Targeting Type Iγ Phosphatidylinositol Phosphate Kinase Inhibits Breast Cancer Metastasis

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Abstract

Most deaths from breast cancer are caused by metastasis, a complex behavior of cancer cells involving migration, invasion, survival, and microenvironment manipulation. Type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) regulates focal adhesion assembly, and its phosphorylation at Y639 is critical for cell migration induced by EGF. However, the role of this lipid kinase in tumor metastasis remains unclear. Here we report that PIPKIγ is vital for breast cancer metastasis. Y639 of PIPKIγ can be phosphorylated by stimulation of EGF and hepatocyte growth factor (HGF), two promoting factors for breast cancer progression. Histological analysis revealed elevated Y639-phosphorylation of PIPKIγ in invasive ductal carcinoma lesions and suggested a positive correlation with tumor grade. Orthotopically transplanted, PIPKIγ-depleted breast cancer cells showed substantially reduced growth and metastasis, as well as suppressed expression of multiple genes related to cell migration and microenvironment manipulation. Re-expression of wild-type PIPKIγ in PIPKIγ-depleted cells restored tumor growth and metastasis, reinforcing the importance of PIPKIγ in breast cancer progression. Y639-to-F or a kinase-dead mutant of PIPKIγ could not recover the diminished metastasis in PIPKIγ-depleted cancer cells, suggesting that Y639 phosphorylation and lipid kinase activity are both required for development.
of metastasis. Further analysis with in vitro assays indicated that depleting PIPKIγ inhibited cell proliferation, MMP9 secretion, and cell migration and invasion, lending molecular mechanisms for the eliminated cancer progression. These results suggest that PIPKIγ, downstream of EGF and/or HGF receptor, participates in breast cancer progression from multiple aspects and deserves further studies to explore its potential as a therapeutic target.

**Keywords**

breast cancer metastasis; PIPKIγ; EGFR; cell migration; invasion

**Introduction**

Despite successful early detection and treatment of primary tumor burden, breast cancer remains one of the most significant malignancies in women because of the frequent occurrence of tumor relapse and metastasis. Understanding the molecular mechanisms underlying breast cancer metastasis is critical to developing therapeutic strategies and defining markers to predict metastatic potential and guide patient care. The phosphatidylinositol 3-kinase (PI3K) pathway is the most frequently altered pathway in breast cancer. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PI4,5P2) to produce phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P3), which then activates AKT and mTOR to promote the growth and survival of primary and metastatic tumors. In addition to being the substrate of PI3K, PI4,5P2 associates with and regulates proteins involved in focal adhesion assembly and actin re-organization; therefore, it might directly participate in the development of tumor metastasis.

Type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) is one of the major enzymes in cells that generate PI4,5P2. PIPKIγ plays a key role in multiple biological processes by controlling PI4,5P2 synthesis. In addition to regulating Ca2+ flux, PIPKIγ targets to focal adhesions via a direct interaction with talin and modulates nascent adhesion formation at the leading edge. PIPKIγ is phosphorylated at Y639 by receptor tyrosine kinases such as EGF receptor (EGFR), which is necessary for cell migration. Additionally, PIPKIγ regulates the assembly of E-cadherin-based intercellular adhesions and epithelial polarization by promoting the association of E-cadherin with the clathrin adaptor AP1B and the exocyst. Considering the established roles of PI3K, EGFR, and E-cadherin in breast cancer, PIPKIγ as a producer of PI4,5P2 could play an important role in breast cancer progression. Indeed, recent work shows that upregulation of PIPKIγ expression inversely correlates with the overall survival of breast cancer patients.

