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Original Articles

PRL-3 engages the focal adhesion pathway in triple-negative breast cancer cells to alter actin structure and substrate adhesion properties critical for cell migration and invasion

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ABSTRACT

Triple-negative breast cancers (TNBCs) are among the most aggressive cancers characterized by a high propensity to invade, metastasize and relapse. We previously reported that the TNBC-specific inhibitor, AMPI-109, significantly impairs the ability of TNBC cells to migrate and invade by reducing levels of the metastasis-promoting phosphatase, PRL-3. Here, we examined the mechanisms by which AMPI-109 and loss of PRL-3 impede cell migration and invasion. AMPI-109 treatment or knock down of PRL-3 expression were associated with deactivation of Src and ERK signaling and concomitant downregulation of RhoA and Rac1/2/3 GTPase protein levels. These cellular changes led to rearranged filamentous actin networks necessary for cell migration and invasion. Conversely, overexpression of PRL-3 promoted TNBC cell invasion by upregulating matrix metalloproteinase 10, which resulted in increased TNBC cell adherence to, and degradation of, the major basement membrane component laminin. Our data demonstrate that PRL-3 engages the focal adhesion pathway in TNBC cells as a key mechanism for promoting TNBC cell migration and invasion. Collectively, these data suggest that blocking PRL-3 activity may be an effective method for reducing the metastatic potential of TNBC cells.

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Introduction

Breast cancer is the principle cause of cancer-related mortality in women worldwide [1]. Gene expression profiling has clustered the disease into five major subtypes based on estrogen receptor (ER) expression, progesterone receptor (PR) expression and human epidermal growth factor receptor 2 (HER2) amplification [2]. Antihormonal therapies are available for breast cancer patients with tumors expressing ER or PR, while targeted therapy with the monoclonal antibodies trastuzumab and pertuzumab are indicated for patients with tumors exhibiting HER2 amplification. Unfortunately, triple-negative breast cancers (TNBCs), which comprise 15–20% of all newly diagnosed cases of breast cancer and are frequently diagnosed as high grade invasive tumors, lack expression of these three molecular markers [3]. As a result, cytotoxic chemotherapy is most frequently utilized as the standard of care for women diagnosed with metastatic TNBC and there are currently no approved targeted agents for these patients. Metastatic TNBC tumors also have a higher risk of distant recurrence and death compared to other breast cancers [4]. Paradoxically, patients with non-metastatic TNBC treated with neoadjuvant chemotherapy have better response rates than other breast cancer subtypes [5], suggesting a relative driver of poor outcome in TNBC is the development of metastatic disease. It is therefore critical to identify new therapeutic agents that can either slow or prevent the metastatic dissemination of TNBC cells.

Our laboratory previously examined the effects of the novel TNBC-specific small molecule inhibitor AMPI-109 on TNBC cell migration and invasion [6]. Our studies revealed that AMPI-109 has a marked ability to block TNBC cell migration and invasion, attributable in-part to its ability to downregulate levels of the metastasis-promoting phosphatase, PRL-3 [6]. We found that AMPI-109 treatment, knock down of PRL-3 or catalytic impairment of PRL-3 activity, blocked the ability of TNBC cells to migrate, whereas overexpression of PRL-3 markedly enhanced both the rate and absolute degree of cell migration in a scratch-wound assay [6]. Moreover, we demonstrated that PRL-3 exerted analogous effects on TNBC cell invasion through Matrigel [6]. These studies suggest that PRL-3 is involved in controlling precursor events for TNBC metastasis. However, the molecular mechanism for how PRL-3 promotes the motility and invasiveness of TNBC cells is unclear.

