Published in final edited form as:

J Cell Physiol. 2009 February ; 218(2): 436–443. doi:10.1002/jcp.21618.

Profilin-1 over-expression upregulates PTEN and suppresses AKT activation in breast cancer cells

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Abstract

Profilin-1 (Pfn1), a ubiquitously expressed actin-binding protein, has been regarded as a tumor-suppressor molecule for breast cancer. Since AKT signaling impacts cell survival and proliferation, in this study we investigated whether AKT activation in breast cancer cells is sensitive to perturbation of Pfn1 expression. We found that even a moderate overexpression of Pfn1 leads to a significant reduction in phosphorylation of AKT in MDA-MB-231 breast cancer cells. We further demonstrated that Pfn1 over-expression in MDA-MB-231 cells is associated with a significant reduction in the level of the phosphoinositide regulator of AKT, PIP₃, and impaired membrane translocation of AKT that is required for AKT activation, in response to EGF stimulation. Interestingly, Pfn1-overexpressing cells showed post-transcriptional upregulation of PTEN. Furthermore, when PTEN expression was silenced, AKT phosphorylation was rescued, suggesting PTEN upregulation is responsible for Pfn1-dependent attenuation of AKT activation in MDA-MB-231 cells. Pfn1 overexpression induced PTEN upregulation and reduced AKT activation were also reproducible features of BT474 breast cancer cells. These findings may provide mechanistic insights underlying at least some of the tumor-suppressive properties of Pfn1.

Keywords

Profilin-1; breast cancer; PIP₃; AKT; PTEN; MDA-MB-231; BT-474

INTRODUCTION

Profilin-1 (Pfn1), ubiquitously expressed in all cell types, was initially identified as an actin-monomer (G-actin) sequestering protein (Karlsson et al., 1977). Later studies revealed that Pfn1 enhances ADP-to-ATP exchange on G-actin, and is actually capable of facilitating actin polymerization by acting as a shuttle to deliver ATP-bound G-actin to the barbed ends of actin filaments (Selden et al., 1999). Dramatic down-regulation of filamentous actin (F-actin) content in various cells induced by Pfn1 depletion in our previous studies further supports its role as one of the major promoters of actin polymerization in vivo (Ding et al., 2006; Zou et al., 2007). Besides actin, Pfn1 binds to various phosphoinositides (PPIs- phosphatidylinositol-4,5-bisphosphate (PIP₂), phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂) and phosphatidylinositol-3,4,5-triphosphate (PIP₃)), at least in vitro, and a number of proline-rich proteins ranging from those participating in cytoskeletal to transcriptional control in cells (Witke, 2004). Recently, Pfn1's role in cancer has been

queried because 1) its expression is down-regulated in several different types of adenocarcinoma (breast, pancreatic, hepatic) (Gronborg et al., 2006; Janke et al., 2000; Wu et al., 2006) and 2) xenograft studies have shown that Pfn1 overexpression completely suppresses tumorigenicity of breast cancer cells in both ectopic and orthotopic model systems (Janke et al., 2000; Wittenmayer et al., 2004; Zou et al., 2007). This correlation of loss of Pfn1 with tumor progression has led to Pfn1 being considered a tumor suppressor. The molecular mechanisms underlying Pfn1's tumor-suppressive action on breast cancer cells, however, remain to be elucidated.

Aberrant PI3K (phosphatidylinositol 3-kinase) /AKT (a serine-threonine kinase) signaling is a recurring theme for both initiation and progression of a variety of cancers including breast cancer (Liu et al., 2007). PI3K-AKT signaling is activated in response to growth factor stimulation and toxic insults. PI3K phosphorylates the D3 position of the inositol ring of PI(4,5)P₂ (PIP₂) to generate PI(3,4,5)P₃ (PIP₃) (Osaki et al., 2004). Generation of this potent lipid second-messenger recruits AKT to the cell membrane through its pleckstrin-homology (PH) domain. Membrane docking induces a conformational change in AKT that results in the exposure of its phosphorylation sites. At the cell membrane AKT is phosphorylated at its T308 and S473 residues by phosphoinositide-dependent kinase-1 (PDK1) and PDK2, respectively, leading to its complete activation. AKT, when activated, translocates to the cytoplasm and nucleus where many of its substrates are located. Downstream signaling initiated from these substrates ultimately influence a plethora of cellular functions including proliferation, motility and survival (Blume-Jensen and Hunter, 2001).

