EFFECTS OF FUNCTIONAL PERTURBATIONS OF PROFILIN ON BREAST CANCER CELL MIGRATION AND INVASION

by

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Breast cancer, one of the most common types of cancer among women, is now the second leading cause of cancer deaths after lung cancer. Since a majority of cancer deaths are due to metastasis of breast tumor cells to distant organs, understanding tumor cell invasion and metastasis at a molecular level will help us in developing therapeutics that will improve the quality of life of breast cancer patients. Cell migration, an integral component of tumor invasion and metastasis, is regulated by the assembly and disassembly of actin cytoskeleton, which involves the coordinated action of several classes of actin binding proteins. It has been shown previously that there is reduced expression of profilin 1 (Pfn1- an ubiquitously expressed actin binding protein) in invasive breast cancer cells. Pfn1 is now considered as a tumor-suppressor protein based on its ability to restrict the growth and tumorigenesis of breast cancer cells when overexpressed. Besides actin, Pfn1 also binds to several families of proline-rich ligands and these interactions have been implicated in several cellular processes including actin assembly, endocytosis and gene transcription. We have previously shown that overexpression of Pfn1 reduces the migration of BT474, a ductal carcinoma breast cancer cell line. The aim of the present work is to determine whether overexpression or selective inhibition of ligand binding of Pfn1 alter the migration and invasion of metastatic breast cancer cells. Specifically, we have studied how stable overexpression of Pfn1 and its mutant forms that are selectively impaired in

binding to either actin or proline-rich ligands affect the migration and invasion of MDA-MB-231, a highly metastatic breast cancer cell line. We show that functional perturbation of Pfn1 affects the F-actin content in MDA-MB-231 cells. Specifically, Pfn1 overexpression stimulates actin polymerization, whereas expression of an actin-binding deficient mutant of Pfn1 decreases the overall level of polymerized actin in MDA-MB-231 cells. Increased focal adhesion formation in MDA-MB-231 cells as a result of Pfn1 overexpression appears to require a functional actin-binding site of Pfn1. We show that cell migration and invasion in response to chemotactic stimulus are inhibited when either fully functional or mutant forms of Pfn1 are expressed in these cells. Finally, we demonstrate that perturbation of Pfn1 affects the secretion of matrix-metalloproteinases (enzymes that are important for matrix degradation during cell invasion) by MDA-MB-231 cells.

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1.0 INTRODUCTION

Cancer is a group of diseases characterized by proliferation and migration of tumor cells. The anticipated cancer deaths in the year 2006 in the United States are 1.4 million (American cancer society). Breast cancer, one of the most common types of cancer among women, is now the second leading cause of cancer deaths after lung cancer. The National Cancer Institute estimates that 1 in 9 women in the United States will develop invasive breast cancer. A majority of these deaths are due to metastasis of breast tumor cells to distant organs. When breast carcinoma cells metastasize and colonize at secondary sites such as lung and brain, 5-year survival rate of patients drops from 90% to 23% (Welch et al., 2000). A very high percentage of patients also suffer from breast cancer that has metastasized to the bone, which leads to osteoporosis, hypocalcaemia and spinal cord compression (Sloan and Anderson, 2002) . Thus understanding tumor cell invasion and metastasis at molecular level will help us in developing therapeutics that will improve the quality of life of breast cancer patients.

Tumor metastasis is a cascade of events (**Figure 1**) where cells from the primary tumor first dissociate and invade through the extracellular matrix (ECM) by both active migration and ECM degradation via proteolytic enzymes such as matrix metalloproteinases that are secreted in the tumor microenvironment (Engers and Gabbert, 2000; Szabo and Singh, 2005; Yamaguchi et al., 2005). A certain population of invading cells then are able to enter the vasculature or lymphatic systems (a process known as “intravasion”) which allow the tumor cells to be

distributed to distant organs. These tumor cells are then either trapped in the smaller blood vessels (a stage known as “tumor dormancy”) or manage to leave the vasculature (a process known as “extravasion”) to enter and invade the target organs where they eventually form secondary tumors.

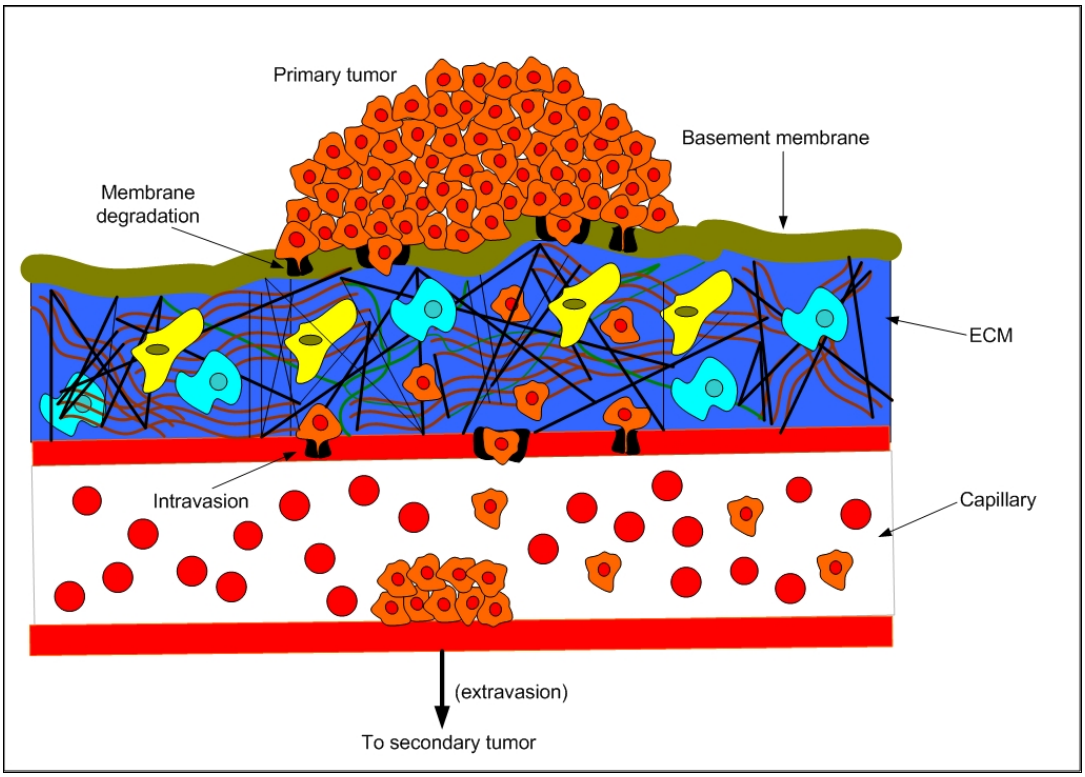

Figure 1. Steps involved in tumor cell metastasis

Cell migration, an integral component of tumor invasion and metastasis, is regulated by the assembly and disassembly of actin cytoskeleton. Regulation of actin cytoskeleton requires a coordinated action of different classes of actin-binding proteins (ABPs) including those involved in monomer sequestering, nucleating, severing, bundling and cross-linking activities. It was observed in malignant cells, that an alteration in the actin cytoskeleton is correlated with altered expression of various ABPs (Button et al., 1995; Clark et al., 2000; Pawlak and Helfman, 2001; Vandekerckhove et al., 1990; Wang et al., 1996). Several actin-associated proteins, including α-

actinin (Gluck et al., 1993), gelsolin (Nikolopoulos et al., 2000; Tanaka et al., 1995), vinculin (Rodrigue-Fernandez et al., 1992), when overexpressed, suppressed the phenotype of transformed cells thus suggesting that altered expression of ABPs can directly contribute to the oncogene-induced transformed phenotype of tumor cells. In the role of cytoskeleton and ABPs in diseases, reduced expression of profilin I (Pfn1, an ubiquitously expressed actin binding protein that links various signaling pathways to cytoskeletal dynamics) (Witke, 2004), has also been reported in invasive breast cancer (Janke et al., 2000), pancreatic cancer (Gronborg et al., 2005), and in astrocytic tumor cells in response to stimulation by S100A4 (a calcium binding protein that facilitates tumor progression, cell migration and metastasis (Belot et al., 2002)). Pfn1 is now considered as a tumor-suppressor protein based on its ability to restrict the growth and tumorigenesis of breast cancer cells when overexpressed (Janke et al., 2000; Wittenmayer et al., 2004). It was previously shown that even a moderate overexpression of Pfn1 alters the actin cytoskeleton and leads to a significant inhibition in EGF stimulated chemotactic migration of BT474, a ductal carcinoma cell line (Roy and Jacobson, 2004). These findings lead us to postulate that **perturbation of Pfn1 alters the migration and invasion of metastatic breast cancer cells** (***HYPOTHESIS***).

