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Research Article

Suppressive Effects of Tyrosine (Y129) Phosphorylation of Profilin I on Breast Cancer Progression

Wasia Rizwani[1*], Aneesa Fasim[2], Surya Satyanarayana Singh[1]

[1]Department of Biochemistry, University College of Science, Osmania University, Hyderabad, Telangana, India

[2]School of Basic and Applied Sciences, Dayananda Sagar University, Bangalore, India

[*]**Corresponding author:** Wasia Rizwani, Department of Biochemistry, Osmania University, Hyderabad, Telangana, India

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Abstract

It gets more and more intriguing to understand the role of multi-faceted Profilin, primarily an actin modulating protein, in breast cancer. Yet again, tyrosine 129 phosphorylation adds another interesting dimension to its role in breast cancer progression. Having discovered that Serine (S137) phosphorylation of profilin I imparts aggressive nature to breast cancer, we wanted to study the effect of Tyrosine (Y129) phosphorylation on the role of profilin I in breast cancer progression. These phosphorylations occur in the residues in the C-terminus of profilin that affect certain functions of profilin such as Polyproline- binding, PIP2 (Phosphatidylinositol 4,5-bisphosphate) and actin interactions. To examine this, we generated phosphorylation mutants PFN-Y129A and PFN-Y129A/S137A by site-directed mutagenesis. We had PFN-WT and PFN-S137A from previous studies. MDA-MB-231 cells and MCF7 cells demonstrated that single mutant PFN-Y129A and double mutant PFN-Y129A/S137A behaved differently than PFN-S137A. Surprisingly, Tyrosine phosphorylation prevents breast cancer progression. Transient transfection of these tyrosine phosphorylation mutants made cells more proliferative, migratory and gave anchorage-independence. They formed larger colonies than PFN-WT and more in number. An interesting fact observed was double phosphorylation mutant showed most aggression in breast cancer. Could this indicate a balance existing in these phosphorylations to prevent breast cancer progression and metastasis? We are yet to solve this dichotomous nature of Serine and Tyrosine phosphorylations and their impact in drug discovery.

Keywords: Profilin I; Serine; Tyrosine; Phosphorylation; Breast cancer

Abbreviations: Pfn/PFN-Profilin I; Ser-Serine (S); Tyr-Tyrosine (Y); HIF-1α- Hypoxia-inducible factor-1α; MMP- Matrix Metalloproteinases

Introduction

Cancer cells exhibit chronic proliferation by disrupting the well-regulated cell-cycle homeostasis that normal cells undergo. They acquire the ability to evade tumor suppressors, enhance migratory signals, increase invasiveness and become immortal by undergoing uncontrolled replication. Growth is further supported by neo-angiogenesis and cell death resistance [1]. Involvement of tumor stroma or the microenvironment has gained importance only recently although extracellular signaling governing cancer cell behavior was fairly well understood. It is usually a combined effect of these activities that drive cancer progression and metastasis. Proliferation and growth involve complex architectural changes that are governed by actin cytoskeleton and actin interacting proteins such as profilin, cofilin, twinfilin, Arp 2/3, ENA/VASP, etc. [2]. Ability of actin to exist as monomeric G-actin and polymeric F-actin enables rapid remodeling for cell integrity, motility and membrane trafficking [3,4]. Profilin (PFN), an actin modulating protein, is an extensively studied cytoskeletal protein yet elusive in its cellular functions in entirety [5]. Profilin isoforms and oligomers are considered to be important regulators of the actin-based cytoskeleton [6].

Profilins contains one polyproline domain and two phosphoinositide binding sites through which it directly interacts with Ena/VASP, N-WASP, WAVE, and formins [7]. Profilins elongate actin either by adding monomeric G actin to preexisting actin filaments or by adding the profilin—actin complex to the barbed ends [8]. Profilin in high amounts is known to inhibit actin polymerization by sequestering actin monomers from F-actin or, inversely when present in low amounts promotes actin polymerization under appropriate environmental stimuli [9-11]. Profilin regulates membrane protrusions by binding to VASP and N-WASP in the leading points of the cells. This increases the intracellular concentration of profilin locally, enhancing the removal of actin monomers at the barbed ends and leading to depolymerization [12]. Profilins have been shown to promote or inhibit membrane protrusion, cell migration and invasion in various cancers based on these protein-protein interactions and actin-regulation [13]. Next, its interaction with PI(4,5)P2 (Phosphatidylinositol 4,5-bisphosphate), a component of the phosphatidylinositol cycle, serves as a link through which the signalling pathways communicate with the dynamics of the actin cytoskeleton [14,15]. Post-translational modifications (PTM), such as nitration and phosphorylation (S137, Y219), of profilin 1 alter its biological functions [16-18]. Profilin is also regulated by phosphorylation at sites T89, S71, Y129 [19-21]. It has been demonstrated that phosphorylation of profilin is counter acted by protein phosphatase-1, and the switch between the two states regulates cell proliferation and survival [22].