Since generation of PIP₃ is critical for activation of AKT and its downstream signaling, the pathway is down-regulated by PTEN (phosphatase and tensin homolog deleted on chromosome 10), a dual-specificity phosphatase as well as a potent tumor suppressor that reduces the amount of PIP₃ by dephosphorylating it back to PIP₂ (Cantley and Neel, 1999). PTEN is transcriptionally upregulated by p53 (Stambolic et al., 2001) and early growth-regulated transcriptional factor-1 (EGR-1; (Virolle et al., 2001)), while Jun (Hettinger et al., 2007) and NFκ-β (Xia et al., 2007) have been shown to repress PTEN transcription. The functioning of PTEN can also be regulated by post-transcriptional modification (phosphorylation, acetylation and oxidation), cellular localization and through interaction with other proteins that limit its ubiquitin-mediated degradation (Tamguney and Stokoe, 2007).

Because of Pfn1's link to breast cancer, in this study we investigated whether AKT activation in breast cancer cells is sensitive to Pfn1 perturbation. We here report a novel finding that Pfn1 overexpression suppresses AKT activation in breast cancer cells via upregulation of PTEN.

MATERIALS AND METHODS

Antibodies and reagents

Polyclonal AKT, phospho-AKT (T308, S473), PDK1, phospho-PDK1 (S241) and PTEN antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Monoclonal EGFR (EGF-receptor), phosphotyrosine, phospho-EGFR antibodies are products of Upstate technology (Lake Placid, NY). Monoclonal PIP₃ antibody was purchased from Echelon Biosciences (Salt Lake city, UT). Monoclonal GAPDH antibody is a product of AbD Serotec (Raleigh, NC). Polyclonal p85 antibody was obtained from Upstate technology. LY294002 was purchased from Calbiochem (Gibbstown, NJ). Monoclonal GFP antibody was obtained from Pharmingen (San Diego, CA). Polyclonal Pfn1 antibody was a generous gift of Dr. Sally Zigmond (University of Pennsylvania). All cell culture reagents are products of Invitrogen (Carlsbad, CA). Recombinant GFP protein was a product of Clontech

(Mountainview, CA). Generation of recombinant (HAT-tagged) Pfn1 protein has been previously described by us (Roy and Jacobson, 2004).

Cell culture and transfection

Generation and culture of MDA-MB-231 (MDA-231) and BT474 breast cancer cell lines stably expressing GFP and GFP-Pfn1 have been described previously (Roy and Jacobson, 2004; Zou et al., 2007). In gene silencing experiments, cells were transfected with either 100 nM of PTEN-siRNA (Cell Signaling technology, Danver, MA) or non-targeting control siRNA commercially available through Dharmacon (Chicago, IL) according to the manufacturer's instructions. For growth factor stimulation experiments, cells were serum-starved for 20-22 hours before stimulating with 100 ng/ml of either EGF (Invitrogen, Carlsbad, CA) or PDGF (Cell Signaling Technologies) for the indicated time-points.

RT-PCR

Total RNA was extracted from cells cultured in normal serum-containing growth medium using the RNeasy mini kit commercially available through Qiagen (Valencia, CA). RT-PCR reactions were performed using a commercial kit from Qiagen. The primer sequences for PTEN were 5'-GGACGAAGTGGTGAATGATATG-3' (sense) and 5'-TCTACTGTTTTTGTGAAGTACAGC-3' (antisense). The primer sequences for GAPDH were 5'-CGGAGTCAACGGATT TGGTCGTAT-3' (sense) and 5'-AGGCTTCTCCATGGTGGTGAAGAC-3' (antisense). The RT-PCR cycle conditions were 94°C (1 min), 55°C (1 min), and 72°C (1 min) with a total of 40 cycles. PCR amplified products were run on a 1.5% agarose gel.

