.69±0.03	0.53±0.06*	0.54±0.05*	0.56±0.07*

Table 1. Testo-BSA casomorphin induces actin polymerization in MCF7 cells: Monomeric (Ts) and total actin (Ts+Ti) were measured by quantitative immunoblot analysis after subcellular fractionation of MCF7 cells treated for different time points with 10^{-7} M testosterone-BSA: * $P<0.05$, $n=4$.

breast cancer (T49D) cells [12, 13, 16]. Here, confocal laser scanning microscopy identified FITC related fluorescence, manifested as specific membrane staining in MCF7 cells treated with the testosterone-BSA-FITC conjugate, indicating the expression of mAR (Figure 1A, b-d). Conversely, in control cells treated with BSA-FITC no apparent staining could be identified (Figure 1Aa). Rapid actin cytoskeleton modifications in MCF7 cells upon testosterone-BSA treatment were investigated as an indicator of the functional status of mAR. For this purpose MCF7 cells were incubated with 10^{-7} M testosterone-BSA for various time-intervals and cellular actin cytoskeleton dynamics were assessed by appropriate quantitative techniques described in [17]. Quantitative immunoblot analysis of Triton X-100 insoluble cytoskeletal pellets and corresponding supernatants revealed that incubation of the cells with 10^{-7} M testosterone-BSA for 5 to 30 minutes resulted in a significant decrease of the Triton-soluble (monomeric) to triton-insoluble (filamentous) actin ratio, indicating rapid actin polymerization (Table 1). Confocal laser scanning microscopy showing potent actin reorganization at 10 and 30 minutes of treatment confirmed these findings (Figure 1B).

Testosterone membrane-binding induces rapid phosphorylation of FAK and PI-3K and activates Rac1 in MCF7 breast cancer cells

In order to investigate whether mAR stimulation may induce FAK activation, MCF7 cells were incubated with 10^{-7} M testosterone-BSA for 5 to 30 min. Cell extracts were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody followed by immunoblot analysis with an anti-FAK polyclonal antibody. As shown in Figure 2A (upper panel, left) the phosphorylation of FAK was significantly increased after 5min of treatment and persisted for at least 30 min. To further examine whether PI-3K is activated upon mAR stimulation, an *in vitro* kinase assay was performed on anti-phosphotyrosine immune complexes isolated from equal amounts of

Fig. 2. Testosterone-BSA induces tyrosine phosphorylation of FAK and PI-3K along with Rac GTPase activation in MCF7 cells: (A): MCF7 cells were stimulated for the indicated time points with testosterone-BSA. Equal protein amounts were immunoprecipitated (IP) with anti-phosphotyrosine (p-Tyr) antibody. Equal amounts of total protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The tyrosine-phosphorylation and total proteins were detected by immunoblotting (IB) with anti-FAK (upper panels), and anti PI-3K (P85) (lower panels) antibodies. (B): Equal protein amounts of MCF7 cell lysates treated with testosterone-BSA for the indicated time points, were affinity precipitated (AP) with GTP-PBD bound to glutathione-agarose beads. Precipitated GTP-Rac1 was detected by immunoblotting (IB) with anti-Rac antibody. The number below each lane indicates the fold phosphorylation of FAK or p85 and Rac1 activation with that of untreated cells taken as 1. Similar results were obtained in three independent experiments.

proteins in control and testosterone-BSA treated MCF7 cell extracts. It was observed that the tyrosine phosphorylation of the p85 regulatory subunit of PI3-K was induced within 5 minutes by testosterone-BSA treatment and persisted for at least 30 minutes (Figure 2A, lower panel, left). Total protein amounts remained unaffected during the course of the experiment (Figure 2A, right panels). Finally, to determine whether PI-3K is an effector of FAK, MCF7 cells were pre-treated with the specific PI-3K inhibitor wortmannin (100nM) and then mAR's were stimulated by testosterone-BSA. As shown in Figure 3A the phosphorylation of FAK remained unchanged by wortmannin indicating that PI-3K is activated downstream of FAK. The phosphorylation of the p85 subunit was only partially inhibited (Figure 3A, lower panel) since wortmannin interacts with the p110 catalytic subunit of PI-3K and would not necessarily be expected to affect tyrosine phosphorylation of the p85 regulatory subunit.