Although increasing evidence suggests the connection between PIPKIγ and tumor metastasis, it remains to be investigated whether PIPKIγ is necessary for the dissemination of tumor cells in vivo. On the other hand, acquired resistance to EGFR inhibition has become a major concern in anti-EGFR therapies. It has been established that resistance to EGFR blockage is related to the deregulation of PI3K, other family members of ERBB, and c-Met. In the context that PIPKIγ functions downstream of EGF and hepatocyte growth factor (HGF) and upstream of PI3K, understanding how PIPKIγ participates in
breast cancer could open avenues for new therapeutic strategies. In the current study, we developed an antibody that specifically recognizes EGFR-phosphorylated PIPKI\textsubscript{\gamma} (pY639) and analyzed the phosphorylation levels of PIPKI\textsubscript{\gamma} in breast cancer biopsies. Utilizing the 4T1 mouse breast tumor model and \textit{in vitro} assays, we determined whether PIPKI\textsubscript{\gamma} is necessary for the metastasis, progression, and invasive behaviors of breast cancer cells. The importance of Y639-phosphorylation in PIPKI\textsubscript{\gamma} to cancer metastasis was also evaluated. Our results support a role for PIPKI\textsubscript{\gamma} in breast cancer progression and suggest this lipid kinase as a potential drug target for breast cancer treatment.

**Results**

**Invasive breast carcinomas exhibit high levels of phosphorylated PIPKI\textsubscript{\gamma}**

As reported previously, hPIPKI\textsubscript{\gamma,i2} (but not hPIPKI\textsubscript{\gamma,i1}) can be phosphorylated by EGFR at tyrosine 639 (Y634 in mPIPKI\textsubscript{\gamma}) and that this phosphorylation is essential for EGF-induced cell migration \textsuperscript{21}. Hyper-activation of EGFR family members is frequently observed in breast cancer and confers a more aggressive clinical behavior \textsuperscript{22}. To explore the role of PIPKI\textsubscript{\gamma} as a key post-receptor cascade of EGF signaling, we first generated an antibody against phosphorylated-PIPKI\textsubscript{\gamma} (pY-PIPKI\textsubscript{\gamma}) and examined the specificity. As shown in Fig. 1A, the pY-PIPKI\textsubscript{\gamma} antibody only recognizes the overexpressed wild-type, but not Y639F, hPIPKI\textsubscript{\gamma,i2} in EGF-treated cells. In 4T1 cells, endogenous mPIPKI\textsubscript{\gamma} could be rapidly phosphorylated 5 min after EGF treatment and then quickly regressed after 15 min (Fig. 1B). Interestingly, HGF stimulation also caused a similar phosphorylation of PIPKI\textsubscript{\gamma} in 4T1 cells (Fig. 1B). HGF functions through the c-Met receptor, which is reported to correlate with poor prognosis and resistance to EGFR/Her2 inhibition \textsuperscript{23,24}. These results established the specificity of this antibody toward Y639-phosphorylated PIPKI\textsubscript{\gamma} and confirmed that endogenous PIPKI\textsubscript{\gamma} can be phosphorylated downstream of EGFR and c-Met, two important players in breast cancer progression.

Because Y639-phosphorylated PIPKI\textsubscript{\gamma} is required for EGF and HGF-induced cell migration \textsuperscript{21}, we next determined the phosphorylation levels of PIPKI\textsubscript{\gamma} in a tissue microarray (TMA) containing 270 invasive ductal carcinoma (IDC) specimens from 160 breast cancer patients. With negative staining in benign tissues, pY-PIPKI\textsubscript{\gamma} antibody displayed clear membrane staining in IDCs (Fig. 1C) as well as ductal carcinoma \textit{in situ} (DCIS) lesions associated with IDC (Supplementary Fig. S1A). The levels of pY639-PIPKI\textsubscript{\gamma} were markedly elevated in IDC (76.3\%, Fig. 1D) and DICS (100\%), suggesting a connection between PIPKI\textsubscript{\gamma} phosphorylation and breast neoplasia. Further analysis reinforced a significant correlation between levels of pY639-PIPKI\textsubscript{\gamma} and the grade of IDC (\(p < 0.001\)) (Fig. 1D, lower panel). However, the global PIPKI\textsubscript{\gamma} levels in tumor tissues did not display a substantial increase compared to normal tissues (Supplementary Fig. S1C) and did not correlate with disease grade when determined using pan-PIPKI\textsubscript{\gamma} antibody \textsuperscript{9,25}. This suggests that Y639 phosphorylation, but not expression, of PIPKI\textsubscript{\gamma} is significantly elevated in breast cancer and positively correlated with breast cancer progression.
Depletion of PIPKIγ attenuates the progression of 4T1 breast cancer