Protein extraction, immunoprecipitation and immunoblotting

Total cell lysate was prepared by extracting cells with either warm 1X sample buffer or modified RIPA buffer (50 mM Tris-HCl -pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with 50 mM NaF, 1mM sodium pervanadate, and protease inhibitors. For immunoprecipitation, 750 µg cell lysate (extracted using a similar lysis buffer without SDS) was first precleared with 25 µl of protein-G/protein A-conjugated agarose beads. Precleared lysate was incubated with 2 µg of polyclonal phosphotyrosine antibody overnight and then with 50 µl of the same beads for an additional 2 hours. Immunoprecipitated protein samples were washed 3 times with the lysis buffer, resuspended in 30 µl of 2X sample buffer and run on a SDS-PAGE. For immunoblotting, all the antibodies were used at 1:500 dilution except those for GAPDH (1:2000), EGFR (1:1000), GFP (1:1000) and phospho-EGFR (1:200).

Immunostaining

For PIP₃ immunostaining, cells were washed twice with warm PBS, fixed with 4% formaldehyde for 20 minutes, permeabilized with 0.2% Triton X-100 in PBS for 10 minutes on ice, and then blocked with 1% BSA in PBS for 1 hour at room temperature (RT). Next, mouse anti-PIP₃ antibody diluted in PBS-1% BSA (1:100) was added and incubated for overnight at 4°C. After washing cells five times with PBS for 5 minutes each, cells were incubated with rhodamine-conjugated goat anti-mouse secondary antibody for 1 hour at RT. Finally, cells were washed four times with PBS and one time with distilled H₂O, each with duration of 5 minutes, before mounting on slides. Images were acquired with a 40X objective on Olympus Fluoview 1000 inverted confocal microscope. All images were background subtracted before quantitative fluorescence intensity analyses. The overall procedure was similar for AKT immunostaining except permeabilization was performed at RT and the images were acquired with a 60X objective.

Biochemical detection of PIP₃

Extraction of acidic lipids of cells and subsequent detection of PIP₃ were performed using a commercial chemiluminescence-based PIP₃ mass-strip kit (Echelon Biosciences, Salt Lake City, UT) completely according to the manufacturer's instructions (loading normalization of lipids between the different experimental groups was based on relative total protein concentration (proportional to the number of cells) measured at one of the intermediate steps during the extraction procedure).

Statistics

All experimental data were analyzed by ANOVA with a 95% level being deemed significant.

RESULTS

Pfn1 over-expression attenuates AKT activation in breast cancer cells

Since AKT-linked signaling plays critical role in tumorigenesis and a few independent studies have now established Pfn1's role as a tumor-suppressor molecule for breast cancer (Janke et al., 2000; Wittenmayer et al., 2004; Zou et al., 2007), we asked whether Pfn1 over-expression has any effect on AKT activation in breast cancer cells. To address this question, we used our previously described stable transfectants of MDA-231 breast cells (our primary choice of cell line) overexpressing either GFP-Pfn1 or GFP (control) (Zou et al., 2007). Our GFP-expressing subline of MDA-231 cells serves as an appropriate control because 1) it is functionally nearly identical to the parental cell line in terms of proliferation (unpublished observation) and motility characteristics, and 2) stably expressing GFP does not impair the tumorigenic ability of MDA-231 cells. We also established that GFP-Pfn1 overexpressing MDA-231 cells are completely impaired in forming tumors when xenografted in nude mice (Zou et al., 2007). Pfn1 immunoblot in figure 1A confirms that the endogenous Pfn1 level in these two cell lines are similar. GFP immunoblot in figure 1A shows comparable expression levels of GFP (lower band) and GFP-Pfn1 (upper band)). Although we and others have shown that GFP to the N-terminus of Pfn1 (as used here) preserves its biochemical functions and cellular localization (Wittenmayer et al., 2000; Zou et al., 2007) and our fusion protein is detectable by Pfn1 antibody in immunoblot, we felt there might still be a difference in sensitivity between endogenous Pfn1 vs exogenous GFP-Pfn1 to recognition by Pfn1 antibody. Therefore, for accurate quantitative estimation of the level of Pfn1 overexpression in our cell line, total cell lysate of GFP-Pfn1 expressers was run on SDS-PAGE with several known amounts of either recombinant GFP or Pfn1 protein, and then immunoblotted with antibodies against Pfn1 (to detect endogenous Pfn1) and GFP (to detect GFP-Pfn1). Densitometric analyses of immunoblots shown in figure 1B revealed that GFP-Pfn1 expressers of MDA-231 has a moderate 1.65-fold overexpression of Pfn1. We next compared the levels of phosphorylated AKT between our two cell lines in regular culture condition (i.e in growth media containing 10% serum) by immunoblotting total cell lysates with different phospho-specific AKT antibodies. AKT, when activated, is known to phosphorylate S9 residue of GSK-3 β (glycogen synthase kinase-3 β) leading to its inactivation. Thus, as a readout for the actual kinase activity of AKT, we also examined the phosphorylation status of GSK-3 β in our cell lines in the same culture condition by immunoblot analyses of cell lysates with a phospho-specific antibody. Figure 1C shows that GFP-Pfn1 overexpressing cells have significantly reduced AKT phosphorylation at both T308 and S473 residues compared to the GFP-expressers. Consistent with these data, we found that the level of phospho-GSK3 β in GFP-Pfn1 expressers was also dramatically less compared to that in control GFP-expressers (figure 1C) thus further confirming an inhibition in the actual kinase activity of AKT in MDA-231 cells as a result of Pfn1 overexpression.

