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Identification of upregulated phosphoinositide 3-kinase γ as a target to suppress breast cancer cell migration and invasion

Yan Xie^a, Peter W. Abel^a, Joseph K. Kirui^a, Caishu Deng^b, Poonam Sharma^b, Dennis W. Wolff^a, Myron L. Toews^c, and Yaping Tu^{a,*}

^aCreighton University School of Medicine, Department of Pharmacology, Omaha, NE 68178

^bDepartment of Pathology, Omaha, NE 68178

^cUniversity of Nebraska Medical Center, Department of Pharmacology and Experimental Neuroscience, Omaha, NE 68198

Abstract

Metastasis is the major cause of breast cancer mortality. We recently reported that aberrant G-protein coupled receptor (GPCR) signaling promotes breast cancer metastasis by enhancing cancer cell migration and invasion. Phosphatidylinositol 3-kinase γ (PI3K γ) is specifically activated by GPCRs. The goal of the present study was to determine the role of PI3K γ in breast cancer cell migration and invasion. Immunohistochemical staining showed that the expression of PI3K γ protein was significantly increased in invasive human breast carcinoma when compared to adjacent benign breast tissue or ductal carcinoma *in situ*. PI3K γ was also detected in metastatic breast cancer cells, but not in normal breast epithelial cell line or in non-metastatic breast cancer cells. In contrast, PI3K isoforms α , β and δ were ubiquitously expressed in these cell lines. Overexpression of recombinant PI3K γ enhanced the metastatic ability of non-metastatic breast cancer cells. Conversely, migration and invasion of metastatic breast cancer cells were inhibited by a PI3K γ inhibitor or by siRNA knockdown of PI3K γ but not by inhibitors or siRNAs of PI3K α or PI3K β . Lamellipodia formation is a key step in cancer metastasis, and PI3K γ blockade disrupted lamellipodia formation induced by the activation of GPCRs such as CXC chemokine receptor 4 and protease-activated receptor 1, but not by the epidermal growth factor tyrosine kinase receptor. Taken together, these results indicate that upregulated PI3K γ conveys the metastatic signal initiated by GPCRs in breast cancer cells, and suggest that PI3K γ may be a novel therapeutic target for development of chemotherapeutic agents to prevent breast cancer metastasis.

Keywords

PI3K γ ; breast cancer; metastasis; migration and invasion; lamellipodia

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*To whom correspondence should be addressed: Yaping Tu, Ph.D., Department of Pharmacology, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178, USA, 001-402-280-2173 (Phone), 001-402-280-2142 (Fax), Yat60399@creighton.edu.

Conflict of interest

The authors declare no conflict of interest.

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1. Introduction

Recent preclinical and clinical studies have demonstrated that specific G-protein coupled receptor (GPCR) systems are excessively activated in malignant breast cancer due to over-expression of receptors [1–4], abnormally elevated levels of ligands for GPCRs [4–6] and/or down-regulation of their regulators [7], which contributes to the progression and spread of breast cancer [8]. For example, signaling initiated by CXC chemokine receptor 4 (CXCR4) and protease-activated receptors (PARs) on breast cancer cells drives cancer cells to migrate and invade through surrounding tissues and spread to distant organs [5,9]. Unfortunately, clinical trials with drugs inhibiting specific GPCR activation show limited efficacy, presumably because metastasis could be driven by several different classes of GPCR simultaneously, thereby generating metastatic signal redundancy.

GPCRs convey signals via heterotrimeric G-proteins (classified into G_s , G_i , G_q , G_{12}) in the form of activated $G\alpha$ -GTP and $G\beta\gamma$ subunits. We recently demonstrated that $G\beta\gamma$ released from G_i -proteins promotes migration and invasion of metastatic breast cancer cells by generating the lamellipodia protrusions at the leading edge of migrating cancer cells [10], suggesting that blockade of $G\beta\gamma$ could attenuate breast cancer metastasis. However, $G\beta\gamma$ cannot be blocked indiscriminately because of its diverse physiological roles. Therefore, the challenge is to target $G\beta\gamma$ effectors that are vital to breast cancer metastasis but inconsequential for physiologically normal cells.