Fig. 3. Effects of PI-3K inhibitor wortmannin on tyrosine phosphorylation of FAK, PI-3K and actin reorganization in MCF7 cells: (A): MCF7 cells were pretreated with wortmannin for 30 min and then incubated for 10 and 30 min with testosterone-BSA. Equal protein amount from cell lysates were immuno-precipitated (IP) with anti-phosphotyrosine antibody. The inhibition of tyrosine phosphorylation was tested by immunoblotting analysis, using specific anti-FAK and anti-PI-3K (p85) antibodies. The number below each lane indicates the fold phosphorylation of FAK or p85 with that of untreated cells taken as 1. (B): MCF7 cells pretreated or not with wortmannin for 30 min and then incubated for 30 min with testosterone-BSA. The redistribution of filamentous actin, determined with rhodamine-phalloidin staining was inhibited by wortmannin. Magnification x400. Representative results from n=3 experiments.

Table 2. α_{s1} casomorphin induce actin polymerization in A375 cells: Monomeric (Ts) and total actin (Ts+Ti) were measured by quantitative immunoblot analysis after subcellular fractionation of A375 cells treated for different time points with 10^{-8} M α_{s1} casomorphin : * $P < 0.05$, n= 4.

Ts/Ts+Ti	Control	15min	30min
A375	0.44±0.02	0.38±0.12*	0.36±0.01*

Rac1 is a member of the Rho-GTPases family involved in actin cytoskeleton remodeling [18]. To investigate whether Rac1 is also implicated in actin reorganization induced by testosterone-BSA in MCF7 breast cancer cells, equal volumes of cell lysates were affinity precipitated with a GST-fusion protein corresponding to the p21-binding domain of PAK1 (GST-PBD) that exclusively binds to the active form of the Rac1-GTP [15, 18]. The presence of Rac1 was assessed by specific anti-Rac1 antibody. Equal volumes of total cell extracts were also analyzed by immunoblotting for total amounts of Rac1. As shown in Figure 2B, left panel, the ratio of GTP-Rac1 to total Rac1, that represents the normalized amount of active Rac1, was significantly increased at 5 to 30 min of testosterone-BSA treatment, indicating that Rac1 activation is involved in actin cytoskeleton remodeling induced by mAR activation. Finally, pretreatment of MCF7 cells with wortmannin blocked testosterone-BSA induced actin reorganization (Figure 3B), indicating a regulatory role of the FAK/PI-3K signaling mechanism in actin reorganization.

Alpha_{s1} (α_{s1}) casomorphin induces rapid actin reorganization in A375 melanoma cells

α_{s1} casomorphin is an opioid agonist derived from human α_{s1} casein with potential anti-tumor activity [19]. We have previously shown that α_{s1} casomorphin induces actin cytoskeleton remodeling in MCF7 breast cancer cells [1]. To investigate whether cancer cells of different origin exhibit actin cytoskeleton rearrangements upon stimulation with opioids, A375 melanoma cells were treated with 10^{-8} M α_{s1} casomorphin for 15min and 30min. Quantitative immunoblot analysis of Triton-soluble and insoluble actin cytoskeleton preparations showed a significant decrease in G/total actin ratio, evident at 15min of treatment, indicating rapid actin polymerization (Table 2). These results were further supported by morphological analysis using confocal laser scanning microscopy. Indeed, remarkable actin reorganization was observed at 15 and 30 min of treatment (Figure 4A) characterized by the formation of stress fibers equally distributed to the cytoplasm of A375 cells.