In order to metastasize, cancer cells must lose attachment with neighboring tumor cells and adopt the ability to migrate, attach to, and invade through the epithelial basement membrane. This is a complex process primarily orchestrated through the formation,

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stabilization and remodeling of focal adhesion (FA) complexes composed of integrins, Src, FAK, ERK and numerous adaptor proteins and downstream effectors, such as RhoGTPases, that collectively regulate epithelial-to-mesenchymal transition (EMT) and trigger activation of cell migration and invasion programs [7,8]. Assembly and disassembly of FA sites within invadopodia control cell migration and invasion by mediating actin assembly and contraction, thereby promoting cell adhesion to various extracellular substrates on which cancer cells can migrate on or invade through [7,8]. Therefore, the identification of key regulators to FA site signaling is critical in order to determine strategies to prevent activation of these metastatic-enabling properties.

Here, we describe PRL-3 modulation of the FA pathway as a potential mechanism by which PRL-3 controls these pro-metastatic phenotypes. Altering PRL-3 expression had a significant impact both on protein expression and on activation levels of a number of FA pathway effectors, including the proto-oncogene tyrosine-protein kinase, *c-src* (Src), ERK, and several RhoGTPases involved in actin cytoskeletal restructuring. We also investigated the role of the matrix metalloproteinase (MMP), MMP-10, which we identified as being upregulated following overexpression of PRL-3. We found that MMP-10 upregulation following forced PRL-3 overexpression coincides with preferential TNBC cell attachment to and degradation of laminin, which is a major basement membrane component in breast tissue and a selective substrate for degradation by MMP-10. Moreover, PRL-3 overexpressing TNBC cells were capable of invading through laminin-rich Matrigel through an MMP-10 dependent mechanism.

Collectively, these data represent new molecular insight on how PRL-3 activates cell migration and invasion programs in TNBC as precursor events to metastasis – the major driver of TNBC-associated deaths.

Materials and methods

Materials

AMPI-109 was synthesized as previously described [9].

Plasmids, transfection and viral transduction

PRL-3 cDNA expression vector was purchased from Origene (Cat. # SC308739). Transfections were carried out using Mirus TransIT LT1 reagent according to manufacturer's instructions (Mirus Bio). Individual pLKO.1 lentiviral shRNA clones were purchased from the University of Colorado Cancer Center Functional Genomics Shared Resource. The RNAi Consortium identifiers are: TRCN0000010661 (shPRL-3 #1), TRCN0000355597 (shPRL-3 #2), TRCN0000378843 (shMMP-10 #1), and TRCN0000372935 (shMMP-10 #2). Transduced cells were selected in a medium containing 2.5 μg/mL puromycin. Specificity of PRL-3 knock down was determined by qRT-PCR. Both PRL-3 shRNAs (#1 and #2) exerted specific knock down of PRL-3 and did not reduce RNA levels of either PRL-1 or PRL-2.

Cell culture and immunoblot analysis

Cell lines were obtained from the University of Colorado Cancer Center Tissue Culture Shared Resource. BT-20 and MDA-MB-468 cells were cultured in DMEM/F-12 medium (Corning #10-092-CV) containing 10% fetal bovine serum. SUM-159 cells were cultured in HAM's F-12 medium (Corning #10-080-CV) containing 5% fetal bovine serum, 1 μg/mL hydrocortisone and 5 μg/mL insulin. All cell lines were authenticated by short tandem repeat DNA profiling performed by the UCCC DNA Sequencing and Analysis Core. Western blot analysis was conducted according to our previous protocol [10]. Antibodies used in the study were: PRL-3 (Cat. # ab82568, Abcam), p-Src (Y416) (Cat. #2101, Cell Signaling), Src (36D10) (Cat. #2109, Cell Signaling), p-ERK 1/2 (T202/Y204) (Cat. #4377, Cell Signaling), ERK 1/2 (44/42) (Cat. #4695, Cell signaling), RhoA (67B9) (Cat. #2117, Cell Signaling), Rac1/2/3 (Cat. #2465, Cell Signaling), MMP-10 (Cat. #SC-9941, Santa Cruz), and β-actin (Cat. # A5441, Sigma-Aldrich).

Immunofluorescence analysis

Immunofluorescence staining was performed as previously described [11] using green Alexa Fluor 488 phalloidin staining for F-actin (Cat. #A12379, Thermo Fisher), β-actin antibody for both filamentous and monomer actin forms (Cat. # A5441, Sigma-Aldrich) and nuclear DAPI stain (Cat. #P-36931, Thermo Fisher).