Previous data from our lab demonstrated that phosphoprofilin (S137) binds to actin monomers with higher affinity and therefore might alter actin polymerization [18]. We also for the first time demonstrated that suppression of serine 137 phosphorylation led to decreased cell motility, invasiveness and anchorage-independence [23]. An elaborate study by Ding et al demonstrated that endothelial cells with loss of profilin expression had defects in membrane protrusion, reduction in cell-cell adhesion and actin filaments. Endothelial cell proliferation and morphogenesis was compromised with silencing profilin 1 [24]. However, phosphorylation of profilin at tyrosine Y129 was found to facilitate adult angiogenesis in tissue wound healing process [21]. VEGFR receptors can phosphorylate profilin directly and has been shown to increase its affinity for actin in adult arteriogenesis. Cancer cells deregulate growth control signals that are largely communicated by growth factors that bind cell-surface receptors, typically containing tyrosine kinase receptor domains, or have direct entry into the cells. Phosphoprofilin (pY129) facilitated glioblastoma progression by endothelial secretion of angiocrine factors that induced hypoxia inducible factor 1α (HIF-1α) stabilization and accumulation in normoxic conditions [25]. However, its role in epithelial cancer cells or solid tumors needs extensive research. Since we had observed the effect of Ser137 phosphorylation on breast cancer progression, we decided to explore Tyr 129 phosphorylation of Profilin I in breast cancer progression in the present study.

Materials and Methods

Generation of GFP-tagged PFN-Y129A and PFN-Y129A/S137A mutants by site-directed mutagenesis

The clone GFP-PFN-S137A has been successfully generated. Profilin-1 single and double phosphorylation mutants mentioned were generated by site directed mutagenesis [23]. Tyrosine 129 residue of PFN-1 was mutated to Alanine using eGFP-PFN1-WT (wild type) clone as a template and (Y129A) primer 5'-GATCAACAAGAAATGTGCTGAAATGG CCTCCCACC TT-3'. A PCR reaction was set up at 95 ˚C for 5min, 1 cycle followed by 95 ˚C for 30sec, 60 ˚C for 30sec and 72 ˚C for 3min using pfu polymerase (NEB) for 30 cycles with a final extension at 72 ˚C for 10min and end hold at 4 ˚C. After PCR, the template was digested using 1 µl of Dpn1 restriction enzyme and incubated at 37 ˚C for 2hr before transforming in *E.coli* DH5α. Positive colonies were confirmed by sequencing. Double mutant was created in the same manner by using eGFP-PFN1-S137A as template and Y129A primer to create eGFP-PFN1- Y129A/S137A clones.

Transient transfection of MCF7 and MDA-MB-231 breast cancer clones with profilin-wild type (PFN-WT) and phosphoprofilin mutants PFN-Y129A, PFN-S137A and PFN-Y129A/S137A plasmids

MCF7 and MDA-MB-231 cells were successfully transfected with a control EGFP-N1 vector or following profilin1 expressing constructs: GFP-PFN1-WT (Wild-Type, human), GFP-PFN1-S137A (serine 137 phosphorylation mutant), GFP-PFN1-Y129A (tyrosine 129 phosphorylation mutant) and GFP-PFN1-Y129A/S137A (double phosphorylation mutant) plasmids. For transfection of 10,000 cells, 0.5 µg plasmid DNA was mixed with 1 µl of transfection reagent (Fugene HD, Promega) in a total volume of 200 µl serum-free DMEM media [26]. Concentrations were adjusted depending on cell number for each experiment. The transfection mix was incubated at room temperature for 30min, following which it was added to cells for various experiments pre-incubated in serum-free DMEM media for 1hr. After 5hr of transfection, media was changed to complete DMEM media and cells were allowed to grow for 24hr for protein expression. PFN-WT was used as control/comparison point in all the experimental analysis.

Immunofluorescence

MCF7 and MDA-MD-231 clones (20,000cells) were plated on cover slips (Corning) in a 6-well plate and cultured for 24hr to visualize localization of overexpressed profilin 1 and its mutants. The cells were fixed in 2% para-formaldehyde for 15min.

The nuclei were stained with Propidium iodide (PI). Cover slips were mounted on glass slides [23]. Images were captured at 40X magnification with a Leica fluorescence microscope. Images are representative of two independent experiments done in duplicate.

Tumorigenesis studies *in vitro*

A. Proliferation Assay/Cell viability Assay: To determine cell growth of transfected clones, 2000 cells of each clone from each cell line were plated in triplicate in 96-well plates and cell viability was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at 48hr after transfection [23]. The assay was repeated three times and analyzed using two-sided Student's *t* test. Data points represent the mean±s.d and $p<0.005$ considered significant.

B. Migration/Wound healing Assay: 5 x 10^4 cells were plated in a 6-well plate in triplicate, transfected with different profilin plasmids and allowed to grow to confluency. Cells were washed and left in serum-free media for 5hr. A scratch was made and media added that was either only serum-free or with serum. Images were captured at 10X magnification using Leica Microscope at zero hour and again at 18/24 hr. The scratch area at zero hour and empty area not covered by migrated cells after 24hr was analyzed with ImagePro software [23]. Data points representing the mean±s.d. from two fields of uncovered scratch area from two independent experiments was analyzed and $p<0.05$ considered significant.