To determine how PIPKIγ might affect tumor progression, we utilized the 4T1 breast cancer model, which closely mimics human breast cancer and shows spontaneous metastasis to multiple distant sites. PIPKIγ was silenced by stably expressing mPIPKIγ-specific shRNA (mPIPKIγ-sh1) (Fig. 2A), which was designed to target to all of the five known splicing isoforms of PIPKIγ. As shown in Fig. 2B, PIPKIγ depletion (mPIPKIγ-sh1) significantly attenuated the size of primary 4T1 tumors. Images taken by PET-CT at day-14 also exhibited reduced tumor volume and isotope uptake ratio in the PIPKIγ-depletion group (Supplementary Fig. S2A and S2B). Similarly, the average weight of PIPKIγ-depleted tumors at day-35 decreased to ~60% of that of control tumors (Fig. 2C), suggesting that loss of PIPKIγ impairs tumor growth in vivo. At day-14 after implantation, control animals showed metastasis in multiple organs with the highest occurrence in lymph nodes (100%) and lung (80%), but metastasis to these two organs in the PIPKIγ-depletion group was only 40% and 20%, respectively (Fig. 2D). PET-CT images at day-28 post inoculation displayed strong lung signals in the control group (green circle) but clear lung in the PIPKIγ-depletion group (Fig. 2E). At the endpoint, PIPKIγ depletion drastically decreased metastatic nodules of the lung surface (Fig. 2F). Moreover, mice inoculated with PIPKIγ-depleted 4T1 showed significantly improved overall survival compared to control mice (Fig. 2G).

To verify that the tumor regression resulted from depletion of PIPKIγ, we used a distinct mPIPKIγ-specific shRNA (mPIPKIγ-sh2) to eliminate the pan-PIPKIγ expression (Fig. 3A). Mice inoculated with PIPKIγ-depleted 4T1 cells showed significantly reduced tumor volume (Fig. 3C). When tumor volume in the control group reached 1500 mm³, an average of 15 large metastatic nodules on the lung surface was observed in control group; however, lungs in PIPKIγ-depletion group were almost free of nodules (Fig. 3D and 3E). These data are consistent with what we observed with mPIPKIγ-sh1 (Fig. 2), suggesting that PIPKIγ is necessary for the progression of 4T1 breast cancer. Both mPIPKIγ-sh1 and mPIPKIγ-sh2 were designed to target all of the six mPIPKIγ splicing isoforms.