Besides actin, profilin also binds to an extensive list of proline-rich motif (PRM) bearing ligands ranging from those that directly stimulate actin assembly in response to extracellular signals (hence important for cell migration) to ones involved in gene splicing and transcription factors (Lederer et al., 2005). Thus related to our overall hypothesis, we form a working postulate that perturbing either actin and/or PRM binding functions of profilin modulate the

migration and invasion of breast cancer cells. To test this hypothesis, we propose the following specific aims:

Specific Aim 1: To determine whether overexpression or selective inhibition of ligand binding of profilin alter the migration of metastatic breast cancer cells.

Specific Aim 2: To determine whether overexpresssion or selective inhibition of ligand binding of profilin alter the invasion of metastatic breast cancer cells.

2.0 BACKGROUND

2.1 PROFILIN - GENE FAMILY

A number of profilin genes have been identified among which Pfn1 is the most ubiquitously expressed and its structure is conserved in protozoa, mammals, yeast, insect, echinoderm and plants. Profilin II (Pfn2) gene is most abundant in brain (Di Nardo et al., 2000; Kwiatkowski and Bruns, 1988; Lambrechts et al., 2000). Although human Pfn1 and Pfn2 genes are known to have similar biochemical properties, it was observed that Pfn1's interaction with actin is higher than Pfn2. The third mammalian profilin (Pfn3) is expressed in kidney and testes (Hu et al., 2001). Recently, a fourth profilin gene (Pfn4) whose expression is testis-specific has now been isolated (Oberman, 2004).

2.2 BIOCHEMICAL ACTION

Although Pfn1 was originally identified as a G-actin sequestering protein (Carlsson et al., 1977) , depending upon the conditions it can either sequester G-actin and hence inhibit actin polymerization, or promote actin assembly (Schluter et al., 1997). Since the intracellular concentration of Pfn1 does not appear to be sufficient to account for the high G-actin content in most cells, its role as a promoter of actin polymerization is currently favored. Pfn1 promotes actin assembly via its ability to accelerate nucleotide (ADP to ATP) exchange on G-actin and

shuttle Pfn1-actin (ATP-bound) complex to free barbed ends of actin filaments (Kang et al., 1999; Pantaloni and Carlier, 1993; Schluter et al., 1997).

Besides actin, Pfn1 has affinity for phosphoinositides (mainly phosphatidylinositol-4,5-bisphosphate [PIP_2] and phosphatidylinositol-3,4,5-triphosphate [PIP_3]) and polyproline stretches. Binding of Pfn1 to actin and polyproline stretches are regulated by its PIP_2 association (Goldschmidt-Clermont et al., 1990). A number of proline-rich motif (PRM) proteins that control actin assembly in response to cAMP or Rho-family GTPase-mediated signaling, such as those belonging to VASP (vasodilator stimulated phosphoprotein – activated at the downstream of cAMP signaling (Huttelmaier et al., 1999; Krause et al., 2002; Kwiatkowski et al., 2003; Reinhard et al., 1995), WASP (Wiskott-Aldrich syndrome protein; example: cdc42-activated Neuronal or N-WASP (Suetsugu et al., 1999; Takenawa and Miki, 2001; Yang et al., 2000), Rac-activated WAVE or WASP-associated verprolin homology (Miki et al., 1998) and Rho-activated Diaphanous (example: mDia; (Evangelista et al., 2003; Higgs, 2005; Severson et al., 2002; Watanabe et al., 1999; Watanabe et al., 1997)) families associate with Pfn1. These PRM proteins localize in various F-actin structures found in actively protruding edges, filopodia, and stress fibers of cells (Reinhard et al., 1995; Takenawa and Miki, 2001; Wallar and Alberts, 2003), and formations of actin-rich structures by at least some of these PRM proteins in response to signals require involvement of Pfn1. One attractive hypothesis is that these PRM proteins may act as molecular scaffolds to spatially target Pfn1-actin complex to the zones of actin remodeling in cells. Thus Pfn1's interactions with these PRM proteins may serve as links between microfilaments and signal transduction pathway. Based on recent findings on Pfn1's association with other types of proline-rich ligands such as splicing and transcription factors, proteins acting in endocytic pathway (example: clathrin) (Witke, 2004), a wide spectrum of Pfn1 function

ranging from control of actin assembly to regulation of gene transcription is currently considered.

2.3 REGULATION OF PFN1 FUNCTION

Pfn1's interaction with actin has shown to be negatively regulated by its PIP_2 association (Reinhard et al., 1995). Phospholipase C-mediated hydrolysis of PIP2 releases Pfn1 from cell membrane, which then becomes available to take part in actin polymerization. More recent studies show that PKC-mediated serine-phosphorylation of Pfn1 could also be another mechanism of modulating Pfn1's association with actin and PRM proteins.

2.4 PFN1 IN CELL MIGRATION

Cell migration is a complex process (**Figure 2)** that is executed through a cycle of events involving 1) cell polarization and lamellipodial protrusion powered by actin polymerization at the leading edge, 2) adhesion of lamellipodia to the substrate by focal adhesions, 3) generation of traction forces (mediated by acto-myosin contractility) to move the cell-body forward, and finally 4) detachment of the rear of the cell.

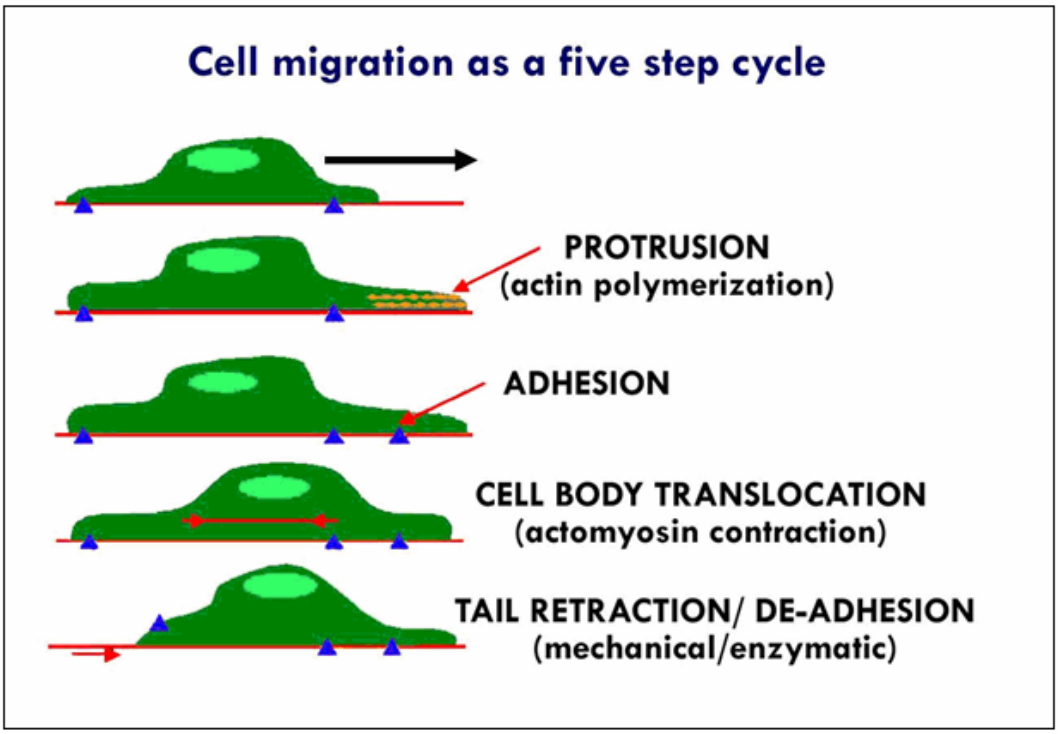

Figure 2. Cell migration as a five step cycle (adapted from (Sheetz et al., 1999))