Fig. 4. Opioids induce actin reorganization and FAK/PI3K/Rac1 activation in A375 cells: (A): A375 cells treated for 10 and 30min with α_1 casomorphin were stained with rhodamine-phalloidin, showing potent actin reorganization. Magnification, x400. (B): A375 cells were incubated with α_{s1} casomorphin for the indicated time points. Equal protein amounts were immunoprecipitated (IP) with anti-phosphotyrosine (p-Tyr) antibody. Equal amounts of total protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The tyrosine-phosphorylation and total proteins were detected by immunoblotting (IB) with anti-FAK (upper panels), and anti PI-3 kinase (lower panels) antibodies. (C): Equal protein amounts of A375 cell lysates treated with α_{s1} casomorphin for the indicated time points, were affinity precipitated (AP) with GTP-PBD bound to glutathione-agarose beads. Precipitated GTP-Rac1 was detected by immunoblotting (IB) with anti-Rac antibody. The number below each lane indicates the fold phosphorylation of FAK or p85 and Rac1 activation with that of untreated cells taken as 1. Similar results were obtained in three independent experiments.

Fig. 5. Effects of PI-3K inhibitor wortmannin on tyrosine phosphorylation of FAK, PI-3K and actin reorganization in A375 melanoma cells: (A): A375 cells were pretreated with wortmannin for 30 min and then incubated for 10 and 30 min with α_{s1} casomorphin. Equal protein amount of cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine antibody. The inhibition of tyrosine phosphorylation was tested by immunoblotting analysis, using specific anti-FAK and anti-PI-3K (p85) antibodies. The number below each lane indicates the fold phosphorylation of FAK or p85 with that of untreated cells taken as 1. (B): A375 cells pretreated or not with wortmannin for 30 min and then incubated for 30 min with α_{s1} casomorphin. The redistribution of filamentous actin, determined with rhodamine-phalloidin staining was inhibited by wortmannin. Magnification x400. Representative results from n=3 experiments.

α_{s1} casomorphin induces rapid phosphorylation of FAK, which in turn activates PI-3K and Rac1 in A375 melanoma cells

To examine whether the FAK/PI-3K/Rac1 pathway regulates the observed actin reorganization in opioid treated A375 cells the possible induction of FAK and PI-3K phosphorylation was initially investigated. A375 cells were

incubated with 10^{-8} M α_{s1} casomorphin for 15min and 30min. As shown in Figure 4B, left panels, the phosphorylation of FAK and the p85 subunit of PI-3K were significantly increased after 15min of treatment and persisted for at least 30 min, indicating that both kinases are activated in opioid treated melanoma cells. In addition, equal volumes of A375 cell lysates were affinity precipi-

Fig. 6. Inhibition of cell migration in MCF7 cells: Representative micrographs from n=3 wound healing experiments. Confluent monolayers of MCF7 cells were wounded with a pipet tip. Following wounding cell culture medium was replaced with fresh medium and cells were pretreated or not with wortmannin for 30min and then stimulated for 12 and 24 h with 10^{-7} M testosterone-BSA. Wound closure was monitored by microscopy at the indicated times. (B) Quantification of the time dependent effects of the indicated drugs on cell motility (% wound width) * $P < 0.05$, n=3.

Fig. 7. Inhibition of cell migration in A375 cells: Representative micrographs from n=3 wound healing experiments. Confluent monolayers of A375 (A) cells were wounded with a pipet tip. Following wounding cell culture medium was replaced with fresh medium and cells were pretreated or not with wortmannin for 30min and then stimulated for 12 and 24 h with 10^{-8} M α_{s1} casomorphin. Wound closure was monitored by microscopy at the indicated times. (B) Quantification of the time dependent effects of the indicated drugs on cell motility (% wound width) * $P < 0.05$, n=3.