Loss of PIPKIγ causes reduced metastasis of breast tumor cells

To define if the impact of PIPKIγ depletion on tumor metastasis correlates with its inhibition of primary tumor growth, we removed the primary tumors when they reached a similar size (Fig. 4A) and then monitored tumor recurrence and lung metastasis. At day-7 post surgery (Fig. 4B), 3 mice in the control group (n = 6) exhibited primary tumor recurrence and 4 mice showed lung metastasis. In the PIPKIγ-depletion group (n = 7), 3 mice exhibited recurrence and only one showed luminescent signal in the lungs. Consistently, mice inoculated with PIPKIγ-depleted cells exhibited significantly improved survival (Fig. 4C), suggesting that the effect of PIPKIγ on tumor metastasis was independent of primary tumor growth. Additionally, three mice in the PIPKIγ-depletion group were sacrificed when their tumor volume reached 1500 mm³ as in the control group. Only 2–3 lung metastatic nodules per animal were found in these mice compared to ~15 lung nodules per control mouse (Fig. 3E). Histological analysis also exhibited many fewer metastatic colonies inside the lungs of the PIPKIγ-depletion group (Fig. 3F), further indicating that PIPKIγ depletion led to fewer 4T1 cells to arrive or survive in lung.
Results of these tumor recurrence studies suggested that lung metastasis of 4T1 cells was more dependent upon PIPKIγ than on local dissemination. To test this, control or PIPKIγ-depleted, luciferase-expressing 4T1 cells were injected into the tail vein instead of the mammary gland to skip the early steps of metastasis. The luminescent signal appeared in the lungs of control mice as early as day-6 and quickly spread by day-10; however mice in the PIPKIγ-depletion group maintained clear lungs until day-18 after injection (Fig. 4D). Animals receiving 4T1 cells by tail vein died quickly once lung metastasis was observed, but mice in the PIPKIγ-depletion group exhibited notably prolonged survival (Fig. 4E). These results indicate that PIPKIγ might promote both the early and late stages of metastasis, but has more striking effects on the later stages.

Wild-type, but not the EGFR phosphorylation defective, PIPKIγ rescues tumor progression abolished by depleting endogenous PIPKIγ

To confirm the role of PIPKIγ in tumor progression, we assessed whether the restoration of PIPKIγ could support tumor metastasis. For this purpose, we introduced the stable expression of HA-tagged wild-type (WT), kinase-dead (KD), or Y639-to-F mPIPKIγ along with luciferase in 4T1 cells. As shown in Fig. 5A, exogenous hPIPKIγ proteins were expressed at comparable levels and were resistant to mPIPKIγ shRNA that efficiently depleted endogenous mPIPKIγ. After being inoculated into mice, mPIPKIγ-depleted 4T1 cells exhibited slower growth compared to control cells (Fig. 5B). However, this phenomenon was not observed between the control and mPIPKIγ-depletion pairs when hPIPKIγ-WT, KD, or Y639F was re-expressed (Fig. 5B). As shown in Fig. 5C and 5D, lung metastasis at day-32 was observed in 4 of 5 control mice but in only 2 of 8 mice inoculated with mPIPKIγ-depleted cells, consistent with previous results (Fig. 2 and 3). With or without mPIPKIγ depletion (8 mice/group), hPIPKIγ-WT-expressing cells displayed lung metastasis in 3 or 4 mice, respectively, suggesting that hPIPKIγ-WT recovered the defective lung metastasis caused by loss of endogenous mPIPKIγ. However, mice inoculated with hPIPKIγ-KD- or hPIPKIγ-Y639F-expressing cells still exhibited much weaker lung metastasis when mPIPKIγ was depleted (Fig. 5, C and D), suggesting that these two mutants cannot compensate the function of endogenous mPIPKIγ on lung metastasis. Therefore, we conclude that the lipid kinase activity and Y639 phosphorylation are both critical for PIPKIγ to promote metastasis, which is consistent with reported results that kinase activity and Y639 phosphorylation are vital for cell migration. These data also reinforce our observation that Y639 is highly phosphorylated in breast cancer, and that its phosphorylation levels correlate with tumor grade.