The most studied type I phosphatidylinositol 3-kinases (PI3Ks), including α , β , γ and δ , play a pivotal role in numerous cellular functions [11]. PI3K γ is especially intriguing because it is normally expressed primarily in hematopoietic cells [12], which have a physiological need to migrate. In addition, PI3K γ is only activated by $G\beta\gamma$ following stimulation of GPCRs, whereas PI3K α , β and δ are stimulated by receptor tyrosine kinases [11, 13]. In fact, GPCR-dependent activation of PI3K γ in neutrophils causes its accumulation at the leading edge of migrating cells, which is a critical determinant of cell migration [12,14]. Although somatic mutations of PI3K α are very common in cancers [15,16], and may promote cancer cell growth and invasion in colorectal and breast cancer [17,18], the contribution of PI3K γ to human cancer is much less clear with different studies showing conflicting results [19–22]. Brazzatti et al. [22] recently reported that knockdown of PI3K γ inhibited lung colonization of human breast cancer MDA-MB-231 cells in xenografts and suppressed primary tumor growth, metastases and lung colonization caused by mouse 4T1.2 mammary carcinoma allografts. While this suggests an important role for PI3K γ in breast cancer tumor growth and metastasis, these studies did not explore the molecular mechanisms associated with PI3K γ signaling or whether PI3K γ protein levels were correlated with the metastatic potential of various human breast cancer cell lines or human breast cancer specimens.

In the present study, we show that PI3K γ is aberrantly expressed in invasive human breast carcinoma and its expression level correlates with the metastatic potential of established breast cancer cell lines. Most importantly, we show that silencing PI3K γ with its siRNA or treatment with a PI3K γ -selective inhibitor, but not with inhibitors or siRNAs of PI3K α and β , attenuates lamellipodia formation and suppresses migration and invasion of breast cancer cells. Thus, targeting dysregulated PI3K γ may provide a novel strategy for development of chemotherapeutic agents to suppress breast cancer metastasis.

2. Materials and Methods

2.1. Cell lines, reagents and plasmids

MCF-10A, MCF-7, T47D, MDA-MB-231 and MDA-MB-436 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). MDA-MB-231 and -436 cells were cultured in DMEM with 10% fetal bovine serum (FBS). MCF-7 cells were cultured in IMEM and 10% FBS with 10 μ g/ml insulin. MCF-10A cells were grown in MEM with additives (ATCC), and T47D cells were maintained in RPMI 1640 with 10% FBS.

N-((1E)-(6-bromoimidazo-[1,2-a]pyridin-3-yl)methylene)-N'-methyl-N''-(2-methyl-5-nitrobenzene)sulfonohydrazide, HCl (PI3K α inhibitor VIII or PIK-75, IC₅₀ = 5.8, 1300, and 78 nM for lipid kinase activity of PI3K α , β and γ isoforms, respectively) [23], 7-methyl-2-(morpholin-4-yl)-9-(1-phenylaminoethyl)-pyrido[1,2-a]-pyrimidin-4-one (PI3K β inhibitor VI or TGX-221, IC₅₀ = 0.005, 5, and 3.5 μ M for PI3K β , α and γ isoforms, respectively) [24], 5-(2,2-difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione (PI3K γ inhibitor II or AS-604850, IC₅₀ = 0.25, 4.5, and > 20 μ M for PI3K γ , α and β) [25], and a broad spectrum PI3K inhibitor 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) were obtained from EMD Biosciences (San Diego, CA). Rabbit PI3K α antibody and PI3K γ antibody were from Cell Signaling Technology (Danvers, MA) and IRDye800-labeled anti-rabbit IgG was from LI-COR Bioscience. Rabbit PI3K β antibody and mouse PI3K δ antibody were purchased from Millipore (Billerica, MA) and Santa Cruz (Santa Cruz, CA), respectively. ON-TARGETplus SMARTpool siRNAs targeting human PI3K α , PI3K β or PI3K γ , were purchased from Thermo Scientific Dharmacon (Lafayette, CO). Epidermal growth factor (EGF) was from BD Biosciences (San Jose, CA). CXCL12 was from R&D Systems (Minneapolis, MN) and protease activated receptor 1 (PAR1) agonist TFLLR was from Peptides International (Louisville, KY). Unless indicated otherwise, remaining reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