Since AKT is known to be activated in response to growth-factor (EGF, PDGF) stimulation, we conducted many of the subsequent experiments in EGF-stimulation settings in order to determine whether activation of some of the key players involved in the PI3K-AKT signaling cascade is altered by Pfn1 perturbation. This growth factor signaling system is relevant in breast cancer as upregulation of the EGF receptor (EGFR) or its related dimerization partner ErbB2 is associated with breast cancer progression (Reid et al., 2007). We initially confirmed that EGF-induced AKT phosphorylation at T308 and S473 residues were also significantly attenuated in MDA-231 cells when Pfn1 was overexpressed (the total AKT level was similar between the two cell lines - figure 1D). Densitometric analyses of the relative levels of AKT phosphorylation (phospho-AKT/AKT) at different time-points after EGF stimulation revealed approximately 2.5 fold inhibition in AKT phosphorylation as a result of Pfn1 overexpression (figure 1E). We asked whether reduced AKT activation in Pfn1 overexpressing cell line could result from a possible impairment in growth-factor receptor activation. Receptor tyrosine kinases (RTKs), such as EGFR, undergo autophosphorylation upon ligand-binding. We therefore examined the tyrosyl-phosphorylation status of EGFR (represents its active state). In fact, we found that GFP-Pfn1 expressers displayed considerably higher EGFR phosphorylation compared to control GFP cells in response to EGF (inset of figure 1E). These data therefore suggests that dampening of AKT activation in Pfn1 overexpressing cells is likely due to alteration in the regulatory pathways downstream of activated growth-factor receptors.

Since PDK1 is directly responsible for T308 phosphorylation of AKT, our next line of inquiry was to assess whether Pfn1 over-expression has any effect on the activation of PDK1 in our cells. PDK1 requires phosphorylation of S241 residue of its activation loop for its catalytic activity, and there is experimental evidence that PDK1 itself catalyzes autophosphorylation of this site (Casamayor et al., 1999). Figure 1F shows that S241 phosphorylation of PDK1 is constitutive i.e. insensitive to EGF treatment (similar to previous findings reported in the literature (Casamayor et al., 1999)) and is comparable between GFP and GFP-Pfn1 expressers. There was no detectable change in the overall expression level of PDK1 between the two cell lines (figure 1G).

Membrane recruitment of AKT is inhibited by Pfn1 over-expression

Since we failed to see any effect of Pfn1 overexpression on the activation status of PDK1 in MDA-231 cells, we postulated that reduced AKT activation in GFP-Pfn1 expressing cells could be due to impaired access of PDK1 to the phosphorylation sites of AKT. It is known that lipid association of AKT facilitates PDK1's access to its phosphorylation sites thereby stimulating the overall kinase activity of PDK1 toward AKT (Anderson et al., 1998). Therefore, we performed AKT immunostaining to determine whether Pfn1 over-expression alters membrane recruitment of AKT in response to growth-factor stimulation. Our data revealed that that EGF treatment leads to increased plasma membrane staining of AKT (arrows) in GFP-expressing cells as expected; however, EGF-dependent membrane translocation of AKT was dramatically less in Pfn1 overexpressing cells (figure 2A). To quantify EGF-induced membrane translocation of AKT, we performed fluorescence intensity analyses of multiple randomly-constructed line-scans across the cells. Specifically, for every cell, the fluorescence intensity on a line-scan measured at the plasma membrane (I_{membrane}) was normalized by the average fluorescence intensity of the line (I_{total}), and then averaged based on the number of line-scans analyzed to obtain a parameter which we termed as "AKT-translocation index" (ATI – a larger value of ATI corresponds to greater membrane localization of AKT). Finally, the mean value of ATI was computed based on the number of cells analyzed for statistical comparison between the different experimental groups (figure 3B). The mean values of ATI for GFP cells in basal and after 30 minutes of EGF stimulation were found to be (1.1 ± 0.17) and (2.5 ± 0.35) , respectively; the