Loss of PIPKIγ inhibits the proliferation, migration, and invasion of 4T1 cells

To define how PIPKIγ participates in tumor progression at molecular levels, we investigated if PIPKIγ regulates the proliferation, migration, and/or invasion of 4T1 cells in vitro. We found that loss of PIPKIγ inhibited the proliferation of 4T1 cells (Fig. 6A) and impaired the activation of MAPK (Fig. 3B). Considering the role of PIPKIγ in promoting cell migration, we examined the impact of PIPKIγ depletion on 4T1 mobility. Compared to the controls, loss of PIPKIγ led to a 60% reduction in wound closure (Fig. 6B) and almost completely blocked EGF-induced directional migration (Fig. 6C). Interestingly, re-expression of WT- but not KD- or Y639F-hPIPKIγ fully rescued the compromised
migration in mPIPKIγ-depleted cells (Fig. 6D). Together with previous findings (Fig. 1 and Fig. 5), these results confirm the importance of Y639-phosphorylated PIPKIγ in cell migration and support its association with metastasis of breast cancer. Additionally, PIPKIγ-depleted cells exhibited a 2-fold decrease in invasion compared to control cells (Fig. 6E). Although control cells exhibited mild matrix degradation (~5%), given time only a few of the PIPKIγ-depleted cells showed matrix degradation (Supplementary Fig. S3B). The area of the degraded region or the average number of degradation foci per PIPKIγ-depleted cell was only ~30% or ~48% of that in a control cell (Supplementary Fig. S3C and S3D). MMP9 is a MMP family member that is essential for matrix degradation and highly correlated with breast cancer progression 31. The expression and secretion of MMP9 were both dramatically decreased in PIPKIγ-depleted 4T1 cells (Fig. 6F). These in vitro results indicate that PIPKIγ depletion altered cell proliferation, migration, and invasion, herein lending molecular explanations to the slow progression of PIPKIγ-depleted 4T1 cells in mice.

**PIPKIγ depletion decreases macrophage infiltration, tumor angiogenesis and EMT**

To gain an insight into the molecular mechanism of weakened tumor progression caused by PIPKIγ-depletion, we performed gene microarray with control or PIPKIγ-depleted tumors. Genes changed more than 2-fold are summarized in Fig. 7A and Supplementary Table 1. The data revealed that several genes involved in cell movement such as myosins and actins were down-regulated in PIPKIγ-depleted tumors, which could lead to reduced cell migration/metastasis. Many tumor-promoting chemokines/cytokines were also down-regulated in PIPKIγ-depleted tumors, including CCL4 which is up-regulated in tissues and correlates with breast cancer grade 32, CCL21 that is involved in metastatic spreading of breast cancer 33, CXCL10 that promotes tumor proliferation in an autocrine manner 34, and leptin which has been implicated in epithelial-to-mesenchymal transition (EMT), metastasis, and poor prognosis of breast cancer 35–37. Down-regulation of these genes in PIPKIγ-depleted 4T1 tumors supports attenuated tumor growth/metastasis and improved survival of tumor bearing animals.

These downregulated chemokines/cytokines such as CXCL10 38, leptin 39,40,41, and IL-6 42 are also involved in the establishment of a tumor-favorable microenvironment, which is vital for implanted tumor cells to survive, proliferate and spread. Secreted frizzled-related protein 2 (SFRP2), a novel angiogenesis stimulator 43 implicated in breast cancer 44, was also down-regulated when PIPKIγ was depleted. To determine if loss of PIPKIγ could result in a less tumor-promoting microenvironment, we examined the tumor-associated macrophages and microvessels that play critical roles in promoting tumor growth and metastasis 45,46. Indeed, a 50% or 30% reduction of infiltrated macrophages or microvessel density was observed in PIPKIγ-depleted tumors, respectively (Fig. 7B and 7C). EMT, a biologic process by which epithelial cells lose their polarity and convert to a mesenchymal phenotype 47, is the hallmark for metastasis 48. As shown in Fig. 7D, PIPKIγ-depleted tumors displayed fewer mesenchymal-like cells (α-SMA-positive) but more epithelial-like cells (CK8-positive) compared to control tumors, suggesting that less EMT occurs when PIPKIγ is lost. Together, these results suggest that PIPKIγ depletion could impair the establishment of a tumor-favorable microenviroment, which could also contribute to the
attenuated in vivo progression of PIPKIγ-depleted 4T1 tumor in addition to inhibiting the migration and invasion of tumor cells.