Although Pfn1 plays an important role in actin polymerization, its exact role in cell migration is still not clear. Mutants of *Dictyostelium* amebae lacking Pfn1 and Pfn2 mutants exhibiting reduced speed of migration, first produced a direct evidence of profilin's involvement in cell migration (Haugwitz et al., 1994). Because of embryonic lethality produced by Pfn1 null-mutation for mammalian development (Witke et al., 2001), similar studies have not been reported so far. However, a large body of literature have demonstrated Pfn1's involvement in host cell-induced actin-based motility of bacterial pathogens (Laurent et al., 1999; Loisel et al., 1999; Mimuro et al., 2000; Sanger et al., 1995; Theriot et al., 1994; Yarar et al., 2002). Pfn1's interactions with both actin and PRM proteins appear to be important for actin assembly in supporting pathogen movement . Since pathogen-induced actin assembly mimics the dynamics of actin polymerization at the leading edge of migrating cells, the current view is that one of

Pfn1's function is to stimulate actin polymerization and facilitate membrane protrusion during cell migration. This is further supported by a number of studies demonstrating Pfn1's preferential localization at the leading edge of migrating cells of various types (Buss et al., 1992; Mayboroda et al., 1997; Moldovan et al., 1996).

Given Pfn1's importance in cell migration, it is thus not clear why Pfn1 expression is downregulated in certain invasive cancers including those originating in breast and pancreas. Whether loss of Pfn1 expression confers increasing migratory potential to mammary carcinoma cells is not known. We previously showed that overexpression of Pfn1 significantly inhibits chemotactic migration of BT474, a ductal carcinoma cell line (Roy and Jacobson, 2004). Whether overexpression of Pfn1 has similar inhibitory effect on the migration as well as invasion of metastatic breast cancer cells is not known **(GAP 1)**. Finally, a recent study has shown that interaction with actin is a key requirement for Pfn1-mediated suppression of breast tumor growth in vivo. The relative importance of Pfn1's interactions with actin and proline-rich ligands in the context of regulation of tumor cell migration and invasion is however not known **(GAP 2)**. These gaps are addressed in the present study by experiments proposed in Specific Aims 1 and 2.

3.0 HYPOTHESIS AND SPECIFIC AIMS

HYPOTHESIS

Perturbation of Pfn1 affects breast cancer cell migration and invasion

SPECIFIC AIM 1: **To test that perturbing either the cellular content or biochemical functions (binding to actin and PRM proteins) alters breast cancer cell migration**.

We specifically determine whether overexpression or selective inhibition of ligand binding of profilin alter

a. actin cytoskeleton (structure) of breast cancer cells.
b. Cell adhesion of breast cancer cells.
c. Migration of breast cancer cells.

SPECIFIC AIM 2: **To test that perturbation of profilin affects breast cancer cell invasion.**

We specifically determine whether overexpression or selective inhibition of ligand binding of profilin alter

a. Invasion of breast cancer cells.
b. Matrix metalloprotease secretion by breast cancer cells.

4.0 MATERIALS AND METHODS

4.1 ANTIBODIES

Polyclonal antibodies against Pfn1 was generously provided by Drs. Sally Zigmond (University of Pennsylvania) and Walter Witke (European Molecular Biology Laboratory, Italy). GFP and VASP monoclonal antibodies were obtained from Pharmingen, (San Diego, CA). Monoclonal antibodies specific for actin and GAPDH are products of Chemicon (Termecula, CA). Monoclonal vinculin antibody is a product of Sigma Aldrich (St.Louis, MO). Polyclonal N-WASP and mDia antibodies were obtained from Abcam(Cambridge, MA). For immunoblotting, the antibodies were used at the following concentration: Pfn1(1:1000), GFP(1:2000), VASP(1:1000), actin(1:1000), GAPDH(1:3000) , Vinculin(1:1000).

4.2 CELL CULTURE

MDA-MB-231 (a metastatic breast cancer cell line) cells were cultured in EMEM media supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate and 1% antibiotics (penicillin/streptomycin/amphotericin). These cells were either transfected with GFP or GFP-fused profilin constructs and stable clones were selected and maintained with G418 at a concentration of 1mg/ml.

4.3 GENERATION OF PFN1 CONSTRUCTS

GFP-Pfn1 expression vectors were constructed previously (Roy, 2004). PCR-based site directed mutagenesis was used to create GFP-fused Pfn1 mutants that are deficient in binding to either actin (GPH119E) or polyproline (GPH133S).

4.4 PROTEIN EXTRACTION

Cells were plated for 48-72 hrs in growth media and extracted with lysis buffer containing 1% NP40, 0.5% deoxycholate, 50mM Tris-HCL (pH 7.5), 150mM Nacl, protease inhibitors (10μg/ml of leupeptin, aprotinin, pepstatin and 1mM phenylmethylsulphonyl fluoride) and phosphotase inhibitors (50mM sodium fluoride and 1mM sodium pervanadate). Prior to extraction cells were washed with cold PBS and lysed on ice for 30 minutes with the buffer. Lysates were clarified in the cold for 15 min at 13,000 rpm and the protein concentration of the supernatants were determined using a coomasie-based protein assay kit (Pierce, Rockford, IL).

4.5 IMMUNOBLOTTING

For protein electrophoresis, approximately 20μg of extracted protein lysates were run on a SDS-PAGE. Samples were run on a 15% gel for 45min at 200V and transferred in cold onto a nitrocellulose membrane for 90 min. After blocking the membrane at room temperature for 1 hr in 5% non-fat dry milk made in TBST, the membranes were incubated overnight in the respective antibodies according to the manufacturers recommended concentration (stated above). After washing with TBST three times at room temperature the membranes were incubated in the respective secondary antibodies and washed three times after which chemiluminescence

(Amersham Biosciences, NJ) was performed to detect the protein bands using the Kodak image station.

4.6 POLYPROLINE BINDING ASSAY

Poly-L-proline was conjugated to cyanogen bromide activated agarose beads according to the manufacturers protocol (Sigma St Louis, MO). Beads were initially washed three times with lysis buffer and 30ul of PLP beads were mixed with 200ug of protein lysates and allowed to rotate in the cold for 1 hr. Samples were centrifuged in the cold for 1min to pellet the PLP beads which were washed with lysis buffer 3 times and resuspended with 20ul of 2X sample buffer and run on a SDS - PAGE.

4.7 IMMUNOSTAINING

Cells were plated on collagen-coated coverslips for 16-18 hrs. Cells were washed with PBS and then fixed with 3.7% formaldehyde for 15 min and permeabilized with 0.5% Triton X for 5 min. For vinculin immunostaining cells were blocked in 10% goat serum and vinculin monoclonal antibody at 1:200 dilution was added for one hour. Cells were washed repeatedly with PBS and PBS containing 0.02% tween after which they were incubated in secondary antibody (Rhodamine goat anti mouse) for 1 hr. Cells were washed in PBS and mounted on slides for fluorescence microscopy on an IX-71 inverted microscope. For F-actin staining the permeabilized cells were stained with rhodamine-phalloidin (Molecular probes, Eugene, OR) at a dilution of 1:100 for 30 min and then washed 5 times with PBS and mounted on slides similarly. For fluorescence-based determination of relative F-actin content between the different cell lines, we acquired images of phalloidin-stained cells at random fields of observation in each

experiment using a 20X objective. After performing background subtraction of the images, the average fluorescence intensity per cell was calculated for each field of observation. These values were then normalized with respect to the average fluorescence value calculated for the control GFP-expressing cells for a given experiment. Normalized fluorescence data of cells were pooled from 3-4 independent experiments, the average values of which were then statistically compared using a Student's T-test.