tated with a GST-fusion protein corresponding to the p21-binding domain of PAK1 (GST-PBD). As shown in Figure 4C, left panel, Rac1 activation became evident at 15 and 30 min of treatment with α_{s1} casomorphin, suggesting that Rac1 is involved in actin cytoskeleton remodeling. To further determine whether PI-3K is an effector of FAK, as indicated in MCF7 cells, A375 cells were pretreated with the specific PI-3K inhibitor wortmannin (100nM) for 30 min and then treated with α_{s1} casomorphin for an additional 15 and 30min. As depicted in figure 5A, the opioid-induced FAK phosphorylation remained unaffected by wortmannin, indicating that PI-3K is activated downstream of FAK in A375 cells. In line with this finding pretreatment of A375 cells by wortmannin blocked casomorphin induced actin reorgani-

zation (Figure 5B), supporting the regulatory role of FAK/PI-3K signaling in actin reorganization. This result is reminiscent to our previous findings in MCF7 and LNCaP cells, which indicated that PI-3K phosphorylation following FAK activation upon opioid or testosterone-BSA treatment regulates actin reorganization [1, 14].

FAK/PI3-K activation affects cell motility

FAK activation has been linked to cell spreading and migration processes [20]. Its function is closely associated with changes in the dynamics of the actin filaments anchored to the focal adhesions. We conceivably tested the effect of the activation of the pathway triggered by FAK phosphorylation and the associated actin cytoskeleton reorganization on the cell motility, using

wound healing assays. To this end, confluent monolayers of MCF7 or A375 cells wounded with a pipet tip were treated in the presence or absence of testosterone-BSA and α_{s1} casomorphin respectively and wound width was evaluated at 12 and 24h of treatment. As shown in Figure 6, a statistically significant reduction of wound width by 18.96% and 22.01% was observed after 12 and 24 hours respectively in untreated cells, whereas in testosterone-BSA treated MCF7 cells the wound width was not significantly changed. Similar results were observed in opioid treated A375 cells. The wound width was significantly reduced by 16.16% and 18.70% after 12 and 24h respectively (Figure 7), while a partial, statistically non significant, inhibition of cell migration was observed at 12h and 24h in the presence of opioids. Interestingly, pretreatment of both cell lines with wortmannin (100nM) blocked the testosterone-BSA or opioid induced inhibition of cell migration in MCF7 (Figure 6) and A375 (Figure 7) cells, respectively. These observations suggest that in both tumor cell models, the FAK/PI-3K/Rac1 induced actin remodeling is involved in the inhibition of cell motility.

Discussion

In previous studies using differentially treated epithelial cancer cells, our group has identified a pathway triggered by FAK phosphorylation that led to actin reorganization through downstream PI-3K and Rac1 activation [1, 14]. In the current study we further demonstrate the activation of this pathway in two malignant cell lines of epithelial (MCF7) and non-epithelial (A375) origin. Interestingly, activation of this signaling cascade is triggered by different extracellular signals through activation of distinct membrane receptors, indicating an important biological role for this mechanism. In fact, our results provide evidence that this pathway is involved in the control of cell migration.

Recently, we have reported the identification of functional membrane androgen receptors (mAR) in the hormone sensitive LNCaP and the hormone insensitive DU145 human prostate cancer cell lines [12, 13]. mAR stimulation by testosterone-BSA was shown to induce rapid actin cytoskeleton reorganization [12]. Activation of this receptor may have anti-tumor potential, as shown in cell proliferation, cell migration and apoptosis experiments in prostate and breast cancer cells [13, 16]. In line with this, *in vivo* studies on prostate cancer xenografts revealed that the administration of testosterone-BSA, resulted in tumor regression [13, 21]. Expression of mAR