**Discussion**

Phosphoinositide signaling mediates multiple biological processes including the adhesion, migration, growth, proliferation, and survival of cells. The altered integrant of this pathway could increase the signaling activation status, thus leading to cellular transformation. For example, the PI3K pathway is commonly dysregulated in human cancers including breast cancer. PIPKIγ, which provides PI4,5P2 as the substrate of PI3K, independently regulates cell migration by affecting focal adhesion turnover and actin reorganization, and therefore is potentially associated with breast cancer. Using a pY639-PIPKIγ antibody, we report here that breast tumor tissues exhibit high levels of phosphorylated PIPKIγ. Utilizing the 4T1 breast cancer model, we for the first time observed that PIPKIγ could regulate behavior of breast cancer cells in vivo by supporting cell proliferation, migration, and invasion, as well as the establishment of a tumor-favorable microenvironment. In particular, the metastasis of 4T1 cells requires the phosphorylation of PIPKIγ at Y639. Our results shed light on the importance of this lipid kinase in breast cancer progression downstream of EGFR/c-Met signaling.

In our hands, PIPKIγ depletion inhibited the proliferation of 4T1 cells, as reported previously in human breast cancer MDA-MB-231 cells. The significance of this inhibition strengthened by the reduced growth of PIPKIγ-depleted 4T1 tumors in mice. Although there was no clear evidence supporting the effect of PIPKIγ in cell proliferation, we did observe decreased MAPK activity in PIPKIγ-depleted cells (Fig. 3B). This might be an indirect result from impaired PI3K activity caused by inadequate production of the PI3K substrate PI4,5P2 when PIPKIγ was lost. Loss of PIPKIγ constrained tumor metastasis independent of its inhibition on tumor growth. Control 4T1 cells relapsed faster than PIPKIγ-depleted cells after tumor resection, suggesting that PIPKIγ is necessary for rapid local dissemination of tumor cells. Indeed, results from in vitro assays revealed that loss of PIPKIγ strongly inhibited cell migration, MMP9 secretion, and matrix degradation, which endorse the diminished local invasion of PIPKIγ-depleted cells. In addition, PIPKIγ depletion also attenuated the lung distribution of 4T1 cells injected by the tail vein, suggesting that PIPKIγ can participate in the late stages of the metastatic process, such as survival in the circulation system, extravasation, and/or survival and proliferation in the lung. These could also result from the impaired MAPK activity, decreased cell adhesion/migration, and/or attenuated MMP9 secretion and invasion.

In addition to influencing cell migration by generating PI4,5P2, our results suggest that loss of PIPKIγ also alters the expression of genes encoding actin, myosin, chemokines and cytokines that are involved in breast cancer progression and metastasis. Although PIPKIγ has not been implicated in regulation of gene transcription, this could ultimately result from attenuated MAPK and/or PI3K activity, retardant small G protein activation and actin reorganization, or the interaction between these altered signaling pathways. In addition, the complex residential environment also contributes to the alteration of gene expression related to PIPKIγ depletion, owing to the fact that we detected many more genes affected by
depletion of PIPKIγ in 4T1 tumors than that in cultured cells (only two genes including MMP9). Nevertheless, down-regulation of these genes clearly contributes to inhibiting cell migration and could eventually change the overall behavior of tumor cells such as their interaction with the surrounding environment. Indeed, the decreased number of macrophages and microvessels in tumors lacking PIPKIγ suggests a failure to form a tumor-supportive microenvironment, which likely inhibits tumor growth and metastasis. Moreover, EMT was also reduced in PIPKIγ-depleted tumors. Therefore, loss of PIPKIγ, in addition to blocking cell migration acutely, can have a tardive effect on the communication between tumor cells with the surrounding environment. Both aspects reduce the growth and metastasis of tumor cells and favor a better survival from breast cancer.