C. Anchorage – independence studies

- Growth of 3D cultures in vitro. The Bio-AssemblerTM System (24-well configuration) from n3D Biosciences, Inc (Houston, TX) was used to construct the 3D in vitro breast tumor model. NanoshuttlesTM (NS) were added to cells at a ratio of 10 ml of NS per 50,000 cells and incubated at 37 ^{0}C overnight, following transfection for 24hr. The cells for 3D cultures were detached and cultured in the Bio-AssemblerTM system. For example, when using a 24-well ultralow-attachment plate (Corning, Inc. Tewksbury, MA), a total of 50,000 cells were added in each well in a total volume of 350 ml of medium. Immediately afterward, a 24-well lid insert will be placed on top of the plate, followed by the magnetic driver which sits inside the insert, and then the 24 well cover on top. The plate will be gently shaken to agitate cells and placed in the incubator. After 4hr, the 24 well plates will briefly be observed with bright field microscopy using a 4X or 10 X objective to determine if the structures will be forming cohesive levitating structures. In brevity, most cells aggregate within a few hours and continue to become denser as more time passes. After 3 days, images of cell clusters were captured for each PFN clone using the inverted microscope and miCAPS software (ICON Biosystems, India) at 4X and 10X magnification.
- Soft agar growth assay. Anchorage independent growth was assayed by the soft agar growth assay as described elsewhere [23]. The first step involved plating a bottom layer of 0.6% agar in serum-free media in 12-well plates. The plates were incubated at room temperature for 30 min to solidify the agar. Cells were harvested by trypsinization following transfection with different profilin plasmids and 3000 cells per well were mixed with 0.3% agarose (made in complete media) and layered carefully on the top of existing 0.6% agarose in triplicate. The plates were covered with 1 ml of medium supplemented with 10% FBS and incubated at 37 ^{0}C in a 5% CO_2 incubator for 10days. Complete media was added every 2 days to the test wells. At the end of 10days, cell colonies with 100 mm in diameter were imaged under a microscopic field at 4X magnification and counted [23]. Mean colony count was based on numbers from all fields imaged with 4X magnification from two independent experiments done in triplicate for each profilin clone and was analysed using two-sided Student's t test.

Immunohistochemistry

Breast TMA were purchased from Biomax, USA (cat # BRC961). Slides were stained following the protocol [26]. Primary antibodies were from ECM Biosciences for specific for Profilin, Pfn-pTyr, Pfn-pSer (Cat #6930, Profilin Phospho-Regulation kit). Tumor sections were imaged at 4X magnification and analyzed (semi-quantitative scoring) by a pathologist using WHO criteria for grading the staining.

Results

Generation of GFP-tagged PfnY129A and -PfnY129A/S137A mutants by site-directed mutagenesis

It is a well-known fact that Profilin I undergoes Tyrosine phosphorylation at various residues by non-receptor tyrosine kinases like Src and receptor tyrosine kinases like VEGFR pathways [21]. Tyrosine kinases are notorious candidates for inducing neoplasm and transformation in cancers. Many have been identified as oncogenes and their enzymes as oncoproteins causing malfunctioning of signaling networks [reviewed in 27]. Hence, to elucidate the consequences of tyrosine 129 phosphorylation of profilin 1 in breast cancer progression, single mutant GFP-PFN1-Y129A and double mutant GFP-PFN1-Y129A/S137A of Profilin 1 were generated by site-directed mutagenesis from GFP-PFN-WT (Wild-Type). GFP-PFN1-WT and GFP-PFN1-S137A were available from previous study (PlosOne2014). They were cloned and transformed plasmids into DH5α bacterial cells for maintenance. Plasmids were isolated in large quantities by maxiprep (Qiagen) and sent for sequencing. Plasmids were sequenced by Eurofins (Bangalore) for confirmation. Results are presented in Figure 1. PFN-S137A sequence is shown in Supplementary figure 1.