corresponding values for GFP-Pfn1 expressers were equal to (1.3 ± 0.28) and (1.53 ± 0.27) , respectively. The difference in the mean ATI value between GFP and GFP-Pfn1 expressers in EGF stimulated state was significant ($p < 0.001$). We also scored the frequency of cells displaying significant membrane staining of AKT with or without EGF treatment for each of the two cell lines, the results of which are summarized in figure 3C. Nearly 77% of GFP cells exhibited conspicuous membrane staining of AKT in response to EGF which was a significantly ($p < 0.001$) greater fraction of cells than the corresponding value calculated for GFP-Pfn1 expressers (equal to 40%). Overall, these data clearly demonstrate that Pfn1 overexpression in breast cancer cells inhibits membrane translocation of AKT in response to growth-factor stimulation.

Pfn1 over-expression decreases PIP₃ level in MDA-231 cells

Since AKT is recruited to cell membrane via its PH domain associating with 3'-phosphorylated lipids e.g. PIP₃ and PI(3,4)P₂, we next asked whether Pfn1 overexpression has any effect on the generation of either of these 3'-phosphorylated PPIs in MDA-231 cells. We focused on the generation of PIP₃ since its precursor substrate PIP₂ is the most abundant PPI in cells. We semi-quantitatively evaluated PIP₃ levels in our cell lines under basal vs EGF-stimulated conditions by immunostaining method, representative images of which are shown in figure 3A. Consistent with its stimulation of PI3K-AKT signaling pathway, we found that EGF treatment leads to increased PIP₃ generation in both cell lines; however, the overall intensity of PIP₃ immunofluorescence was conspicuously higher for GFP-expressing cells compared to GFP-Pfn1 expressers for both serum-starved and EGF-stimulated states. Image analyses revealed that there is a nearly 2-fold decrease in the average intensity of PIP₃ immunofluorescence as a result of Pfn1 over-expression in MDA-231 cells (figure 3B). To determine the specificity of antibody-mediated detection of PIP₃, we pre-treated our cells with either 25 μ M LY294002 (an inhibitor of PI3K which should inhibit PIP₃ generation) or its diluent DMSO before challenging with EGF. Immunostaining data showed that LY compound treatment dramatically decreases the average intensity of PIP₃ staining thus validating the specificity of our immunostaining-based findings (figure 3C; the immunoblot in the inset confirms that 25 μ M LY compound treatment is effective in inhibiting PI3K activity of MDA-231 cells as judged from near complete suppression of EGF-induced AKT phosphorylation). To additionally verify reduced PIP₃ generation in Pfn1 overexpressers by biochemical methods, we conducted further experiments in PDGF (a highly potent activator of PI3K/AKT pathway) stimulation settings. Time-course of AKT activation of GFP and GFP-Pfn1 expressing MDA-231 cells in response to PDGF treatment, as shown in figure 3D-i, first confirmed that similar to our observation in EGF-based experiments, PDGF-induced AKT activation is also dramatically suppressed in Pfn1 overexpressers. We next extracted acidic lipids from PDGF-treated GFP and GFP-Pfn1 expressers, spotted on a membrane and probed with a PIP₃-specific detector. Results shown in figure 3D-ii demonstrate that consistent with reduced AKT activation, GFP-Pfn1 overexpresser present dramatically reduced PIP₃ level compared to control GFP cell line (absence of chemiluminescence signal in PI and PI3P spotted areas confirms specificity of PIP₃ detection). These biochemical data validate our immunostaining-based findings.