4.8 TRANSWELL MIGRATION ASSAY

The underside of the 8µm transwell membranes (BD Biosciences) were precoated with 25µg /ml of collagen for 1 hour at room temperature. Cells were serum starved overnight and 25000 cells were plated on the upper chamber of the transwells in 0.15% BSA containing EMEM media in triplicates and the lower chamber contained the same media with 10% FBS which serves as a chemoattractant. After 3 hours the transwells were removed and the nonmigrated cells were gently removed using a cotton swab and the underside of the membrane was fixed in 3.7% formaldehyde and stained with Hoechst staining solution (Molecular Probes) in order to count the number of transmigrated cells in 5 random fields of observations. These experiments were repeated 3 times and t-test ($p<0.05$) was performed in order to obtain statistical significance.

4.9 INVASION ASSAYS

Cells were plated for 24 hours in culture media and serum starved for 12 hours prior to performing the experiment. The bottom surface of the transwells of matrigel-coated invasion chambers (BD Biosciences) was coated with 25µg /ml of collagen for 30-45 minutes at room temperature, after which the membranes were rehydrated with 500µl of serum-free EMEM

media for 2 hours in the 37^{o}C incubator. The media was gently aspirated and 25000 cells were plated in 500 µl serum-free media containing 1% BSA. The bottom chamber had 500µl of the same media with 10% FBS which acts as an attractant for cells to invade through the membrane. Cell proliferation was blocked with 10µg/ml of mitomycin C. After 24 hours the non- invading cells were gently scraped off from the upper surface using a cotton swab and the invaded cells on the underside of the membrane were fixed and stained with Hoechst. Five random fields of cells were taken and the average number of invading cells for different experimental groups were counted using the Metamorph imaging software and statistically compared by Student's t-test.

4.10 GELATIN ZYMOGRAPHY

Cells were plated to confluence and serum starved for 24 hours with phenol red free media. Supernatants of the various cell types were concentrated using the Amicon 10Kd filters (Millipore) for 30 minutes at 4000g. Supernatants containing equal amount of total protein were then run on a 10% gelatin Zymogram gel where MMP2 and MMP9 bands were detected.

5.0 RESULTS

5.1 STABLE OVEREXPRESSION OF PFN1 AND ITS FUNCTIONAL MUTANTS IN MDA-MB-231 CELLS

It was shown earlier that point mutations, H119E and H133S (histidines at 119 and 133 positions were replaced by glutamic acid and serine) could abolish Pfn1's interactions with actin and polyproline, respectively. Previous biochemical work in our laboratory had confirmed that these two mutations are also effective in maintaining selective loss of functions of GFP-tagged Pfn1 as well. We thus expressed GFP-Pfn1, GFP-Pfn1-H119E (GPH119E) and GFP-Pfn1-H133S (GPH133S) in MDA-MB-231 cells and generated stable clones expressing these constructs. As a control, a stable clone of GFP-expressing cells was created. A GFP-immunoblot in (**Figure 3**) shows the expression levels of exogenous GFP–Pfn1 and its mutants in MDA-MB-231 cells. Pfn1-immunoblot shows that the levels of endogenous Pfn1 in these mutant cell lines are comparable to that in the WT and GFP-expressing control cells.

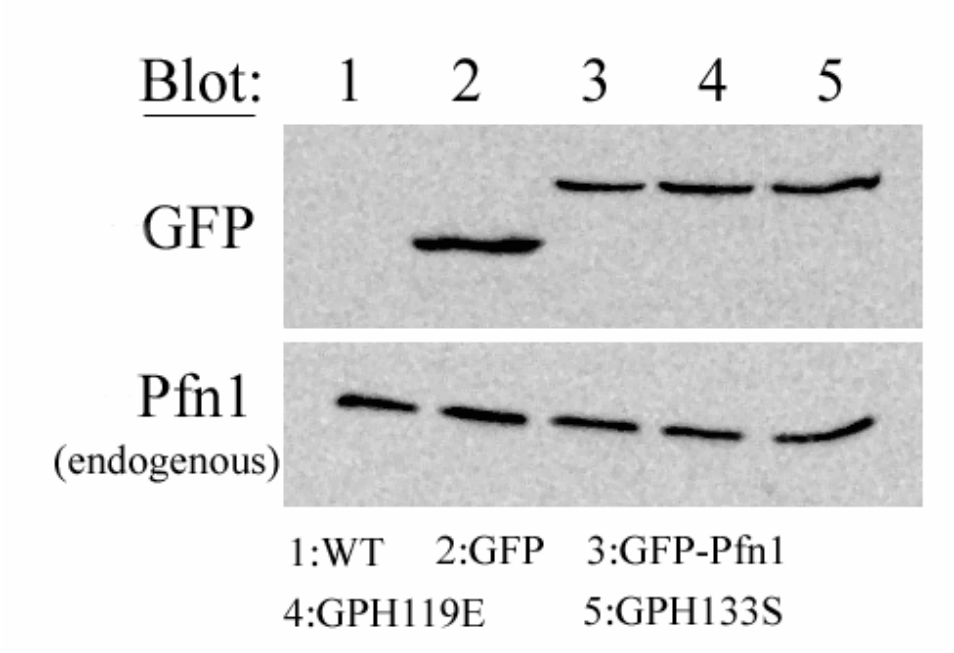

Figure 3. Total cell lysate (15µg) from MDA-MB-231 cells show exogenous expression of GFP-Pfn1 in the various cell lines. Endogenous Pfn1 levels are comparable between the cell lines.

5.2 PERTURBATIONS OF PFN1 AFFECT THE MORPHOLOGY OF BREAST CANCER CELLS

Most of the WT (data not shown) and GFP expressing control cells show a typical spindle shaped, bipolar morphology within 24 hrs of plating (**Figure 4A**). The Pfn1-overexpressers show dramatically increased cell spreading represented by a distinct, flattened and polygonal morphology (**Figure 4B**). Pfn1-induced enhanced cell spreading is not seen in either of the mutant cell lines despite comparable levels of exogenous Pfn1 in all of these cell lines (**Figure 4C and 4D**). These data suggest that functional actin and polyproline binding sites are required for Pfn1-induced enhanced cell spreading.

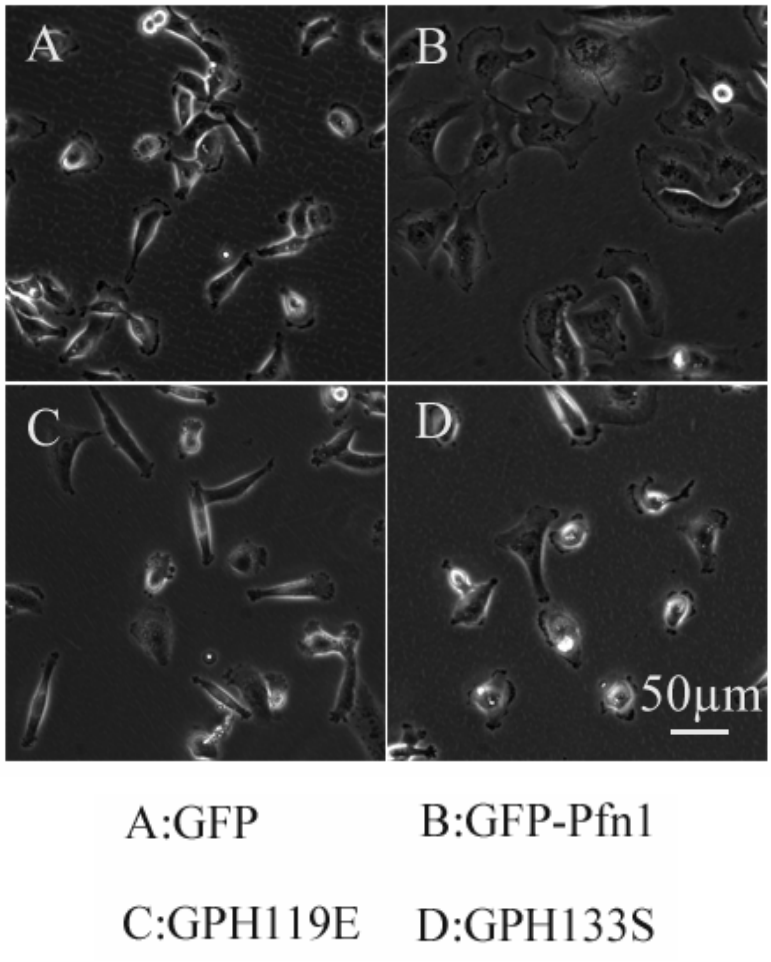

Figure 4. GFP-Pfn1(B) expressers of MDA-MB-231 cells show dramatically increased cell spreading compared to WT and GFP control cells. GPH119E and GPH133S (C-D) mutants look similar to control cells (morphology of GFP and WT (data not shown) cells are similar).