The pEYFP-PI3K γ -CAAX plasmid encoding YFP-tagged PI3K γ with the CAAX motif for constitutive association with the plasma membrane was a gift from Dr. Bernd Nürnberg (University of Tübingen, Germany) [26]. PI3K γ consists of the catalytic subunit p110 γ and two regulatory subunits, p101 and p84 [27–29]. A constitutively membrane targeted PI3K γ (p110 γ -CAAX) could compensate for the lack of its regulatory subunits [26].

2.2. Conventional RT-PCR and quantitative real-time RT-PCR analysis

The procedures for conventional RT-PCR and real-time RT-PCR were described previously [30]. β -actin was used as the internal control. The PI3K γ primers resulting in a 98-bp product are: 5'-ttgtgatgggaactctgga-3', 5'-ggttgtgtgat gacgaagg-3'. The conventional RT-PCR cycling conditions were 1 cycle at 94°C for 3 min; 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, followed by 1 cycle of 72°C for 7 min.

2.3. Western blot analysis

Protein was extracted from cultured cells using 1x RIPA lysis buffer (Santa Cruz). Samples (40 μ g) were electrophoresed and subjected to Western blot using β -actin and PI3K antibodies as we previously described [31]. Images were captured with an Odyssey (LI-COR Biosciences).

2.4. Immunofluorescence staining

MDA-MB-231 cells and MCF-7 cells transfected with control vector or vector encoding PI3K γ were seeded on coverslips overnight, and then fixed and stained with PI3K γ

antibody as previously described [31]. To investigate the effects of different inhibitors on lamellipodia formation, serum-starved MDA-MB-231 cells seeded on coverslips were pretreated with PI3K α and PI3K β inhibitors or PI3K γ inhibitor for 1 h, and then stimulated without or with TFLLR (25 μ M), CXCL12 (50 ng/ml) or EGF (5 ng/ml) for 30 min at 37°C. F-actin was visualized with rhodamine-labeled phalloidin (Cytoskeleton, Denver, CO) as previously described [7,10]. Lamellipodia, the flattened F-actin-rich leading edge of migrating cells, were outlined and measured in length using the Image-Pro Plus software from Media Cybernetics, Inc. (Rockville, MD). The summed length of lamellipodia was expressed as a percentage of total cell circumferences [7].

2.5. Immunohistochemistry analysis of human breast tissues

Archived formalin-fixed, paraffin-embedded human breast tissue blocks were from the Creighton University Department of Pathology as approved by the Creighton University Institutional Review Board. Immunohistochemistry was performed as we previously reported [7], using a rabbit PI3K γ antibody (Cell Signaling Technology). The negative control used non-immune rabbit IgG as the primary antibody. Expression levels of PI3K γ protein were graded using a four-tier system from 0 (negative) to 3 (high) based on overall staining intensity by two pathologists, independently. A total of 40 sets of pair-matched patient samples were included.

2.6. RNA interference and overexpression of PI3K γ

MDA-MB-231 cells (1×10^6 in 100 μ l) were electroporated with 300 nM of control siRNA (negative control #1 siRNA, Ambion) or ON-TARGETplus SMARTpool siRNA for human PI3K α , PI3K β or PI3K γ using Nucleofector kits with an Amaxa Nucleofector System (Lonza) (Allendale, NJ). Cells were seeded on 6-well plates. The following day, adherent MDA-MB-231 cells were re-transfected with the same siRNA using Lipofectamine 2000 as previously described [32]. Two days later, cells were harvested for Western blot analysis of PI3K α , PI3K β or PI3K γ expression or migration and invasion assays.

MCF-7 cells (2×10^6) electroporated with 4 μ g of plasmid encoding YFP or YFP-tagged PI3K γ were seeded on 6-well plates. Stable MCF-7 cell lines were established with standard methods, and selected with G-418 (800 μ g/ml) for three weeks as we previously described [7]. Positive clones were amplified and verified by Western blotting for PI3K γ expression.

