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Prolactin-Induced Protein regulates cell adhesion in breast cancer

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ABSTRACT

Prolactin-Induced Prolactin (PIP) is widely expressed in breast cancer and has key cellular functions in this disease that include promoting invasion and cell cycle progression. Notably, we have recently identified a strong association between PIP-binding partners and a number of cell functions that are involved in cell adhesion. Therefore in this study, we investigated the effect of PIP on the regulation of cell adhesion using *PIP*-silencing in breast cancer cell lines T-47D, BT-474, and MFM-223. Our findings suggest that PIP expression is necessary for cell adhesion in a process that shows variation in the pattern of PIP regulation of cell-matrix and cell–cell adhesions based on the types of adhesion surface and breast cancer cell line. In this respect, we observed that *PIP*-silencing markedly reduced cell adhesion to uncoated plates in all three cell lines. In addition, in T-47D and MFM-223 cells fibronectin matrix induced baseline adhesion and reversed the *PIP*-silencing mediated reduction of cell adhesion. However, in BT-474 cells we did not observe an induction of baseline adhesion by fibronectin and *PIP*-silencing led to a marked reduction in cell adhesion to both uncoated and fibronectin-coated plates. Furthermore, we observed a significant reduction in cell–cell adhesion of BT-474 cell line following *PIP*-silencing. To explain an underlying mechanism for PIP regulation of cell adhesion, we found that PIP expression is necessary for the formation of α-actinin/actin-rich podosomes at the adhesion-sites of breast cancer cells. In summary, this study suggests that PIP expression regulates the process of cell adhesion in breast cancer.

1. Introduction

Prolactin-Induced Protein (PIP) is highly expressed in luminal A, luminal B, and molecular apocrine subtypes of breast cancer and is used as a characteristic biomarker for breast cancer [1–5]. Furthermore, expression of *PIP* in breast cancer is regulated by androgen and prolactin hormones through a number of transcription factors and signaling cross-talks including STAT5, Runx2, and CREB1 [6–8]. In this respect, we have previously demonstrated that *PIP* transcription is induced by CREB1 in a positive feedback loop between AR and extracellular signal-regulated kinase (ERK) [8].

Available data suggest that PIP is both cytoplasmic and secreted with key cellular functions that include promoting invasion and cell cycle progression in breast cancer cells [2–4,8]. Notably, secreted PIP has an aspartyl protease activity that can degrade fibronectin [9]. In addition, we have shown that PIP mediates invasion of breast cancer cells in a process that partially depends on the degradation of fibronectin by this protein resulting in the outside-in activation of integrin-β1 signaling [8]. Furthermore, PIP expression promotes cell proliferation and cell cycle progression [3,7,8,10]. Importantly, PIP is required for the progression through G1 phase, mitosis, and cytokinesis in breast cancer cells [3]. Moreover, defects in mitotic transition and cytokinesis following *PIP*-silencing are accompanied by an increase in aneuploidy and abnormal formation of actin and tubulin cellular structures [3].

To investigate the underlying molecular mechanisms of PIP function, we have carried out mass spectrometry analysis to identify protein-binding partners for endogenous PIP in breast cancer cells [3]. In this process, we have discovered a total of 156 PIP-binding partners and shown that PIP binds to β-tubulin and is necessary for microtubule polymerization [3]. Furthermore, our data suggest that PIP interacts with actin-binding proteins including Arp2/3 and is needed for inside-out activation of integrin-β1 mediated through talin [3]. Functional classification and pathway analysis of PIP-binding partners have revealed several PIP-associated cellular functions that include regulation of actin and cytoskeleton, remodelling of epithelial adherens junctions, and integrin signalling [3]. These findings suggest that PIP has a

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versatile function in breast cancer resulting from a diverse range of both intracellular and extracellular binding partners [3].

Further studies are required to elucidate the role of PIP in cellular and molecular processes associated with PIP-binding partners. Notably, PIP interaction with actin-binding proteins and modulation of integrin signalling are suggestive of a possible function for this protein in the regulation of cell adhesion. Therefore in this study, we investigated the effect of PIP on cell adhesion in breast cancer. Our results demonstrate that PIP promotes the process of cell adhesion and formation of α-actinin/actin-rich podosomes in breast cancer cells.

2. Materials and methods

2.1. Cell culture

Breast cancer cell lines T-47D and BT-474 were obtained from American Type Culture Collection (Manassas, VA). Breast cancer cell line MFM-223 was obtained from (Sigma–Aldrich, St. Louis, MO). All cell cultures were carried out as we previously published [3].

2.2. Quantitative Real Time-Polymerase Chain Reaction

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) to assess the expression level of *PIP* (assay ID: Hs00160082_m1) was carried out using Taqman Gene Expression Assays (Applied Biosystems, Grand Island, NY). Housekeeping gene *RPLP0* (Applied Biosystems) was used as a control. Relative gene expression was calculated as gene expression in the *PIP*-silenced group/average gene expression in the control group.