MMP array

A human MMP antibody array kit was purchased from Abcam (Cat. # ab134004). BT-20 cells were transiently transfected with PRL-3 cDNA expression vector 48 hours prior to cell lysis and the array developed according to the manufacturer's protocol. Membranes were developed using enhanced chemiluminescence (Perkin Elmer) and autoradiography.

Cell adhesion and spreading assay

We utilized the impedance-based xCELLigence Real-Time Cell Analysis system (ACEA Biosciences) for the detection of BT-20 and SUM159 TNBC cell adhesion and spreading on the following substrates: Laminin (Cat. #L4544, Sigma-Aldrich), Elastin (Cat. #E1625-5G, Sigma-Aldrich), Fibronectin (Cat. #F1141, Signa-Aldrich) and Collagen (Cat. #C2124, Sigma-Aldrich). Briefly, each substrate was diluted to 10 μg/mL in appropriate TNBC cell media and added to wells on a 96X E-Plate (ACEA Biosciences) and incubated for 1 hour at 37 °C. The coated plates were then washed with PBS and incubated in 0.5% BSA solution in PBS for 20 minutes at 37 °C. Wells were washed again with PBS and 5000 cells were added per well. Cell adhesion and spreading was measured as changes in impedance with the RT-CES system every 3 minutes for 3 hours. The assay expresses impedance in arbitrary cell index (AU) units. The cell index at each time point is defined as $(R_n–R_b)/15$; where R_n is the cell-electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the media alone.

Cell invasion assay

Cell invasion was assessed by IncuCyte Zoom Kinetic Live Cell Imaging (Essen BioScience). 96 well ImageLock Plates (Essen Bioscience) were coated overnight with 100 μg/mL Matrigel Basement Membrane Matrix (Cat. # 356231, Corning). The following day, Matrigel was aspirated and 25,000 cells were plated per well and allowed to adhere prior to making a scratch with the 96-pin WoundMaker (Essen Bioscience). 50 μL of Matrigel Matrix (8 mg/mL) was then added to the wells, and covered in 100 μL media containing the appropriate treatments (refer to Fig. 6 legend for details). Migration and invasion were quantified using the "Relative Wound Density" metric generated by IncuCyte software.

Reverse phase protein array analysis

The results outlined in Table 1 are in whole or part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. Protein expression values from the "Provisional TCGA Invasive Breast Carcinoma" dataset as assessed by the reverse phase protein array (RPPA) tool on the Memorial Sloan-Kettering Cancer Center's cBio Cancer Genomics Portal. Protein expression values are indicated by the Z-score (mean RPPA score/std) and were obtained using a Z-score threshold of +/– 2.0.

Results

PRL-3 copy number amplification positively correlates with high levels of Src protein in human breast cancers

To identify PRL-3 associated pathways potentially involved in migration and invasion phenotypes, we analyzed the provisional cancer genome atlas (TCGA) invasive breast carcinoma cohort and looked

Table 1
Protein expression changes in invasive human breast carcinoma samples exhibiting PRL-3 copy number amplification (>2 copies). Significance values were calculated based on Student's t-test.

Protein Expression Z-score (RPPA)			
Protein	PRL-3 Unaltered	PRL-3 Amplified	P-value
AR	0.15	–0.47	8.33E-08
GATA3	0.12	–0.37	3.15E-05
ESR1	0.11	–0.32	2.41E-04
PRKAA1	0.09	–0.28	3.48E-04
STK11	0.09	–0.28	3.00E-03
BCL2	0.08	–0.25	3.00E-03
MAPK9	0.08	–0.25	4.00E-03
PGR	0.07	–0.22	7.00E-03
SRC	–0.08	0.23	6.00E-03
CCNE1	–0.08	0.25	4.00E-03
SYK	–0.09	0.26	2.00E-03
CCNB1	–0.10	0.29	3.35E-04

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