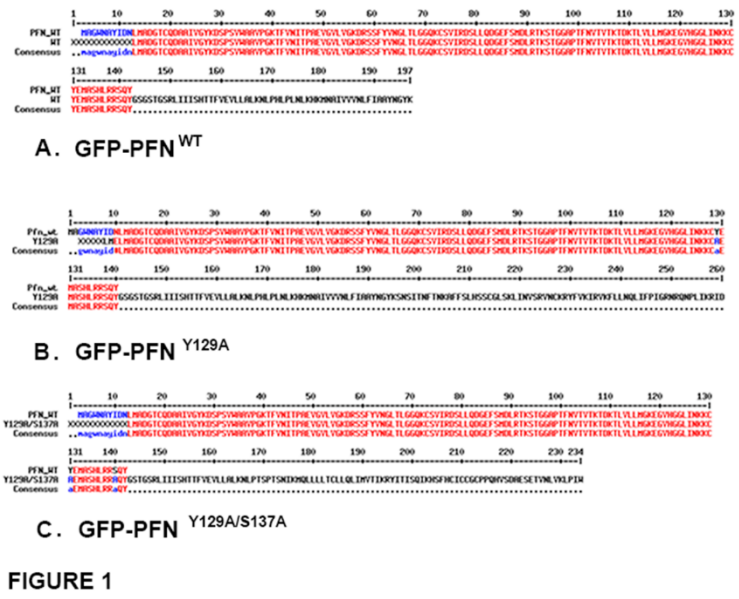
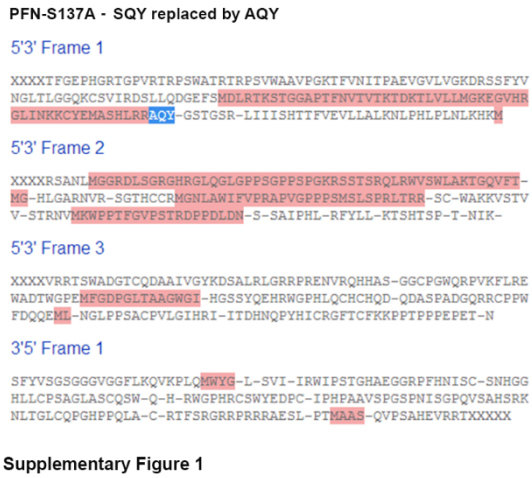


Figure 1: Site-directed mutagenesis. Single tyrosine phosphorylation mutant PFN-Y129A and dual phosphorylation mutant PFN-Y129A/S137A were created using eGFP-PFN-WT (Profilin wild-type) plasmid. The mutations were confirmed by sequencing.



Supplementary Figure 1

Supplementary Figure 1: Plasmid Sequencing was done to confirm mutation of S to A in PFN-S137A mutant.

Expression of PFN clones in MDA-MB-231 cells and visualization by fluorescence microscopy.

Emphasis was given to expression of new clones GFP-PFN-Y129A and GFP-PFN-Y129A-S137A in MDA-MB-231 cells. Cells were transfected and grown for 24hr before fixing them in paraformaldehyde and staining with PI. Interestingly, single mutant PFN-Y129A was more cytoplasmic whereas double phosphorylation mutant PFN-Y129A-S137A had more nuclear presence compared to PFN-WT or PFN-Y129A, along with cytoplasmic expression (Figure 2). PFN-S137A was mostly cytoplasmic, similar to previous results from MCF7 cells [23]. Results indicate presence of cellular signaling to be controlling the variation in expression between compartments.

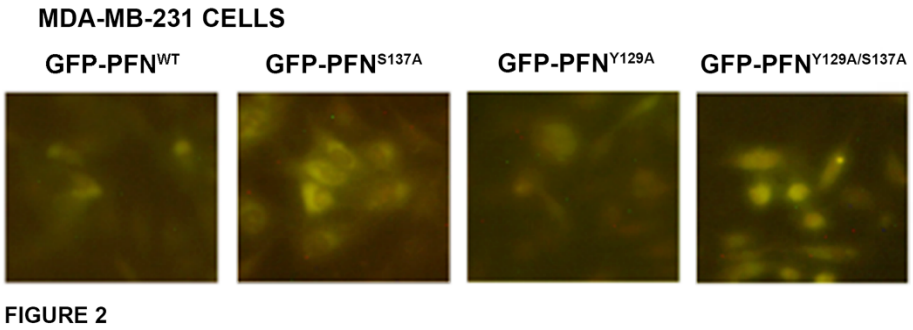


Figure 2: Overexpression and localization of profilin 1 and its mutants in MDA-MB-231 breast cancer cells. All plasmids had both cytoplasmic and nuclear expression, evident from GFP expression. PI staining was done for nuclear staining. Images were overlaid. PFN-WT (profilin 1 Wild-type), PFN-Y129A were more evenly distributed in the cell and PFN-Y129A/S137A had slightly more nuclear presence while PFN-S137A (serine phosphorylation mutant) was predominantly cytoplasmic. Magnification-40X.

Anchorage-independence in profilin phosphorylation mutants in breast cancer cells.

Transfected MDA-MB-231 breast cancer cells were incubated with magnetic nano beads overnight, trypsinized and plated into low attachment plates with a magnetic plate on top. Magnetic levitation allows cells to form 3D clusters in 3 days. We found that PFN-S137A formed smaller and lesser number of colonies than PFN-WT, similar to MCF7 cells from previous studies [23]. There were few large colonies but significantly lesser than WT. An interesting observation was PFN-Y129A (50%, $p<0.005$) formed larger colonies than PFN-WT and the double mutant, PFN-Y129A/S137A, had comparitively the largest and most number (90% more, $p<0.005$) of colonies (Figure 3).