2.7. Transwell invasion and migration assays

Matrigel invasion assays were carried out at 37°C for 16h using 24-well Transwell inserts (Corning) (Tewksbury, MA) coated with 30 μ g of Matrigel (BD Biosciences) as we previously described [7,10]. Transwell cell migration assays were performed similarly without Matrigel, but only for 5h for MDA-MB-231 and MDA-MB-436 cells or 12 h for MCF-7 cells. Conditioned medium (CM) from NIH-3T3 cells was collected and used as a chemoattractant as previously reported [5,7].

2.8. 3D invasion assay

A collagen solution was prepared by mixing NIH 3T3 CM and collagen (2.5 mg/ml) at pH 7.3. Collagen gels were prepared by pipetting 100 μ l of this solution into Transwell insets, allowing it to polymerize, and then equilibrating at 37 °C with 5% CO₂. MCF-7 cells (50,000/well) were added to the upper chamber in 200 μ l serum free DMEM while the lower chamber received 0.6 ml of NIH-3T3 CM. After 48 h, cells were fixed in 3% glutaraldehyde in PBS, stained with 0.1% toluidine blue in 30% methanol and destained. Thin cross-sections were photographed using an upright Nikon Eclipse 80i microscope. Three random fields at 100x magnification were selected and the number of invading cells

per high power field (HPF) was counted manually. Data are presented as average numbers of invading cells per HPF.

2.9. Statistical analysis

Tissue immunohistochemistry staining scoring was analyzed with a Kruskal–Wallis test and Dunn posttest. Other results are mean $\pm$ S.E. of at least three determinations, with statistical comparisons done by Student's *t*-test with $p < 0.05$ considered to be significant.

3. Results

3.1. PI3K γ is upregulated in human breast tumors

We first performed immunohistochemistry staining for PI3K γ protein in histologically benign and neoplastic cells of 40 archival human breast tissue blocks using a PI3K γ specific antibody. PI3K γ protein expression was elevated in ductal carcinoma *in situ* and invasive breast carcinoma when compared to adjacent benign mammary tissue (Fig. 1). Expression levels of PI3K γ were graded from 0–3 based on overall staining intensity. Table 1 shows that average PI3K γ staining intensities in ductal carcinoma *in situ* were increased compared to normal or adjacent normal breast tissues (0.73 ± 0.17 vs. 0.19 ± 0.08 , $p < 0.01$). PI3K γ protein expression in invasive breast carcinoma was significantly higher than that in ductal carcinoma *in situ* (1.60 ± 0.18 vs. 0.73 ± 0.17 , $p < 0.001$). These data show that up-regulation of PI3K γ protein is correlated with the degree of tumor invasiveness, and suggest possible relevance to aberrant migration and invasion of breast cancer cells.

3.2. PI3K γ is overexpressed in metastatic breast cancer cells

Quantitative real-time PCR and Western blot analysis were further performed to investigate the levels of PI3K γ mRNA and protein in established human breast cancer cell lines. As shown in Fig. 2A and 2B, PI3K γ mRNA and protein were almost undetectable in an immortalized human breast epithelial cell line (MCF-10A) or in non-metastatic breast cancer MCF-7 and T47D cells, but were significantly increased in metastatic breast cancer MDA-MB-231 and MDA-MB-436 cells. Immunofluorescent staining also showed expression of PI3K γ protein in the cytosol of MDA-MB-231 cells but not in MCF-7 cells unless the MCF-7 cells were transfected with a recombinant PI3K γ plasmid (Fig. 2C). In contrast, other type I PI3Ks were ubiquitously expressed in these breast cell lines, except that PI3K α was not detected in MDA-MB-436 cells (Fig. 2B).