Pfn1 over-expression suppresses AKT activation in a PTEN-dependent manner

Since PIP₃ is generated by the action of PI3K and this pathway is negatively regulated primarily by the action of PTEN phosphatase in non-hematopoietic cells, we next interrogated whether either of these regulatory pathways is altered in MDA-231 cells when Pfn1 is overexpressed. Activation of receptor-tyrosine kinases causes tyrosyl-phosphorylation of the regulatory p85 subunit of PI3K and this is a necessary step for PI3K activation leading to PIP₃ generation. We therefore probed the status of p85 phosphorylation of our two cell lines in basal versus EGF-stimulated conditions by immunoblotting anti-

phosphotyrosyl immunoprecipitates from cell extracts with a PI3K p85-specific antibody. Our data showed increased tyrosine phosphorylation of p85 in response to EGF stimulation as expected. Also, consistent with increased EGFR activation, we found slightly elevated tyrosyl-phosphorylation of p85 subunit in GFP-Pfn1 expressing cells compared to control cells (figure 4A). Immunoblot of total cell lysates showed that the expression level of p85 subunit of PI3K is comparable between our two cell lines (figure 4A). However, when we probed the PTEN expression status of our cell lines, we found significantly higher PTEN level in GFP-Pfn1 expressing cells compared to GFP cells (figure 4B). RT-PCR data showed comparable levels of PTEN transcripts between the two cell lines suggesting that PTEN upregulation in Pfn1 overexpressing cells occurs by post-transcriptional mechanisms (figure 4C).

Since PTEN inhibits AKT activation, we asked whether PTEN upregulation could be responsible for attenuation of AKT-activation in GFP-Pfn1 expressing cells. To address this question, we suppressed PTEN expression in our cell lines by siRNA treatment (as control, each of the two cell lines was also transfected with non-targeting control-siRNA) and then re-evaluated phospho-AKT levels. Figure 4D shows that PTEN expression can be significantly downregulated within 72 hours after siRNA treatment. Control siRNA treatment did not alter the phospho-AKT level differential between GFP and GFP-Pfn1 expressers as expected. For GFP cells, there was only a minor increase in phospho-AKT level when PTEN expression was further knocked-down. This was most likely due to very low initial expression level of PTEN in this cell line. However, when we suppressed PTEN expression in GFP-Pfn1 expressing MDA-231 cells, AKT phosphorylation markedly increased and became comparable to the level observed for GFP-expressing cells. These data clearly demonstrate that PTEN upregulation accounts for reduced AKT activation in Pfn1-overexpressing MDA-231 cells.

Finally, we asked whether Pfn1 overexpression induced PTEN upregulation and reduced AKT phosphorylation is only specific to MDA-231 cells or applicable to other breast cancer cell line. We previously reported generation of stable transfectants of BT-474 breast cancer cell line overexpressing either GFP-Pfn1 (has a 2-fold overexpression of Pfn1) or GFP (Roy and Jacobson, 2004). When we probed PTEN and pAKT levels between these two sublines of BT474, we found a similar PTEN upregulation and reduced AKT phosphorylation in Pfn1 overexpressers, thus demonstrating that our findings are not cell line specific.

DISCUSSION

Hyperactivation of PI3K-AKT signaling that promotes cell proliferation and survival has been implicated in a wide variety of cancers. Phosphorylation of AKT (closely parallels AKT activation) is elevated in highly aggressive tumors and thus serves as a prognostic marker for tumor grade (Perez-Tenorio and Stal, 2002). Loss of expression or mutation of PTEN (a feature commonly observed in various carcinomas) leading to increased AKT phosphorylation promotes tumorigenicity in mouse models, and there is a strong inverse correlation of PTEN level with the tumor stage in human cancers (Salmena et al., 2008). The data presented in this study reveal a novel finding that over-expression of Pfn1, which has been previously shown to elicit a strong anti-tumor effect on breast cancer cells, leads to a significant reduction in AKT activation in breast cancer cells, and this effect is mediated through PTEN upregulation. Given the importance of PI3K-AKT signaling in cancer development, our results could provide for the first time insights into the molecular basis of Pfn1's tumor-suppressive actions.