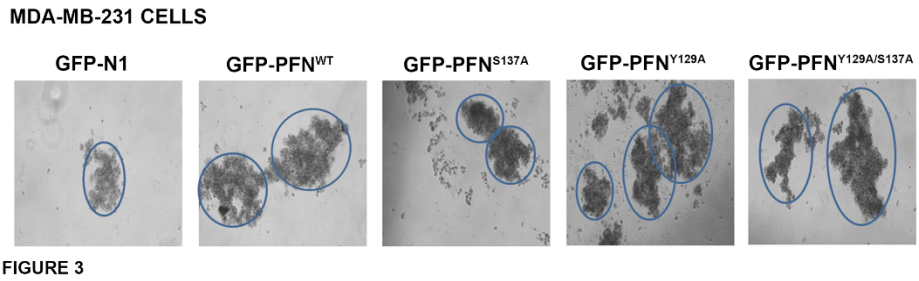


Figure 3: Overexpressed profilin 1 and its mutant clones exhibit different cell growth and show difference in 3D cultures using n3D Bio-assembler kit in MDA-MB-231 cells. Representative phase contrast images from n3D colony formation assay demonstrating that PFN-Y129A/S137A formed the most number of colonies followed by PFN-Y129A when compared to PFN-WT. PFN-S137A formed smaller and significantly less number of colonies (***$p<0.005$). Magnification-10X. Sale bar-300 µm.

MCF7 cells were also transfected with different profilin plasmids and soft agar assay was performed. Colonies were imaged and counted. Both PFN-Y129A and PFN-Y129A/S137A formed more number of colonies and they were larger in size compared to PFN-WT (Figure 4). PFN-S137A formed 44% ($p<0.005$) less colonies of size 300 µm compared to PFN-WT, whereas PFN-Y129A formed 41% ($p<0.005$) more and PFN-Y129A/S137A formed 105% ($p<0.005$) more colones in number. It could be deduced that inhibiting serine phosphorylation could result in cancer regression whereas inhibiting tryrosine phosphorylation could make cancer aggressive. A very contraindicating result that could result in complicating treatment regimen for breast cancer.

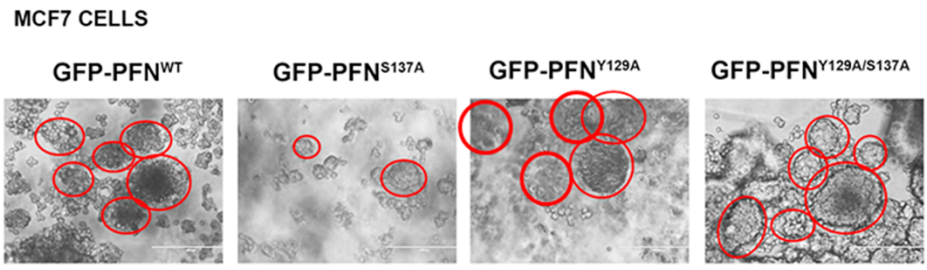


FIGURE 4

Figure 4: Overexpressed profilin 1 and its mutant clones exhibit different cell growth and show difference in 3D cultures with soft agar assay in MCF7 cells. Representative phase contrast images from 3D colony formation assay demonstrating that PFN-Y129A/S137A formed the most number of colonies followed by PFN-Y129A when compared to PFN-WT. PFN-S137A formed smaller and significantly less number of colonies (***p<0.005). Magnification-10X. Sale bar-300 μm.

Inhibition of tyrosine phosphorylation results in enhanced proliferation and migration

Having observed anchorage-independence with various profilin plasmids under study, we next assessed effects of tyrosine 129 profilin phosphorylation in breast cancer progression. Both MDA-MB-231 and MCF7 cells were used for the assays. Comparison was mostly concentrated on PFN-WT and tyrosine 129 phosphorylation mutants. We observed that both proliferation and migration were affected and there was a significant increase in proliferative as well as migratory activities of breast cancer cells in PFN-Y129A and PFN-Y129A/S137A mutants over PFN-WT. Figure 5A represents proliferation assay for MDA-MB-231 cells and Figure 5B depicts proliferation assay for MCF7 cells. Similarly, Figure 6 represents migration assay for MDA-MB-231 cells and Figure 7 shows migration assay using MCF7 cells. Combined data suggested that inhibition of tyrosine phosphorylation either by targeting VEGF pathway directly or other tyrosine kinsases indirectly that phosphorylate profilin could be detrimental to controlling breast cancer progression.

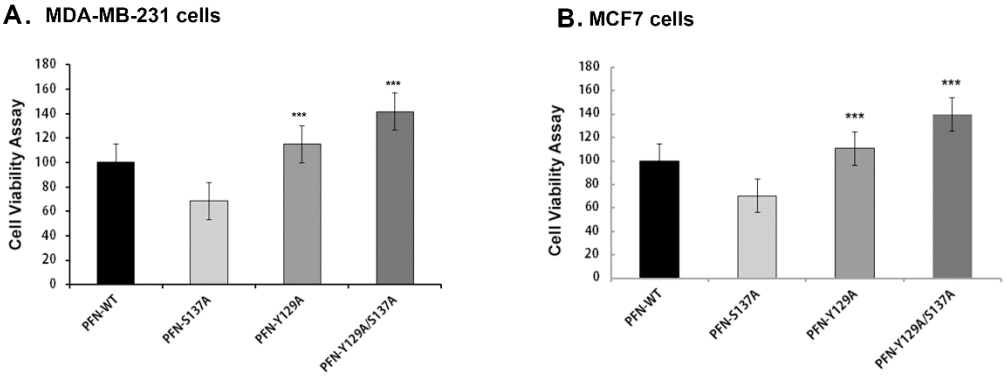


FIGURE 5

Figure 5: Proliferation/Cell viability assay was conducted on MDA-MB-231 cells (A) and MCF7 cells (B) following transient transfection of profilin plasmids. MTT assay of MDA-MB-231 and MCF7 cells transfected with profilin I and its phosphorylation mutants demonstrate that PFN-Y129A/S137A had the highest viability followed by PFN-Y129A when compared to PFN-WT, and PFN-S137A had least viability at 48hr post transfection (***p<0.005).