5.3 EFFECTS OF FUNCTIONAL PERTURBATION OF PROFILIN ON ACTIN CYTOSKELETON

We proposed that the GPH119E and GPH133S mutants would compete with the endogenous Pfn1 in binding to proline-rich ligands and actin, respectively. Since many proline-rich proteins (example: VASP, N-WASP, mDia) utilize Pfn1 to stimulate actin assembly, we postulate that inhibition of endogenous Pfn1's interactions with either actin or proline-rich ligands by the

action of the mutants should thus interfere with actin polymerization and alter the F-actin content in MDA-MB-231 cells. To test this postulate, we performed rhodamine-phalloidin staining to visualize actin cytoskeleton as well as to quantify the relative F-actin contents between various cell lines. WT (data not shown) and control GFP-expressing cells mainly exhibit only cortical actin that is highly enriched at the leading edge (**Figure5A**) . A striking feature of GFP-Pfn1 expressing cells is the presence of prominent actin-stress fibers, which are not detected in either of the mutant cell lines (**Figure5B**). GPH119E cells (**Figure5C**) in general, display a much weaker phalloidin-fluorescence and also fail to exhibit the enrichment of cortical actin at the leading edge that is typically seen in the control GFP-expressers. The GPH133S mutant cell lines show strong cortical actin staining throughout the periphery without any particular directional bias (**Figure 5D**). The results of fluorescence-based analyses of relative F-actin content between various cell lines (normalized with respect to GFP cells) are summarized in the form of a bar-graph (**Figure 6**) that shows comparable levels of F-actin between the WT and GFP expressing cells. GFP-Pfn1 expressers have a nearly 66% increase in the overall level of polymerized actin compared to the control cells thus suggesting Pfn1-overexpression stimulates actin assembly in MDA-MB-231 cells. A dramatic 68% decrease in the F-actin content is noticed in the H119E mutant cells. The GPH133S mutant cells only show a slight reduction of 13% in the total F-actin level compared to the GFP control cells. Polyproline bead pull down of these cell lysates probed with GFP antibody showed binding of GFP-Pfn1 and GPH119E (deficient in binding actin) but not GPH133S (deficient in binding PRM proteins) as seen in (**Figure 7**). We also performed actin immunoblot of whole cell lysates that show similar levels of actin expression between various cell lines (**Figure 8**). Since Pfn1 has been recently implicated in gene transcription, we also wanted to confirm whether or not the expression levels of VASP, N-WASP and mDia-1,

some of the key actin-binding proteins that are important for growth-factor stimulated actin assembly, are altered as a result of perturbations of Pfn1. Immunoblot data show comparable levels of these three proteins between the various cell lines (**Figure9**). Overall, these data suggest that overexpressed Pfn1 stimulates actin assembly most likely via its direct effect on actin polymerization and that Pfn1-induced enhanced actin assembly require both functional actin and polyproline binding sites.

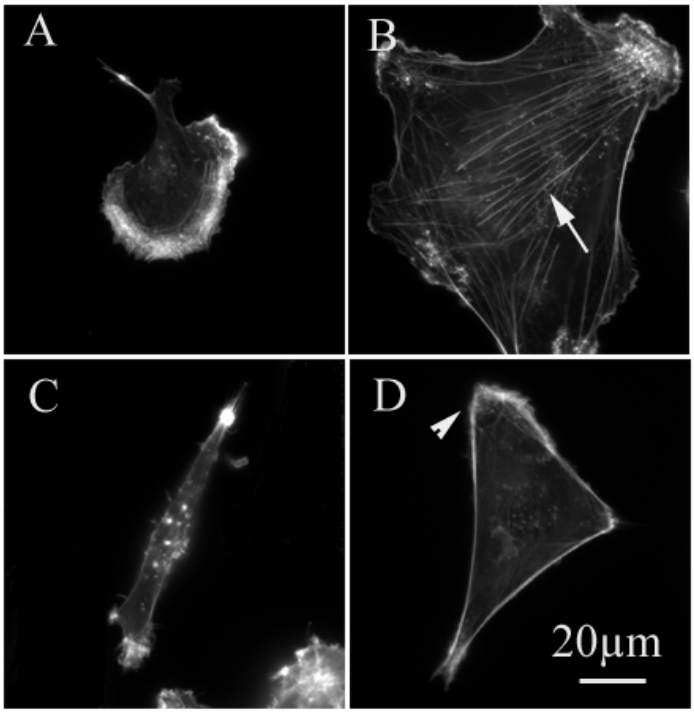

Figure 5. Phalloidin staining shows dramatically enhanced actin stress fibers in GFP-Pfn1 overexpressers compared to GFP control cells. GPH133S expressing cells show strong cortical

actin which is not seen in the GPH119E expressing cells. Staining of WT (data not shown) and GFP cells are similar.

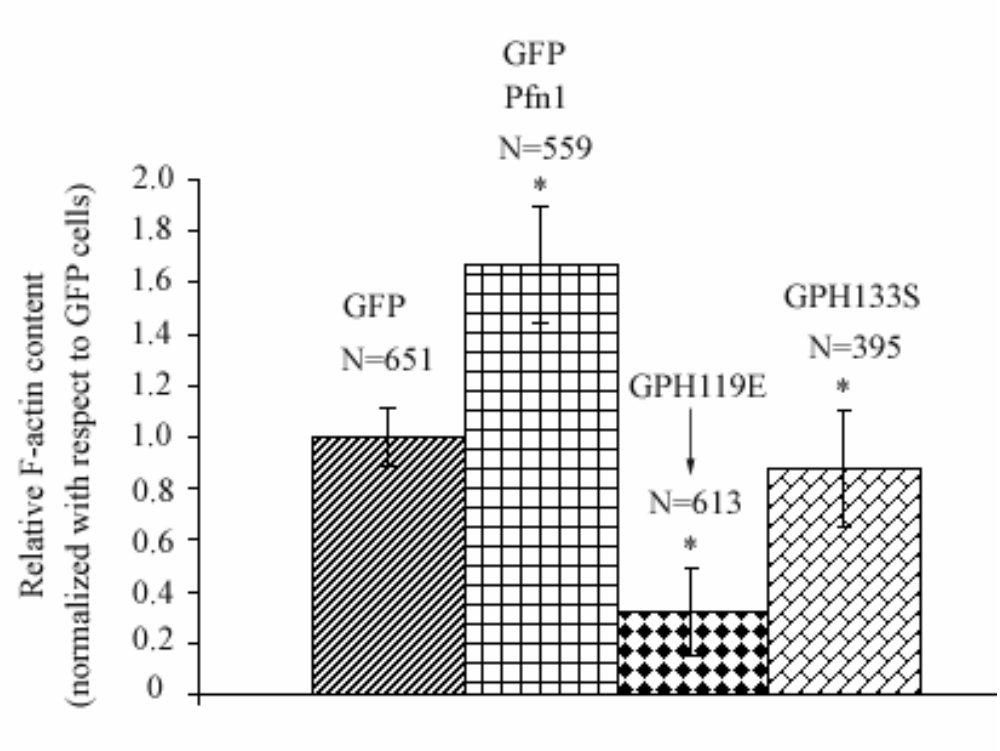

Figure 6. Fluorescense based relative F-actin content (normalized with respect to GFP cells) shows a 66% increase in GFP-Pfn1 expressers but only a slight reduction (13%) in the GPH133S expressing mutants. GPH119E expressing cells show 68% decrease in F-actin level. There is no significant difference between WT (data not shown) and GFP expressing control cells.