3.3. Blocking PI3K γ activity attenuates metastatic breast cancer cell migration and invasion

Directed cell migration and invasion are critical steps in the tumor metastasis cascade [5]. NIH-3T3 fibroblast CM contains numerous chemokines and growth factors, and is widely used for inducing cancer cell migration and invasion in Transwell assays [5,7]. Thus, the effect of PI3K inhibitors on the NIH-3T3 CM-stimulated migration and invasion of metastatic breast cancer MDA-MB-231 and MDA-MB-436 cells was examined (Fig. 3). PI3K γ -selective inhibitor attenuated MDA-MB-231 cell migration in a dose-dependent manner with a maximum inhibition of 60% and IC_{50} of $1.2 \pm 0.3 \mu M$ (Fig. 3A, inset). $3 \mu M$ PI3K γ -selective inhibitor or $10 \mu M$ LY294002 (LY), a broad-spectrum PI3K inhibitor [33], inhibited migration and invasion of both MDA-MB-231 and -436 cells by 50–60% (Fig. 3A and 3B). In contrast, inhibitors of PI3K α or β at concentrations 5-fold or 20-fold higher than the IC_{50} reported for their primary targets *in vitro* [23,24] had no significant effect.

CXC chemokine receptor 4 (CXCR4) plays an important role in breast cancer metastasis [7,34,35], and initiates signaling through G_i -proteins when its ligand, CXCL12, is bound [36]. As shown in Fig. 3C, the PI3K γ inhibitor attenuated CXCL12-stimulated cell

migration in a dose-dependent manner with a maximum inhibition of 80% and IC_{50} of $2.7 \pm 0.3 \mu\text{M}$. In contrast, the PI3K γ inhibitor only had a very modest effect (<20%) on cell migration stimulated by epidermal growth factor (EGF), a process dependent on the EGF receptor tyrosine kinase. These data suggested that upregulated PI3K γ has an important role in GPCR-stimulated migration and invasion of metastatic breast cancer cells.

3.4. siRNA-mediated knockdown of PI3K γ , but not PI3K α or PI3K β , attenuates metastatic breast cancer cell migration and invasion

To exclude any non-specific effects of PI3K γ inhibitor, siRNA was used to individually silence the endogenous PI3K α , PI3K β or PI3K γ expression in metastatic breast cancer cells (Fig 3D, top panel). Dual-transfection of a PI3K γ -specific siRNA into MDA-MB-231 cells reduced PI3K γ protein levels by $70 \pm 11\%$ when compared to a negative control siRNA. Knockdown of endogenous PI3K γ attenuated migration and invasion of MDA-MB-231 cells in response to NIH-3T3 CM by about 60% (Fig. 3D). In contrast, 80% reduction of endogenous PI3K α or PI3K β by their specific siRNAs (Fig. 3D, top panel) had no significant effect on migration and invasion of MDA-MB-231 cells.

3.5. PI3K γ blockade attenuates GPCR-dependent lamellipodia formation of metastatic breast cancer cells

Formation of lamellipodia, the flattened F-actin-rich leading edge of migrating cells, is required for cancer cell migration and invasion during metastasis [37]. The effect of PI3K γ blockade on CXCR4-dependent lamellipodia formation was investigated. As shown in Fig. 4, CXCL12 increased lamellipodia extent by 2.7-fold, which was blocked by the PI3K γ inhibitor but not by inhibitors of PI3K α and β . In addition, the PI3K γ inhibitor, but not inhibitors of PI3K α and β , also blocked lamellipodia formation induced by TFLLR, an agonist of G-protein coupled protease-activated receptor 1 (PAR1) known to promote breast cancer cell invasion [5,7]. In contrast, the 3-fold increase in lamellipodia formation in response to EGF was blocked by inhibitors of PI3K α and β but not by the PI3K γ inhibitor. These data suggest that PI3K γ functions as a key molecule linking GPCRs to lamellipodia formation in metastatic breast cancer cells.

3.6. PI3K γ overexpression enhances MCF-7 cell migration and invasion in 3D collagen matrices

Non-metastatic breast cancer MCF-7 cells were further used as an *in vitro* model to investigate the effect of exogenously expressed PI3K γ on breast cancer metastasis. MCF-7 cells were stably transfected with vector encoding YFP or YFP-tagged, membrane-targeted PI3K γ protein. Transwell assays with these cells indicated that PI3K γ overexpression increased migration and invasion of MCF-7 cells by about 3-fold (Fig. 5A).