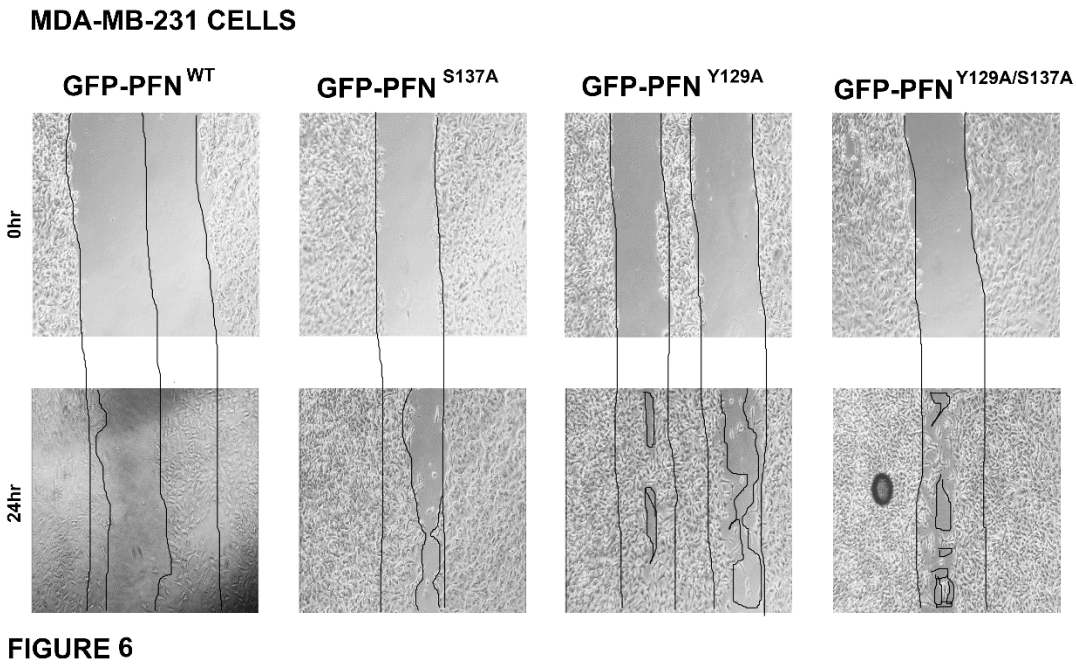


Figure 6: Inhibition of tyrosine phosphorylation of profilin 1 increases the migratory ability of MDA-MB-231 cells. Upper panel images are representative of MDA-MB-231 cells overexpressing profilin 1 and its mutants in serum containing media at 0hr of making the scratch. Lower panel shows cells that migrated into the scratched area at 24hr. Magnification-10X. Scale bar-200 μm.

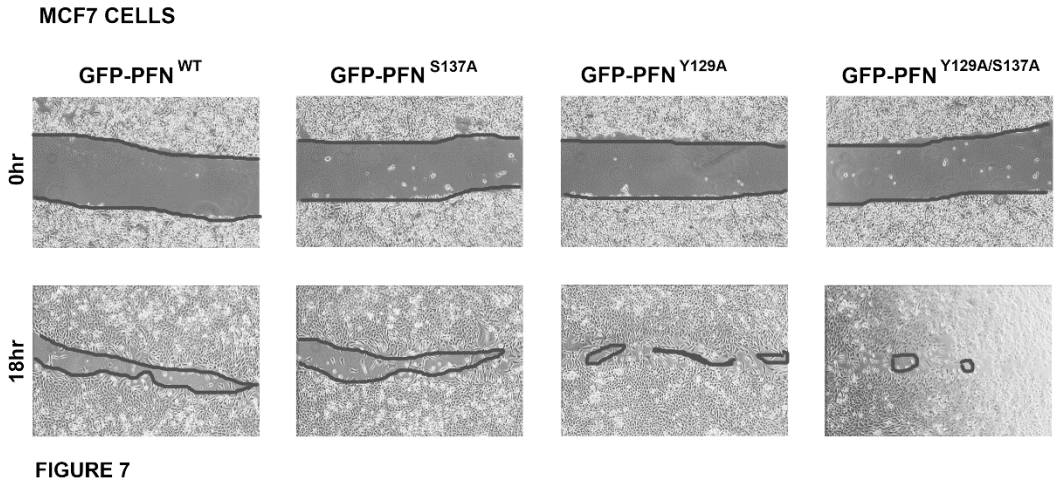


Figure 7: Inhibition of tyrosine phosphorylation of profilin 1 increases the migratory ability of MCF7 cells. Transient transfections were done hence a repeat of PFN-WT and PFN-S137A from previous study was carried out. Comparison was drawn with PFN-WT transfected cells. Upper panel images are representative of MCF7 cells overexpressing profilin 1 and its mutants in serum containing media at 0hr of making the scratch. Lower panel shows cells that migrated into the scratched area at 18hr. Magnification-10X. Scale bar-200 μm.