was also reported for T47D human breast cancer cells [16], indicating that mAR may be functional in cancer cells of different origin. The detection of functional mAR in MCF7 breast cancer cells shown in the present study (Figure 1) is in agreement with this observation. In accordance to previous reports [14], the stimulation of mAR led to FAK and PI-3K phosphorylation and subsequent Rac1 activation, which in turn regulate actin remodeling via rapid actin polymerization. The FAK/PI-3K/Rac1 signaling pathway seems to be active not only in cancer cells of epithelial origin, as has been already observed in LNCaP and MCF7 cells, but also in non-epithelial A375 melanoma cells. Indeed, treatment of A375 cells with α_{s1} casomorphin, an opioid agonist with anti-tumor activity [1, 20], showed potent and rapid actin cytoskeleton rearrangements (Table 2, Figure 4A) along with FAK/PI-3K/Rac1 activation (Fig 4B,C). These findings suggest a key functional role of these signaling transducers, linking extracellular signals with actin remodeling in cancer cells. Interestingly, the specific inhibitor of the p110 catalytic subunit of PI-3K (wortmannin), failed to inhibit FAK phosphorylation in both cell systems (Figures 3A, 6A), indicating that PI-3K is an effector of FAK. This is in accordance with our previous observations in LNCaP and MCF7 cancer cell lines [1, 14].

An abundance of experimental evidence points to FAK as an important player in signaling cascades associated with cancer progression and metastasis [22]. Increased FAK expression has been correlated with increased cell motility, invasiveness and proliferation [23-25]. Moreover, FAK phosphorylation has been demonstrated in different tumor cell types [26-28] but not in normal epithelium [29]. FAK activation has been linked to integrin clustering and is considered a critical step in the initiation of cell migration [20]. In contrast to most of the published studies, the evaluation of cell migration in breast cancer and melanoma cells presented here revealed that the activation of FAK/PI-3K/Rac1/actin pathway was associated with decreased cell motility. In addition, blocking this pathway by the specific PI-3K inhibitor wortmannin revealed that cell motility was at least partially restored. This may reflect differential activation of FAK via phosphorylation of additional sites. To date, six tyrosine phosphoacceptor sites have been identified in FAK, namely tyrosines 397, 407, 576, 577, 861 and 925 [30, 31]. Most of them seem to play positive regulatory roles. However, tyrosine phosphorylation of FAK at Tyr-407 has been recently reported to negatively regulate kinase activity and cell migration [32]. In addition, in a human breast cancer cell line, a bioactive sphingolipid

metabolite, sphingosine 1-phosphatase, inhibited cell migration through FAK Tyr-397 phosphorylation [33]. In accordance, treatment of squamous cancer cell lines with the proteasome inhibitor bortezomib resulted in decreased cell migration associated with FAK Tyr-397 phosphorylation [34].

In our systems, the inhibition of cell migration besides FAK activation was also associated with the activation of Rac1. Rac1, a member of the Rho-GTPase family of intracellular signal transducers is considered one of the key signaling components controlling actin cytoskeleton reorganization [18]. Rac1 has been shown to mediate lamellipodia formation and membrane ruffling in response to serum and PDGF stimulation [35]. In addition, Rac1 is also essential for actin stress fiber and focal adhesion complex formation [36]. Rho-GTPases have been shown to mediate actin cytoskeleton changes and cell migration [37, 38]. However, the functions of Rac1 on migration and invasion are at least in part cell type- and cell-substrate dependent. Thus, it has been reported that sustained activation of Rac induces invasion and metastasis of murine T-lymphoma cells [39], whereas, in other studies it was shown to inhibit migration and invasion of epithelial cells [40]. Our results showing inhibition of cell motility upon Rac1 activation in cancer cells of epithelial or

melanoma origin are in line with these observations.

In conclusion, the results presented here indicate that actin reorganization through FAK/PI3-K/Rac-1 signaling operates in various human cancer cell systems. Activation of this pathway inhibits cell motility, supporting its broader role in cancer progression. It has been reported that increased FAK expression is associated with poor clinical outcome [28, 41], indicating that FAK could be a therapeutic target. Thus, several means of pharmacological inhibition of FAK function are being investigated [22] and it is anticipated that inhibition of FAK should result in the control of neoplastic cell invasion. However, the possibility that at least in some tumors FAK inhibition could potentially result in increased cell motility, as shown in our study, implies that additional more detailed analysis is necessary in order to further evaluate the clinical implication of these findings.

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