Because tumor cell invasion depends on proteolysis and dispersion through 3D extracellular matrix barriers largely composed of type I collagen [38], invasive abilities of these MCF-7 stable cell lines were compared in an *in vitro* 3D invasion assay [39]. Cells were seeded on top of a gel composed of type I collagen. The cells first adhered, forming a monolayer, and then invaded the 3D gel in response to chemoattractants. As shown in Fig. 5B and 5C, NIH-3T3 CM stimulated invasion of MCF-7 control cells expressing YFP into 3D collagen matrices, which was inhibited by a broad-spectrum PI3K inhibitor LY but not by the PI3K γ inhibitor. Compared to MCF-7 control cells, the number of invaded MCF-7/YFP-PI3K γ cells was 2.5-fold higher, and this increase was largely abolished in the presence of the PI3K γ inhibitor or LY.

4. Discussion

Although several lines of evidence indicate a role of PI3K γ in pancreatic cancer and breast cancer [19–22], our results provide the first detailed evidence that expression levels of PI3K γ are correlated with the metastatic potential of established human breast cancer cell lines. In contrast, PI3K α , β and δ were ubiquitously expressed in these breast cell lines. More importantly, our data showed that PI3K γ protein expression was modestly increased in noninvasive ductal carcinoma *in situ* as compared to normal breast tissues but was markedly increased in invasive breast carcinoma. These results suggest that PI3K γ upregulation may be an early event in the development of an invasive breast cancer phenotype.

PI3K γ is well established as a focal point for activation when chemokine sensors are stimulated, and thus it plays a key role in the directional migration of leukocytes toward chemoattractants [40]. A lack of PI3K γ has been shown to block the ability of neutrophils and macrophages to respond to stimuli from several GPCRs [41]. Similarly, chemokines and their receptors are involved in cancer metastasis [1], raising the possibility that mechanisms underlying cancer epithelial cell migration and invasion are similar to those underlying leukocyte trafficking. Thus, several breast cancer cell lines were selected as a model system to establish the role of PI3K γ in breast cancer metastasis. Our data showed that blocking endogenous PI3K γ in metastatic MDA-MB-231 and MDA-MB-436, either with the selective PI3K γ inhibitor or by siRNA-mediated knockdown, attenuated migration and invasion abilities of these cells. Conversely, overexpression of recombinant PI3K γ in non-metastatic breast cancer MCF-7 cells that lacks endogenous PI3K γ expression, significantly increased cell migration and invasion in transwell assays. Similar results were observed from a 3D invasion assay demonstrating that overexpression of PI3K γ enhanced cellular invasion of MCF-7 cells into collagen matrices. It should be noted that the PI3K γ -selective inhibitor used in our studies attenuated metastatic breast cancer cell migration with an IC₅₀ of $1.2 \pm 0.3 \mu\text{M}$. Loss of metastatic abilities of breast cancer cells after treatment with $3 \mu\text{M}$ of the PI3K γ inhibitor might also be due to partial inhibitory effects on PI3K α (*in vitro* IC₅₀ = $4.5 \mu\text{M}$). However, selective inhibitors of PI3K α or β at concentrations much higher than the IC₅₀ reported for their primary targets *in vitro* had no significant effects. More importantly, siRNA knockdown of PI3K α or β did not attenuate metastatic abilities of breast cancer cells. Thus, these data together suggest that upregulated PI3K γ , but not PI3K α or β , plays an important role in breast cancer invasiveness and metastasis.