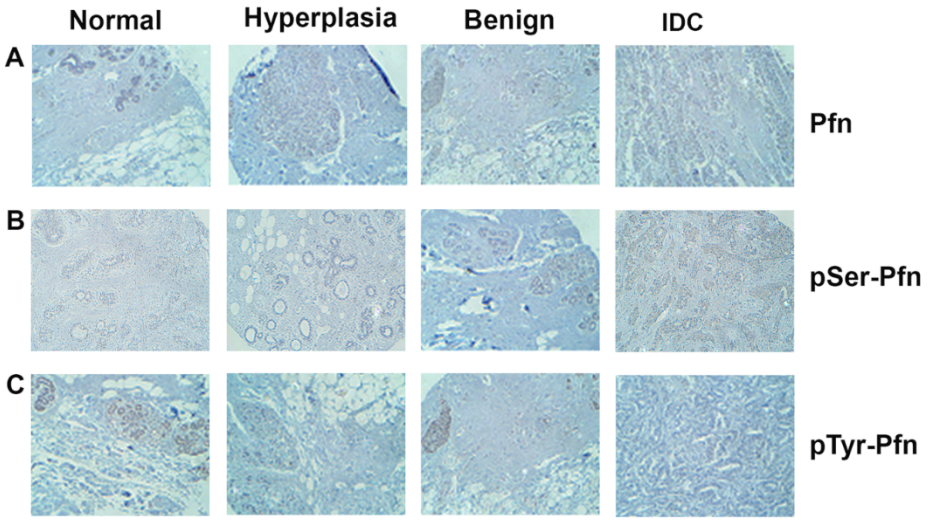
Discussion

Profilin I undergoes phosphorylation on various sites that seem to control its functional aspects in cancer cells and endothelial cells [2,13,21]. It has been shown that profilin phosphorylation on Tyrosine 129 is mediated by VEGFR2 and VEGFR2-activated Src kinase. VEGFR1 may also be contributing to this, as it was found that its depletion led to inhibition of VEGF-dependent profilin I phosphorylation, but through an indirect unknown pathway. We had previously revealed that Serine 137 phosphorylation is mediated by PKCzeta [28]. ROCK (Rho-associated Kinase) and PP1 (Protein phosphatase 1) are also known to regulate phosphorylation and dephosphorylation of profilin 1 at Serine 137 respectively [29,30]. Our studies also provided clarity with respect to Serine phosphorylation and its effects on breast cancer progression. We proved that phosphorylation of profilin on Serine 137 residue promoted proliferative, migratory and invasive abilities in breast cancer cells [23]. Understanding the significance of another phosphorylation site in the C-terminus of Profilin on Tyrosine 129 residue was intriguing. We used breast cancer cell lines MDA-MB-231 and MCF7 to elucidate its role in breast cancer progression.

In our current studies, we find that inhibiting profilin phosphorylation at Tyrosine 129 residue made breast cancer cells more aggressive. Breast cancer cells are well known to stimulate angiogenesis by Vascular Endothelial Growth Factor (VEGF) and TGF-β [31]. Studies have shown reciprocal angiogenic effects by vascular endothelial cells upon co-culturing with breast cancer cells demonstrating increased VEGF expression, endothelial proliferation, migration and organization [32]. Detection of VEGF-C, which induces lymph-angiogenesis in lymphatic endothelial cells, in breast cancers indicated shorter disease-free survival. VEGF-C overexpressing breast cancers are associated with lymphatic vessels invasion and lymph node metastasis [33-35]. Additionally, adipocytes also interact with breast cancer cells. Visceral, but not subcutaneous, adipocytes promote tumor proliferation and induce epithelial-to-mesenchymal transition via IL-6 and IL-8 [36]. The effects of visceral adipocytes on the breast cancer microenvironment are especially pronounced due to abundant fatty tissue in the breast [37].

Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) control the initiation and progression of tumor angiogenesis [38-41]. Varying levels of angiogenic factors, and the subsequent number of vascular networks triggered, could predict a thriving breast cancer [42-44]. Poor prognosis and aggressive tumors are synonymous with elevated levels of growth factors [45-47]. Coupled with activating angiogenesis, these factors also dictate the rate and extent to which the blood vessels permeate- a reason for survival of cancer cells. Hence, compounds that target the angiogenesis pathway are under investigation as therapeutic agents in research in breast cancer [48]. However, our results throw caution to this notion of inhibiting tyrosine kinases to suppress breast cancer progression and metastasis.