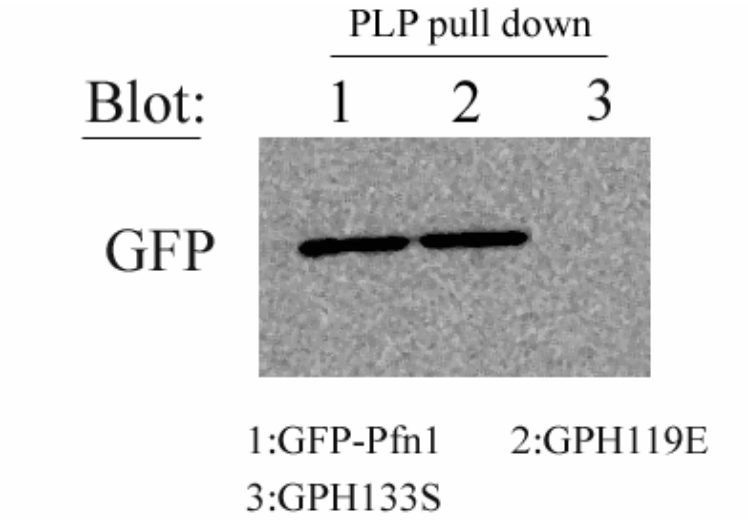

Figure 7. PLP pull down of different cell lysates probed with GFP antibody show binding of GFP-Pfn1 and GPH119E but not GPH133S.

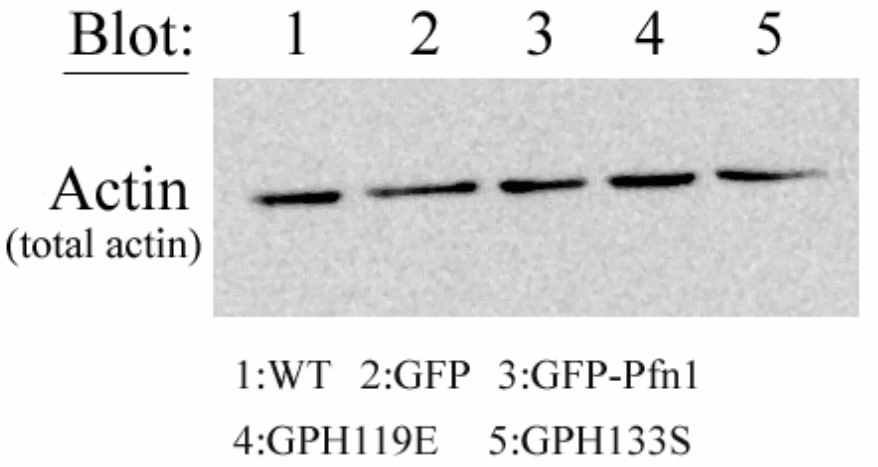

Figure 8. Total cell lysate of various cell lines probed with actin antibody show similar levels of total actin.

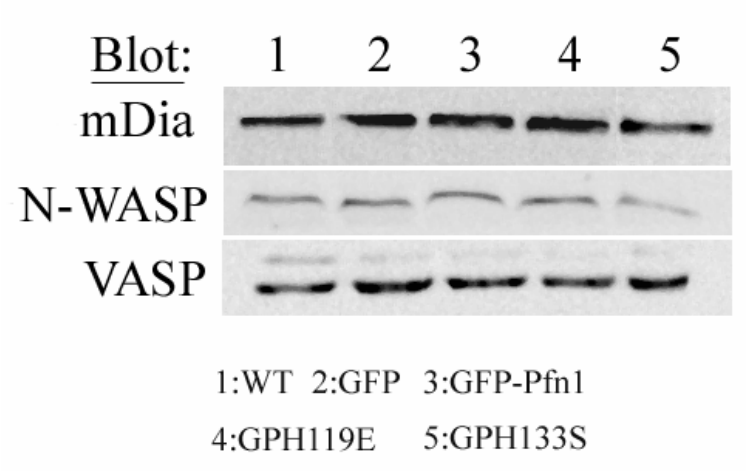

Figure 9. Total cell lysate of the various MDA-MB-231 cell lines probed with the indicated antibodies show comparable level of expression of the proteins.

5.4 EFFECTS OF FUNCTIONAL PERTURBATION OF PFN1 ON CELL ADHESION.

To determine whether perturbations of Pfn1 affect cell adhesion, we performed vinculin (a marker for focal adhesion) immunostaining of the different cell lines, the results of which are shown in (**Figure 10**). While control cells typically have a few small focal contacts, large focal adhesions that are consistent with the appearance of stress fibers are seen in the GFP-Pfn1 expressing cells implying an overall increase in cell adhesion induced by overexpressed Pfn1. GPH133S expressing cells also exhibit focal adhesions that are distributed mostly in the cell periphery. No focal adhesion plaques are however seen in the GPH119E mutant cells thus suggesting that Pfn1-induced formation of focal adhesions requires a functional actin-binding site.

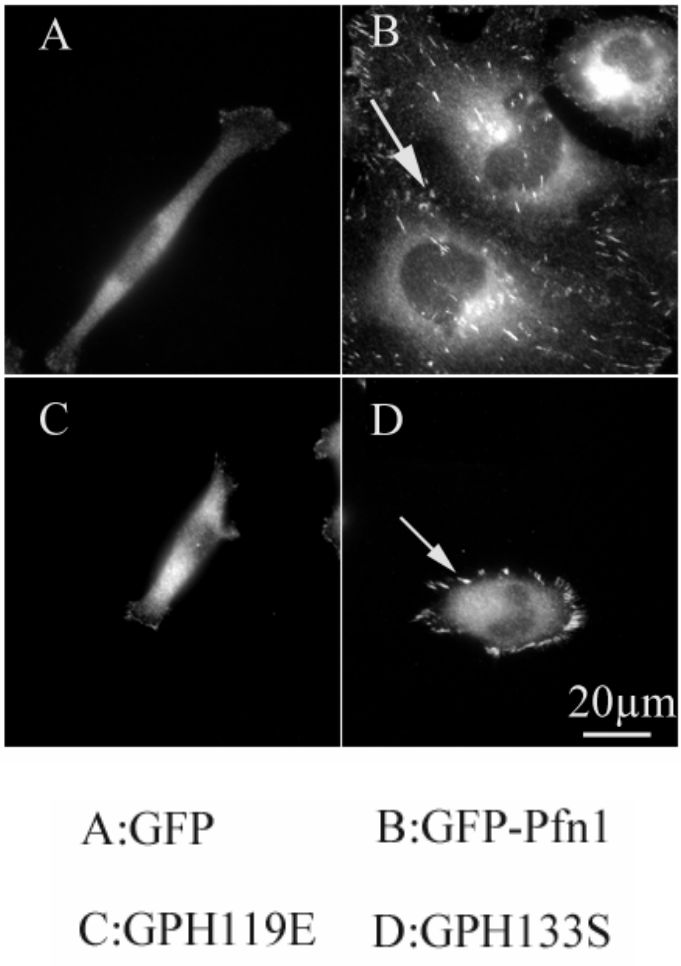

Figure 10. Vinculin immunostaining shows increased focal adhesions throughout the cell in the case of GFP-Pfn1(B) expressing cells compared to GFP(A) control cells. GPH133S(D) expressing cells show focal adhesions on the periphery, while no focal adhesions are observed in H119E (C) expressing cells. Staining of WT (data not shown) and GFP expressing cells are similar.