The molecular mechanism underlying PI3K γ -facilitated breast cancer cell migration and invasion was also characterized. Lamellipodia are a key structure required for cancer cell migration and invasion during metastatic progression [37], and chemoattractant activation of receptors triggers reorganization of the actin cytoskeleton to form lamellipodia [7,42]. The PI3K γ inhibitor, but not PI3K α and β inhibitors, blocked lamellipodia formation of MDA-MB-231 cells induced by agonists of G-protein coupled CXCR4 and PAR1. In contrast, the PI3K γ inhibitor had little effect on lamellipodia formation and cell migration induced by EGF, a process not dependent on G-proteins, but mediated by EGF receptor tyrosine kinase. It is well established that the small G-protein Rac controls lamellipodia formation in cells. Aberrant Rac activity is found in some human cancers and mediates cancer metastasis [43,44] in part, by generating lamellipodia protrusions at the leading edge of migrating cancer cells. Inhibition of Rac activity blocked breast cancer cell migration and invasion [44]. Rac acts as a molecular switch and can cycle between an inactive GDP-bound form and an active GTP-bound form catalyzed by guanine nucleotide exchange factors (GEF) [45]. Interestingly, in many cell types, PI3K activity is necessary and sufficient for receptor-driven Rac activation via RacGEFs [46]. Thus, our data suggest that PI3K γ may function as

a key molecule specifically linking GPCRs to Rac-dependent lamellipodia formation to promote breast cancer cell migration and invasion.

In summary, our data are the first to establish a correlation between PI3K γ expression and the metastatic potential of human breast cancer cell lines and breast cancer specimens. More importantly, either blockade or knockdown of PI3K γ was sufficient to inhibit the migration and invasion of metastatic breast cancer cells, whereas overexpression of recombinant PI3K γ in non-metastatic breast cancer cells increased their migratory and invasive abilities. These findings support a crucial role for upregulated PI3K γ in breast cancer metastasis. We recently demonstrated that G $\beta\gamma$ released from G-proteins following stimulation of GPCRs promotes migration and invasion of breast cancer cells. However, G $\beta\gamma$ cannot be blocked indiscriminately because of its vital physiological role. Since PI3K γ is only activated by G $\beta\gamma$ and was not expressed in normal breast tissue but upregulated in invasive breast cancer, PI3K γ may provide a desirable target for blocking metastatic GPCR signals in breast cancer cells. A further study of *in vivo* effects of PI3K γ inhibitors on breast cancer metastasis will potentially lead to new avenues for cancer drug design. In addition, PI3K γ protein expression in human breast cancer specimens varied significantly, even within the same category. Since this may be related to breast cancer sub-type, we are currently examining the expression patterns of ER, PR and HER2 to precisely classify these breast cancer specimens. If PI3K γ over-expression is more common in a particular sub-type of breast cancer, this may become an important marker for planning treatment and developing new therapies.

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Fig. 1.

Upregulation of PI3K γ protein expression in human invasive breast carcinomas. Sections of formalin-fixed, paraffin embedded breast tissue were immunohistochemically stained for PI3K γ protein as described in "Materials and Methods". Images shown are representative of 40 matched breast cancer specimens. (A) Benign breast tissue, (B) Ductal carcinoma *in situ*. (C and D) Invasive breast carcinoma.

Fig. 2.

Aberrant expression of PI3K γ in human metastatic breast cancer cell lines. (A) Analysis of PI3K γ mRNA expression in breast cell lines by quantitative real-time PCR. Bars represent the mean $\pm$ S.E. of PI3K γ mRNA levels, normalized by β -actin expression levels (n=4).

* $p < 0.01$ compared to MCF-10A cells. Inset: Conventional PCR for PI3K γ (35 cycles) and β -actin (25 cycles). (B) Western blot analysis of PI3K α , PI3K β , PI3K γ and PI3K δ protein expression levels in breast cell lines. β -actin was used as an internal control. Images shown are representative of four separate experiments. (C) Immunofluorescence staining of PI3K γ protein (green) with DAPI staining of the nuclei (blue) in MDA-MB-231 cells and MCF-7 cells transfected with vector or PK3K γ . The negative control used non-immune rabbit IgG as the primary antibody. Images shown are representative of at least 50 cells from three separate experiments.

Fig. 3.