More interestingly, our data support a tight association between EGFR and PIPKIγ in the progression of breast cancer. EGFR directly phosphorylates PIPKIγ at Y639. We show here that this phosphorylation is not only necessary for EGF-induced migration of breast cancer cells, but also required for the metastasis of 4T1 tumors in mice. These results suggest that PIPKIγ is a key player downstream of EGFR. EGFR and/or ERBB2 are overexpressed in >30% of breast cancer patients and have become the most important drug targets. However, lack of appropriate biomarkers to predict which patients would most likely respond to EGFR/ERBB2 inhibition limits the application of these EGFR/ERBB2-targeting drugs. Although the commonly used EGFR and ERBB2 antibodies can determine the expression levels of these receptors in patients, diagnostic antibodies efficiently recognizing the constitutively activated EGFR are still not available. In this context, our anti-pY639-PIPKIγ antibody could be used as a marker for the hyper-activated EGF pathway and help the diagnosis and treatment decision. Further study will be necessary to confirm whether the staining of anti-pY639-PIPKIγ antibody is correlated with patient survival independent of EGFR/ERBB2. Furthermore, EGFR/ERBB2 inhibition often causes resistance. Recent studies hypothesized that c-Met as a substitute player of EGFR/ERBB2 when the latter is inhibited mediates the drug resistance. Interestingly, Y639 of PIPKIγ can also be phosphorylated upon HGF stimulation, suggesting c-Met also needs PIPKIγ to function. This raises a possibility that inhibiting PIPKIγ may block both EGFR/ERBB2 and c-Met pathways and might provide an alternative prospect for therapeutic design.

Materials and Methods

Cell lines

The 4T1 murine breast cancer cell line was kindly provided by Dr. Vijayalakshmi Shridhar (Mayo Clinic). 4T1 cells stably expressing PIPKIγ-specific shRNA or wild-type or mutated human PIPKIγ were created by infecting cells with lentivirus carrying relevant shRNA or cDNA. 4T1 or 293T cells were cultured using MEM-alpha or DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Invitrogen, Carlsbad, CA) with 5% CO2 at 37°C.

Constructs and lentivirus

The pLKO.1 constructs encoding two distinctive short hairpin RNA (mPIPKIγ-shRNA1: GAGAGGAUGUGCAGUAUGA; mPIPKIγ-shRNA2: GUGGUUGUCAUGAACAAG)
were constructed. A pLKO.1 construct with nonfunctional shRNA (TAAGGTTAAGTGCCCTCG) was used as negative control. The cDNA sequence encoding human wild-type or mutated PIPKIγ was cloned into pCDH lentiviral vector. Lentivirus production and transduction were performed following the protocol as described previously 14.

**Antibodies**

Polyclonal PIPKIγ anti-serum was generated by immunizing rabbits using purified Histagged mouse PIPKIγ and purified using a human PIPKIγ-conjugated affinity column to generate the anti-pan-PIPKIγ antibody as described 9. To generate the phosphorylated-PIPKIγ antibody, we immunized rabbits with a PIPKIγ phosphopeptide (CDIpYFPTDERSWVYSPLHYSA). The anti-sera were collected, pre-cleaned by a non-phosphopeptide (CDIYFPTDERSWVYSPLHYSA) affinity column, then purified using an affinity column conjugated with the phosphopeptide. Antibodies: anti-HA and MMP9 antibodies (Millipore, Billerica, MA); pERK1/2 and ERK1/2 antibodies (Cell Signaling, Danvers, MA); β-actin antibody and monoclonal anti-α smooth muscle actin antibody (Sigma, St. Louis, MO); Rabbit anti-Cytokeratin 8 antibody, Rat monoclonal anti-CD34 antibody and monoclonal anti-CD68 antibody (Abcam, Cambridge, MA); Alexa Fluor 488 goat anti-mouse antibody, Alexa Fluor 555 goat anti-rabbit antibody and Alexa Fluor 555 goat anti-rat antibody (Molecular probes).