5.5 EFFECT OF PERTURBATIONS OF PFN1 ON THE MIGRATION OF BREAST CANCER CELLS

Transwell migration experiments were next performed to determine if serum induced chemotactic migration of MDA-MB-231 cells are dependent on Pfn1's functions (Specific Aim

1), the results of which are shown in the form of a bar graph (**Figure 11**). No significant difference in cell migration was observed between the WT and GFP-expressing control cells thus confirming that presence of GFP does not produce any non-specific change in cell migration. Overexpression of both Pfn1 and its mutants inhibit transwell migration of MDA-MB-231 cells although the % inhibition in cell migration seen in the case of GFP-Pfn1 expressing cells (72%) is higher than that achieved by the actions of GPH119E (49%) and GPH133S (61%) mutants, respectively. These data suggest that overexpression of a fully functional Pfn1 is required for the maximum inhibitory effect on breast cancer cell migration and also support our overall hypothesis that perturbations of Pfn1 alter the migration of breast cancer cells.

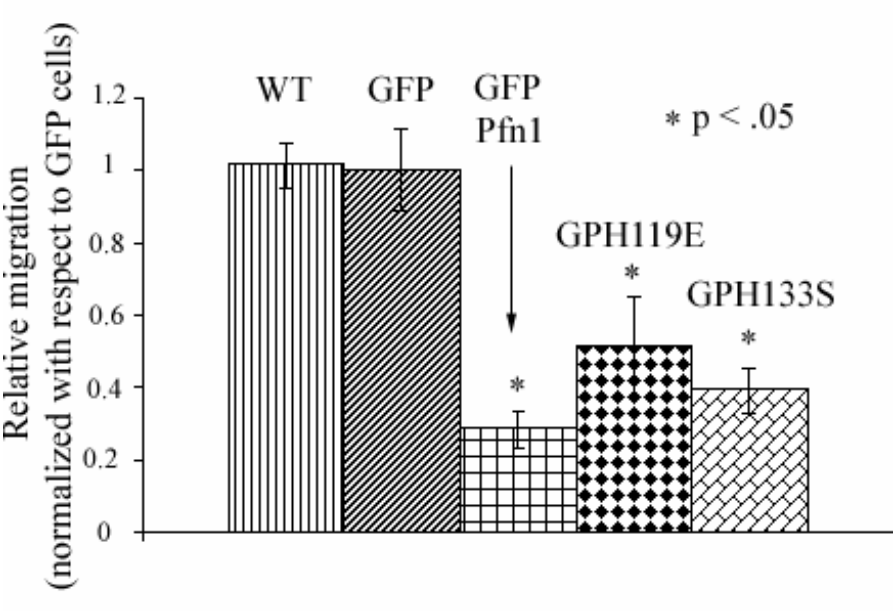

Figure 11. Relative chemotactic migration (normalized with respect to GFP cells) of various MDA-MB-231 cell lines show inhibition in migration with perturbation of Pfn1. There is no significant difference between WT and GFP cells.

5.6 EFFECT OF PERTURBATIONS OF PFN1 ON THE INVASION OF BREAST CANCER CELLS

Since our data show that perturbations of Pfn1 alter the migration of breast cancer cells, it suggests that Pfn1 should have an effect on breast cancer cell invasion (Specific Aim 2), a process that critically depends on cell migration. Similar transwell experiments were performed to assay cell invasion with the exception that cells in this case were plated on transwells that are already pre-coated with a thin layer of matrigel to mimic cell invasion through the extracellular matrix. The results of transwell invasion experiments are shown in the form of a bar graph (**Figure 12**). No significant difference in cell invasion was observed between the WT and GFP-expressing control cells thus confirming that presence of GFP does not produce any non-specific change in cell invasion. Our data show that overexpression of both Pfn1 and its mutants inhibit transwell invasion of MDA-MB-231 cells. The average % inhibition in cell invasion in GFP-Pfn1, GPH119E and GPH133S expressers were 75%, 62% and 70% respectively. Overall these data support our hypothesis that perturbations of Pfn1 alter the invasion of breast cancer cells.

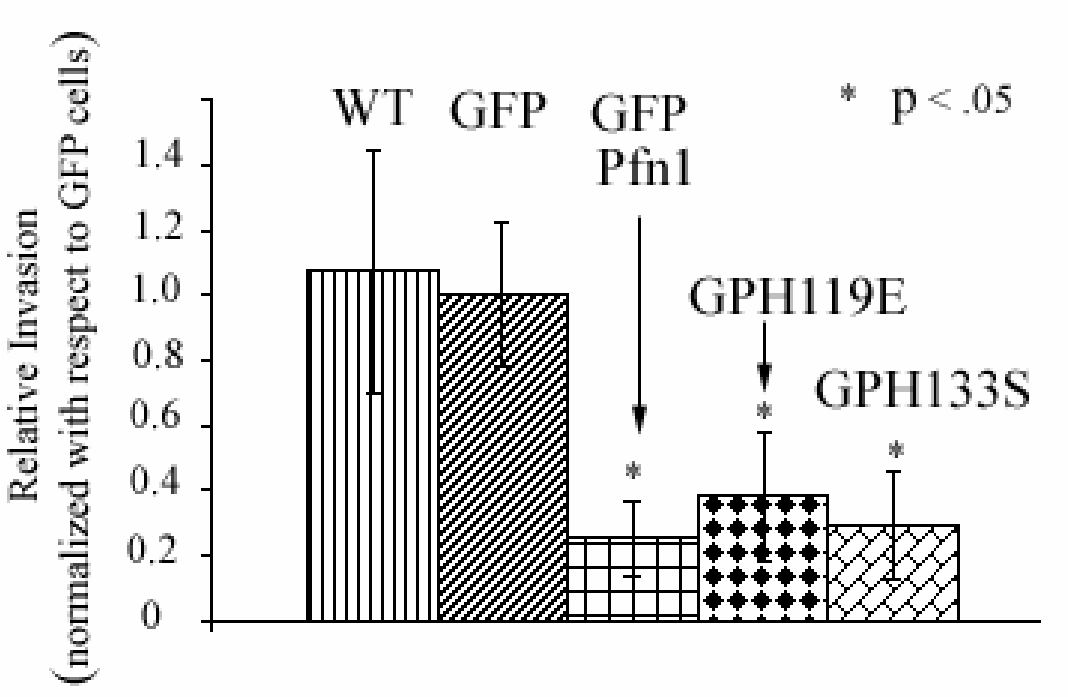

Figure 12. Relative invasion (normalized with respect to GFP cells) of various MDA-MB-231 cells show inhibition in invasion with perturbation of Pfn1. There is no significant difference in the invasive ability between WT and GFP expressing cells.

5.7 FUNCTIONAL PERTURBATIONS OF PFN1 AFFECT THE SECRETION OF MATRIX-METALLOPROTEINASES

Since proteases secreted by carcinoma cells degrade the basement membrane matrix and facilitate cell invasion, we next investigated whether the secretion of MMP2 and MMP9 (gelatinases that belong to the matrix metalloproteinase family of enzymes) by MDA-MB-231 cells are altered as a result of perturbations of Pfn1. Gelatin zymogram of conditioned media derived from the various cell lines was performed, and the results of which is shown in (**Figure 13**). Our data show two interesting features: 1) a decrease in MMP9 secretion when either Pfn1 or its mutants are expressed, and 2) an increases in MMP2 secretion specifically by the

GPH133S mutant cells. Overall these data suggest that secretion of MMP2 and MMP9 by MDA-MB-231 cells are modulated by Pfn1 function.

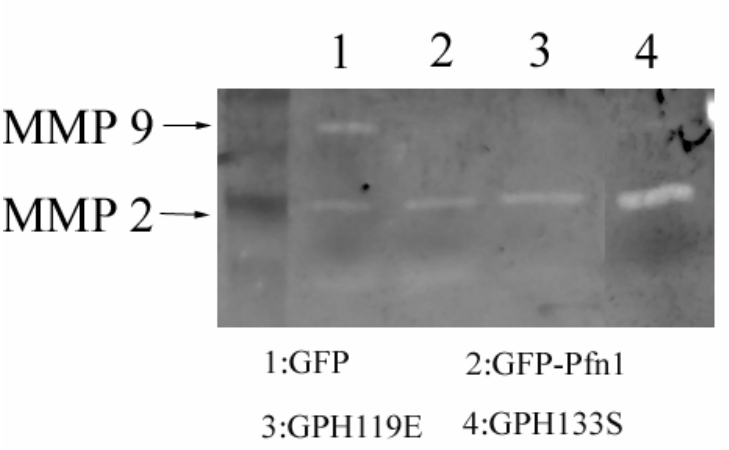

Figure 13. MMP secretion of the various cell lines of MDA-MB-231 cells show i) decreased MMP9 secretion for cells with perturbed Pfn1, and ii) increased MMP2 secretion specifically in GPH133S expressing mutant cells (The apparent discontinuity between the last and the penultimate lanes of the gel is because of cropping of intermediate lanes representing samples from other experiments).