Using EGF-stimulation as a model system for studying AKT activation, we showed that Pfn1 over-expression does not lead to impaired activation of any of the positive regulators of

AKT activation including EGFR itself, PI3K or PDK1. On the contrary, we found higher EGFR activation in Pfn1 over-expressing cells compared to control GFP cells, as judged by the level of its tyrosine-phosphorylation of EGFR in response to EGF. How Pfn1 overexpression increases EGFR activation in MDA-231 cells is not clear. Since ligand (EGF)-induced clustering of EGFR is required for its activation, one likely explanation is that Pfn1 overexpression may augment EGFR activation by somehow increasing the clustering ability of EGFR. Since Pfn1 binds to multiple molecules of PPIs, it may increase EGFR clustering via promoting lipid aggregates, a model that has been recently proposed in the literature based on biophysical evidence (Moens and Bagatolli, 2007).

Our finding of lower PIP₃ levels is also consistent with impaired membrane targeting and reduced phosphorylation of AKT in Pfn1 overexpressing cells. This effect is most likely the direct result of Pfn1-induced PTEN upregulation since silencing PTEN expression fully rescues the inhibition of AKT phosphorylation in Pfn1-overexpressing cells. Our data showed that PTEN upregulation in Pfn1-overexpressing cells occurs at a post-transcriptional level. How this happens is not clear although at least two possibilities exist which need to be explored in the future. First, lipid binding influences PTEN stability (Das et al., 2003). Since literature has implicated that Pfn1 may play a role in controlling PIP₂ turnover (Goldschmidt-Clermont et al., 1990), PTEN stability can be altered by Pfn1 overexpression. Second, there has been evidence of contact-inhibition induced PTEN upregulation, at least, in endometrial carcinoma cells thus suggesting cell-cell adhesion dependent modulation of PTEN (Uegaki et al., 2006). Interestingly, we previously demonstrated that both MDA-231 and BT474 breast cancer cell lines display some degree of cell clustering when overexpress Pfn1 therefore suggesting increased cell-cell interaction (Roy and Jacobson, 2004; Zou et al., 2007), and this may also account for Pfn1 overexpression-induced PTEN upregulation. As a future study, it will be also important to determine whether Pfn1-dependent PTEN upregulation and suppression of AKT activation are i) generalized phenomena in other variants of adenocarcinoma, and ii) accounts for growth inhibition and impaired tumorigenicity of Pfn1-overexpressing breast cancer cell lines.

Finally, PTEN status has been shown to be a critical determinant of apoptotic sensitivity of cancer cells to chemotherapeutic agents. Cancer cells with low levels of PTEN expression are resistant to chemotherapy and conversely, over-expression of PTEN sensitizes cancer cells to chemotherapeutic-drug induced apoptosis (Frattini et al., 2007; Jiang et al., 2007; Yan et al., 2006). Thus, it will be interesting to further explore whether Pfn1 overexpression has a negative impact on the survival characteristics of breast cancer cells.

Acknowledgments

The authors wish to thank Vaishnavi Panchapakesa for technical assistance. This study was funded by a grant from the National Cancer Institute (R01-CA108607) to P.R.

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Fig. 1. Effect of Pfn1 overexpression on AKT activation in MDA-231 cells

A) GFP and Pfn1 immunoblots of total cell lysates extracted from GFP and GFP-Pfn1 expressers of MDA-231. **B)** GFP and Pfn1 immunoblots of GFP-Pfn1 expresser lysate with known amounts of purified recombinant GFP (rGFP) or Pfn1 (rPfn1) proteins, respectively, were analyzed for quantitative estimation of the expression level of GFP-Pfn1 relative to that of endogenous Pfn1. **C)** Comparative profiles of phospho-AKT (T308 and S473), phospho-GSK3β (S9) between GFP and GFP-Pfn1 expressing MDA-231 cells in regular culture condition. **D)** Phospho-AKT profiles (T308 and S473) of GFP and GFP-Pfn1 expressers under basal and EGF-stimulated conditions confirm Pfn1-overexpression induced inhibition of AKT phosphorylation. AKT immunoblot shows comparable expression level of AKT between our two cell lines. **E)** Quantitative representation of the ratio of phospho-AKT (S473) to total AKT for GFP and GFP-Pfn1 expressers at different time points after EGF stimulation (data summarized from 3 independent experiments and * denotes $p < 0.05$). The inset shows a representative phospho-EGFR immunoblot from one such experiment revealing higher tyrosine phosphorylation of EGFR in Pfn1 overexpressing cells. **F and G)** Immunoblots reveal that S241 phosphorylation (*panel F*) as well as the total expression level (*panel G*) of PDK1 for GFP and GFP-Pfn1 expressers are similar. PDK phosphorylation is insensitive to EGF treatment (*panel D*). For all experiments, GAPDH immunoblots serve as the loading controls.