It is a well-known fact that surgical intervention creates a hypoxic environment [49] that could promote metastasis of breast cancer. Oxygenation of the regional tumor microenvironment has significant impact on breast tumor biology [50,56]. A hypoxic environment induces expression of aggressive phenotype (estrogen receptor negativity), local tumor progression and nodal metastasis [50,51]. HIF-1α is a critically important transcription factor instrumental in regulating diverse cellular responses to hypoxia, inducing expression of glycolytic enzymes and multiple angiogenic growth factors that drive aberrant vascularization during tumorigenesis [52-55]. HIF-1α is upregulated in most malignant tumors, primarily by hypoxia-induced protein stabilization [54]. Endothelial cells could stabilize HIF-1α independent of hypoxic environment by competitive inhibition of VHL binding to HIF-1α due to phosphorylation of Pfn-1 at Tyr129. In turn, elevated HIF-1α induced expression of multiple angiogenic factors, eventually leading to aberrant vascularization and GBM progression [25]. The exact mechanism of how breast cancer cells are promoting tumorigenesis upon inhibition of tyrosine 129 phosphorylation of profilin is not understood. Localized tumors versus metastatic sites could have varied expression after eliciting a certain signalling network in various cancers. We found elevated levels of profilin pSer (137) as tumor progresses and decreased levels of profilin pTyr (129) from hyperplasia to benign to metastasis in a breast tumor microarray through immunohistochemistry. We did not compare Profilin versus phosphorylated profilin expression due to differences in antibody specificity. Extensive analysis and studies are in progress. Presence of phosphorylated profilin in the nucleus, cytoplasm and some membranous staining (Supplementary figure 2) indicated a continuous signalling process that breast cancer cells must be undergoing for survival and spread. It could involve genetic control, translocation and actin-binding functions of profilin. Tumor infiltrating cells with positive profilin expression is another interesting fact to be investigated with much detail. Complexity of signalling between tumor microenvironment and actual tumor cells could be the reason why a popular drug, Bevacizumab (a humanized anti-VEGF monoclonal antibody) successful in some cancers is being rescinded by FDA for the treatment of metastatic HER2-negative breast cancer due to contradictory results in further trials beyond pre-clinical work. Although one of the reasons cited is elevated toxicity [56-60].



Supplementary Figure 2

Supplementary Figure 2: Expression of Profilin, pSer (137)-Profilin and pTyr (129)-Profilin in breast tumor microarray. Three same microarrays (cat # BRC961, Biomax USA) were independently stained with profilin, pSer-profilin and pTyr-profilin antibodies. Staining was visualized with vectastain kit and representative images are presented here of normal, hyperplasia, benign and Invasive ductal carcinoma (malignant) tissue sections. Both nuclear and cytoplasmic expression of profilin was observed. Magnification is 4X, scale bar = 50 μm. (A) Profilin antibody staining (B) Profilin pSer antibody staining and (C) Profilin pTyr antibody staining. Profilin phospho-regulation kit from EMD Biosciences was used.

Whether VEGF inhibition in tumor microenvironment is leading to reduced phosphorylation or absence of tyrosine phosphorylation of profilin is leading to more aggressive tumors needs to be explored? We have already shown the involvement of MMPs in Pfn-pSer signaling pathway in MCF7 cells [23]. What gene expression changes in the nucleus and cytoplasm are leading to increased interaction with tumor suppressor proteins upon phosphorylation to keep cells from undergoing metastasis? The regulatory loop running to keep a check on Ser/Thr kinases as well as Tyr kinases and respective phosphatases to keep profilin phosphorylations from creating havoc in a normal cell; and what leads to aberrant pathways related to profilin's wider role in breast cancer warrants further investigation. Multiple kinases and phosphatases affect profilin phosphorylation thereby increasing the complex nature of profilin I function; its interactions with its many isoforms and oligomers [61]. These in turn will affect the cascade of protein interactions further and the biological consequences thereafter in cancers.

Conclusion

Our studies highlight one of the reasons for profilin's tumor suppressor activity; it could be phosphorylation of Tyr (129), whereas tumor promoting activity could be due to Ser (137) phosphorylation. An imbalance in VEGF levels could reduce levels of Tyr-phosphoprofilin and lead to enhanced tumorigenesis or increase in PI3K activity and PKCs could overtake Pfn-Ser phosphorylation and promote tumorigenesis. Hormone dependency in breast cancer development and progression could add another level of regulation towards metastasis. It is possible that not all profilin undergoes phosphorylation and the extent to which it undergoes phosphorylation at Tyr or Ser residues could be a determining factor for tumorigenesis and metastasis. This study opens up many areas to explore in profilin phosphorylations and interaction with Polyproline containing tumor -suppressing/ -promoting proteins. What levels of VEGF or PKC are needed to maintain homeostasis in profilin function in normal cells? Presence of profilin Y129A/S137A mutant in the nucleus suggests another complicated aspect to profilin's function in stimulating gene expression. Probably, a threshold level exists within cells for profilin phosphorylation to show its effects in cancer.

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