6.0 DISCUSSION

The exact role of Pfn1 in cell migration is still not clear although there is previous evidence of Pfn1's contribution in the intracellular movement of pathogenic organisms and migration of lower eukaryotic organisms such as *Dictyostelium* (Haugwitz et al., 1994; Sanger et al., 1995). It is believed that the Pfn1-actin complex is recruited to the barbed end of actin filaments at the protruding lamellipodia of migrating cells where actin polymerization is initiated. Cell migration in its entirety is however more complex than just the lamellapodial protrusions and particularly how perturbations of ligand-binding of Pfn1 affects the migration of mammalian cells has not been reported in the literature. This work evaluates for the first time the effects of functional perturbations of Pfn1 on the migration and invasion (involves active migration) of MDA-MB-231, a metastatic breast cancer cell line where interestingly the expression of Pfn1 is much less compared to that in the normal mammary epithelial cells (unpublished observation).

We perturbed cellular Pfn1 by overexpressing either GFP-Pfn1 (source of cDNA: mouse) or its mutants that are selectively deficient in binding to actin and polyproline ligands. Since previous studies show that GFP-fusion to N-terminus of Pfn1, maintains its cellular localization and preserves its biochemical interactions with actin and polyproline ligands similar to that of endogenous Pfn1 (Wittenmayer et al., 2000) , use of GFP-fusion in the overexpression construct is a valid approach. Although subtle differences in the ligand-binding properties between human and mouse Pfn1 cannot be absolutely ruled out, overexpressing mouse Pfn1 in human cells is

still a reasonable approach since the amino acid sequences of mouse and human Pfn1 are 96% identical and our mutational studies show that amino acids important for actin and polyproline binding for mouse Pfn1 are exactly the same as those reported for human Pfn1 in the literature (Suetsugu et al., 1998). Also, since our immunoblot data confirms that the endogenous Pfn1 level is not altered in any of the stable clones of MDA-MB-231 cells used in the study, we feel confident that any changes in the cytoskeletal structure, cell migration and invasion that we observe after perturbations of Pfn1 are strictly due to the action of exogenous Pfn1 in the cells.

Previous work by our group reported that Pfn1 overexpression causes a net actin depolymerization in BT474, a ductal carcinoma cell line (Roy and Jacobson, 2004). Actin depolymerization in BT474 cells was characterized by loss of cytoplasmic actin filaments; however thicker F-actin-rich cortical rim was observed in these Pfn1-overexpressers, similar to other previous reports (Finkel et al., 1994; Moldovan et al., 1996; Rothkegel et al., 1996), thus implying Pfn1-induced selective stabilization of actin filaments. By contrast, we have now found that Pfn1 overexpression significantly stimulates actin polymerization in MDA-MB-231 cells as exemplified by the appearance of prominent actin stress-fibers in these cells (**Figure 5**).This observation is consistent with the presumed role of Pfn1 as a promoter of actin polymerization. Differences in the absolute Pfn1 content between these two cell types may partially account for the differential effects of Pfn1 on the overall actin cytoskeleton. Also, whether Pfn1 would promote a net actin polymerization or G-actin sequestration depends on its concentration relative to that of available G-actin and free barbed ends of actin filaments. These parameters are controlled by other ABPs (sequestering, severing, and capping), expression of which can vary between cell lines. Pfn-1 stimulated actin polymerization in MDA-MB-231 cells is completely

abolished when either its actin or polyproline binding ability is abolished thus implying that Pfn1 must bind to both actin and proline-rich motif proteins for efficient actin polymerization. A dramatic reduction in the overall F-actin content in GPH119E-expressers can be due to inhibition of endogenous Pfn1's interaction with PRM-proteins that are important for actin assembly (example: VASP, N-WASP, mDia). We also observe higher nuclear localization of the GPH119E mutant of Pfn1 (data not shown). Since nuclear export of actin requires its binding to Pfn1 (Stuven et al., 2003), possibility of additional nuclear sequestering of G-actin can further inhibit actin polymerization. We have observed only a slight reduction in F-actin content in the GPH133S mutant cells. This result is not surprising since, because of a functional actin-binding site, Pfn1-H133S mutant can still polymerize actin in a PRM-independent manner. An interesting cytoskeletal feature of these cells is however the lack of polarized enrichment of cortical actin at the leading edge that is usually observed for the control cells. Although the reason is not clear, one can postulate the following scenario. Because of functional competition, the H133S mutant of Pfn1 (GPH133S) is expected to sequester G-actin, at least in part, from the endogenous Pfn1. If PRM-proteins are activated spatially in response to signals and then act as molecular scaffolds to induce actin assembly utilizing Pfn1, GPH133S-actin complex can then be prevented from being recruited to correct spatial locations because of lack of PRM-binding of the mutant. Further work is needed to definitively address this issue.

Similar to our previous finding for BT474 cells (Roy and Jacobson, 2004) , we find that overexpression of Pfn1 suppresses the chemotactic migration of MDA-MB-231 cells. Pfn1-induced inhibition in migration of MDA-MB-231 cells can be partly due to excessive actin polymerization, since a significant depletion of cellular G-actin pool as a result of this may retard the rate of actin-treadmiling that is necessary for rapid migration for carcinoma cells. We also

find that Pfn1 overexpression dramatically enhances the formation of focal adhesions in MDA-MB-231 cells suggesting a possibility of increased cell adhesion which can further contribute to inhibition in cell migration (Indeed we find that GFP-Pfn1 expressing cells also more resistant to trypsinization compared to the control GFP cells).

Although from the inhibition in cell migration induced by the functional mutants of Pfn1, one could potentially interpret that both actin and polyproline binding of Pfn1 is required for MDA-MB-231 migration, we will not do so. This is because of unpublished results in our laboratory that silencing Pfn1 expression actually increases MDA-MB-231 migration. We have considered several alternative explanations of our data to clarify an apparent paradox that the cellular effects of selective functional blocking of Pfn1 by overexpressing mutants may not be the same as those achieved under gene-silencing condition. For example, overexpressing the GPH119E mutant (actin-binding deficient) can sequester PRM proteins from endogenous profilin and prevent Pfn1-independent PRM-mediated actin polymerization (possible because the PRM proteins have direct actin-binding sites) that could otherwise be required for tumor cell migration. Indeed we see an overall decrease in the F-actin level in this mutant cell line. Other possibilities such as increased nuclear sequestering of G-actin and alteration in gene expression (since Pfn1 has been recently implicated in gene transcription and splicing) cannot be ruled out. Similarly, GPH133S mutant can potentially inhibit cell migration by 1) interfering with the development of spatial asymmetry of cells (a key feature for migrating cells), and 2) possibly altering gene expression (since a recent study shows that Pfn1 binds to transcription factors via PRM interactions). Overall, these possibilities imply that the cellular effects of selective functional blocking of Pfn1 by overexpression of mutants cannot be mimicked by loss of Pfn1 in

cells. To critically determine the role of actin and polyproline-binding of Pfn1 in cell migration, one needs to express these mutant constructs in a null background at a concentration similar to that of endogenous Pfn1 normally present in these cells and study the resulting effects on cell migration.

Since cell invasion is critically dependent on cell migration, we were able to observe inhibition in cell invasion by overexpression of both fully functional and either of the mutant forms of Pfn1. Interestingly, we found that perturbation of Pfn1 affects the MMP secretion by MDA-MB-231 cells. Future work is needed to determine whether Pfn1 is involved in the transcriptional regulation of MMPs.

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