Fig. 2. Membrane targeting of AKT is inhibited by Pfn1 overexpression

A) AKT immunostaining shows much more pronounced EGF-dependent membrane targeting of AKT (marked by arrow) in GFP-expressing cells compared to Pfn1-overexpressers (scale bar – 20 μ m). B) Fluorescence intensity analyses of AKT-immunostaining images are summarized in a bar graph to reveal the relative ATI values of GFP and GFP-Pfn1 expressers in basal and EGF-stimulated conditions (n - number of analyzed cells pooled from 2 independent experiments). C) A bar graph summarizes the % of cells displaying membrane staining of AKT. These data are summarized from 2 independent experiments and * indicates $p < 0.001$.

Fig. 3. Pfn1 overexpression suppresses PIP₃ generation

A) PIP₃ immunostaining images of GFP and GFP-Pfn1 expressers at basal and 5 minutes after EGF stimulation (scale bar – 20 μ m). **B)** Quantification of the average intensity of PIP₃ staining of the two cell lines with or without EGF treatment (these data were normalized to the intensity value calculated for GFP cells in basal state; ‘n’ denotes number of cells for each experimental group pooled from 2 independent experiments). **C)** Quantification of the average intensity of PIP₃ staining of GFP and GFP-Pfn1 expressers which were first pretreated with either 25 μ M LY294002 or DMSO (vehicle control) for 30 minutes and then stimulated with EGF for 5 minutes shows a dramatic decrease in the intensity of PIP₃ staining as a result of LY treatment (these data were normalized to the intensity value calculated for GFP cells under DMSO treatment; ‘n’ denotes number of cells for each experimental group). The inset shows a phospho-AKT (S473) immunoblot of GFP-expressers under both basal and EGF stimulation (30 min) in the presence of either 25 μ M LY compound or DMSO (AKT immunoblot serves as the loading control). LY compound treatment completely suppresses EGF-dependent AKT phosphorylation thereby confirming inhibition of PI3K activity. **D) i)** Time-course of AKT phosphorylation of GFP and GFP-Pfn1 expressing MDA-231 cells in response to PDGF treatment show downregulation of AKT activation in Pfn1 overexpressers (GAPDH blot serves as the loading control). **ii)** Lipids extracted from GFP and GFP-Pfn1 expressers following 5 min of PDGF stimulation, when spotted on a membrane and probed with PIP₃-specific detection system, show significantly reduced PIP₃ level in GFP-Pfn1 overexpresser compared to GFP cell line. Known amounts of PIP₃ (positive control) and other PIs (PI, PI3P – negative control) were pre-spotted on the membrane to demonstrate the specificity of the detection procedure. The dotted circles represent the spotted area. For all analyses * represents $p < 0.05$.

Fig. 4. Pfn1 overexpression inhibits AKT activation in a PTEN-dependent manner

A) Phosphotyrosine immunoprecipitates from GFP and GFP-Pfn1 expressers, either in basal or EGF stimulated (10 minutes) conditions, were probed with antibodies specific for p85 and EGFR to reveal their tyrosine phosphorylation status. EGF-induced tyrosine phosphorylation of both p85 and EGFR were found to be higher in Pfn1 over-expressing cells. Immunoblot of total cell lysates shows comparable expression level of p85 between the two cell lines. **B)** Pfn1-overexpressing cells show marked increase in PTEN level. **C)** RT-PCR data show similar levels of PTEN transcript between GFP and GFP-Pfn1 expressers (GAPDH RT-PCR products serve as the loading control). **D)** PTEN immunoblot of total extracts from siRNA (either control or PTEN-specific)-transfected cells shows strong silencing of PTEN expression. Phospho-AKT (S473) immunoblot demonstrates that PTEN silencing rescues the inhibition of AKT phosphorylation in GFP-Pfn1 expressers. **E)** Comparative profiles of PTEN and pAKT (T308) between GFP and GFP-Pfn1 overexpressers of BT474 breast cancer cells. For all experiments, GAPDH blots serve as the loading controls.