2.3. Western blot analysis

Rabbit monoclonal PIP antibody (Novus Biologicals, Littleton, CO) was applied at 1:1000 dilution using 20 μg of each cell lysate. Rabbit α-tubulin antibody (Abcam, Cambridge, UK) was applied to assess loading. To extract protein from conditioned media, cell lines were cultured for 48 h in serum-free media followed by concentration using Amicon Ultra-15 (3K) centrifugal filters (Millipore, Billerica, MA). A total of 100 μg from each concentrated sample was precipitated and used for immunoblotting. Western blots were performed in three replicates and average fold change was shown for each cell line.

2.4. RNA interference

PIP-silencing using siRNA duplex oligos was performed as we published before [3,8,11]. The following two siRNA-duplexes (Sigma–Aldrich) were applied; duplex-1 (PIP-D1): sense, 5′CUCUACAAGGUGCAUUUAA; antisense, 5′UUAAAUGCACCUUGUAGAG; duplex-2 (PIP-D2): sense, 5′CCUCUACAAGGUGCAUUUA, antisense, 5′UAAAUGCACCUUGUAGAGG. Transfections with siRNA Universal Negative Control # 1 (CTL-siRNA, Sigma–Aldrich) were used as controls. The effect of *PIP*-silencing was assessed 72 h following transfections.

2.5. Cell-matrix adhesion assay

Baseline cell adhesion assays were performed on uncoated tissue culture plates. The optimal seeding cell density for the adhesion assays was initially established for each cell line. *PIP*-silencing in T-47D, MFM-223, and BT-474 cell lines was carried out using PIP-D1 and PIP-D2, and control experiments were performed using CTL-siRNA. Seventy-two hours following siRNA transfection, cells were seeded in a 96-well plate at 40,000 (BT474) or 60,000 cells/well (T47D and MFM223) density and allowed to attach for 6 h at 37 °C. At the end of this incubation, cells were rinsed with PBS, fixed with absolute ethanol for 10 min, and stained with 0.1% crystal violet (Fisher Scientific) for 30 min. Cells were then washed with water and solubilized in 10% acetic acid. Finally, cell adhesions were measured by reading absorbance at 570 nm.

To assess fibronectin cell-matrix adhesion, 96-well plates were first pre-coated with 50 μl of PBS-fibronectin 20 μg/ml (Sigma–Aldrich) overnight at 4 °C. Before seeding cells, wells were blocked with 1% bovine serum albumin (Sigma Aldrich) for 30 min. Seventy-two hours following siRNA transfection, cells were seeded in a 96-well plate at 40,000 (BT474) or 60,000 cells/well (T47D and MFM223) density and allowed to attach for 6 h at 37 °C. At the end of the incubations, breast cancer cells were stained as explained before.

2.6. Cell–cell adhesion assay

Seventy-two hours following transfections with *PIP*-siRNA or CTL-siRNA, T-47D, BT-474, and MFM-223 cells were re-suspended at the dilution of 1,000,000 cells/ml and stained with 5 μM Vybrant CM-Dil (553/570 nm, Life Technologies) for 20 min. Each siRNA-transfected cell line was then seeded at 20,000 cells/well density in a 24-well plate containing a monolayer of matching non-transfected breast cancer cells at 80% confluence. Cells were incubated for 6 h at 37 °C, rinsed, and placed in fresh media. Next, images of 12 fields per well were taken with an Olympus IX81 Inverted Fluorescence Microscope (Center Valley, PA) using a 10X lens. Subsequently, labelled cells were counted for each field and the total number of cells in all 12 fields was calculated.

2.7. Immunofluorescence

Immunofluorescence (IF) staining was performed using the methodology we published before [3,11,12]. Seventy-two hours following siRNA transfections, breast cancer cells were seeded at 200,000 cells/well on poly-D-lysine-coated coverslips (BD Biosciences, Franklin Lakes, NJ). Cells were then incubated at 37 °C for 6 h before IF staining. Primary antibody incubation was carried out with rabbit monoclonal α-actinin-4 (Millipore) at 1:100 dilution and Alexa-594 secondary antibody (Life Technologies). Quantification of α-actinin-stained podosomes was performed on 250 cells in two replicate experiments for each condition. To assess co-localization between α-actinin and F-actin, we performed α-Actinin-IF staining followed by incubation with Alexa Fluor-488 Phalloidin (Cell Signaling Technology, Danvers, MA) at 1:20 dilution for 20 min. Finally, in order to localize α-actinin-stained podosomes at the adhesion-sites, a Z-stack analysis was performed on five control BT-474 cells using a Carl-Zeiss LSM 710 confocal microscope (Thornwood, NY).

2.8. Statistical analysis

Biostatistics was carried out using IBM SPSS Statistics 22. Paired sample t-test and Student's t-test were applied to calculate the statistical significance. All error bars depict ± 2SEM.

3. Results

3.1. PIP-silencing in breast cancer cells

To investigate the effect of PIP on cell adhesion, we employed breast cancer cell lines T-47D (ER+/luminal A), BT-474 (ER+/luminal B), and MFM-223 (ER-/molecular apocrine), representing

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