PI3K γ blockade attenuates migration and invasion of metastatic breast cancer cells. MDA-MB-231 (A) and MDA-MB-436 (B) cells were pre-treated with PI3K α inhibitor (30 nM), β inhibitor (100 nM), γ inhibitor (3 μ M), or the general PI3K inhibitor LY (10 μ M) for 15 min prior to Transwell migration and invasion assays in response to NIH-3T3 CM. Data are the % of cells migrated or invaded compared to untreated cells (None). Bars show the mean $\pm$ S.E. (n=3) with * p <0.01 compared to untreated cells. Inset, migration assay of MDA-MB-231 cells pre-treated with various concentrations of PI3K γ inhibitor (n=3). (C) PI3K γ inhibitor attenuated CXCR4-dependent migration of MDA-MB-231 cells. Cells were treated with various concentrations of PI3K γ inhibitor prior to Transwell migration assays in response to 50 ng of CXCL12 (O) or EGF (Δ). Data shown are the mean $\pm$ S.E. (n=3). (D) MDA-MB-231 cells were dual-transfected with ON-TARGETplus SMARTpool PI3K α , PI3K β or PI3K γ siRNA or control siRNA as described in "Materials and Methods". Cells were harvested for Western blot analysis of PI3K α , PI3K β or PI3K γ expression (upper panel) or migration and invasion assays in response to NIH-3T3 CM. Bars show the mean $\pm$ S.E. (n=4) with * p <0.01 compared to cells transfected with control siRNA.

Fig. 4.

Blocking PI3K γ activity attenuates GPCR-dependent lamellipodia formation of breast cancer cells. MDA-MB-231 cells cultured in serum-free medium were treated with inhibitors of PI3K α (30 nM) and β (100 nM) or γ (3 μ M) 1 h prior to 30 min incubation with CXCL12 (50 ng/ml), TFLLR (25 μ M) or EGF (5 ng/ml). Cells were then fixed and subjected to F-actin staining using rhodamine-labeled phalloidin. (A) Images shown are representative of at least 50 cells. Arrows indicates lamellipodial regions. (B) Lamellipodial extent at cell leading edges was quantified as a fraction of cell circumference on 50 randomly selected cells in each group. Columns, means; bars, S.E., * $p < 0.001$ compared to cells without inhibitor treatment (None).

Fig. 5. Overexpression of PI3K γ promotes MCF-7 cell migration and invasion. Non-metastatic breast cancer MCF-7 cells were stably transfected with YFP or constitutively membrane-targeted YFP-PI3K γ . (A) Transwell migration and invasion assays. Data shown are numbers of cells migrated or invaded per 10,000 loaded cells. Bars show the mean $\pm$ S.E. (n=5) with *p<0.01 compared to cells transfected with YFP. Inset: Western blot of PI3K γ and β -actin in MCF-7 cell lines. (B and C) PI3K γ promotes breast cancer MCF-7 cell invasion of 3D collagen matrices. MCF-7 cells were incubated with type I collagen gels for 48 h in the presence of DMEM or NIH-3T3 CM without (none) or with 10 μ M PI3K γ inhibitor or LY. Gels were fixed, stained and cross-section images were photographed. (B) Images of representative fields, showing the cell monolayer and the underlying collagen matrix containing invading cancer cells. (C) Quantitation of MCF-7 cell invasion. Data are expressed as mean numbers of invading cells per field $\pm$ S.E. (n=4) under 10X magnification from ten fields. *p<0.01 compared to cells transfected with YFP or cells in the absence of inhibitors (None).

PI3K γ expression by immunohistochemistry staining in human breast cancer specimens

Table 1

Breast specimens	n	Staining intensity				Average $\pm$ S.E.
		0	1	2	3	
Normal/Adjacent Normal	37	32	3	2	0	0.19 $\pm$ 0.08
Ductal Carcinoma <i>in Situ</i>	26	13	8	4	1	0.73 $\pm$ 0.17 *
Invasive Carcinoma	40	9	9	11	11	1.60 $\pm$ 0.18 **,#

Statistical significance was determined using a Kruskal-Wallis test and Dunn posttest.

* $p < 0.01$,
** $p < 0.001$ vs. Normal/Adjacent Normal
$p < 0.001$ vs. Ductal carcinoma *in Situ*