**Cell proliferation, migration, and invasion assays**

4T1 cells (2,000/well) were seeded into 96-well culture plates and cell proliferation was determined by MTT assay at different time points. Migration and invasion assays were performed using Transwell according to the manufacturer’s instruction. The detailed procedures are described in Supplementary Methods.

**4T1 breast cancer model**

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic. 1 × 10⁶ 4T1 cells were implanted subcutaneously into the mammary fat pad of female BALB/c mice (6–8 weeks of age). Tumor volume (V) was measured with calipers every several days and calculated by using the standard formula V = 0.5 × LW² (L, Length; W, width). For the tumor resection experiment, the primary tumor was removed when tumor size reached 3 mm (millimeter) in diameter after implantation. For experimental lung metastasis, 5 × 10⁵ control or PIPKIγ-depleted 4T1 cells were injected into the tail veins of BALB/c mice. Mice were monitored using a bioluminescent imaging system to follow the growth of metastasis at indicated times.

**Immunohistochemistry and Immunofluorescence**

TMA samples containing benign and breast cancer tissues from patients with invasive ductal carcinomas were provided by Dr. Wilma Lingle (Mayo Clinic). Patient studies were approved by the Institutional Review Board (IRB) at Mayo Clinic. Immunohistochemistry and immunofluorescence were performed according to standard protocols.
Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting assays were performed as described previously ⁹.

In vitro cell migration and invasion assay

The migration assay and invasion assay were performed as described ²¹ using 2×10⁴ and 5×10⁴ 4T1 cells, respectively. Cells were incubated for 4 hr in the migration assay and 16 hr in the invasion assay. Wound healing assays were performed as described ²¹. Phase contrast images of the wound area were acquired with a 10× objective at 0 and 12 hr after the wound was created. The area of wound in each picture was determined by ImageJ software.

Matrix degradation assay

Coverslips were sterilized with 100% ethanol and then coated with 50 μg/ml poly-L-lysine for 20 minutes at room temperature, washed with PBS, and fixed with ice-cold 0.5% glutaraldehyde for 15 minutes followed by extensive washing. Coverslips were then inverted on an 80 μl drop of fluorescent gelatin matrix (0.2% gelatin and Alexa Fluor 488 gelatin at an 8:1 ratio) and incubated for 15 minutes at room temperature. Coverslips were washed with PBS and the residual reactive groups in the gelatin matrix were quenched with 5 mg/ml sodium borohydride in PBS for 10 minutes followed by further washing in PBS. 1×10⁵ cells were plated on the coated coverslips and incubated at 37°C for 6–8 hours. To assess the ability of cells to degrade matrix, at least 10 randomly chosen fields were imaged per trial and evaluated for degraded matrix foci, which appear as dark ‘holes’ in the bright fluorescent matrix field.

PET-CT and Bioluminescence imaging

For positron emission tomography-computed tomography (PET-CT), mice were fasted for 6 h before 18F-fluorodeoxyglucose (¹⁸F-FDG) injection. The injected dose of each mouse was 200 μCi ¹⁸F-FDG. This was then followed by a 60 min uptake period under continuous isoflurane anesthesia before PET images were acquired. CT and PET scanning were performed using an Inveon microPET/CT scanner (Siemens). Bioluminescence imaging was conducted using a Xenogen IVIS 200 imaging system (Caliper LifeSciences, Hopkinton, MA). Mice were intraperitoneally injected with 200 μl of 15 mg/ml D-Luciferin (Glod Biotechnology, St Louis, MO) in PBS. Bioluminescence imaging with a chargecoupled device (CCD) camera was initiated 10 minutes after injection. The signal intensity was quantified as sum of all detected photons within the region of interest per second using Living Image software (Xenogen Corp, Almeda, CA).

Microarray analysis

Total RNA was isolated from control and PIPKIγ-depleted 4T1 tumors using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction and submitted to Advanced Genetics Technology Center at Mayo Clinic (Rochester, MN). Microarray analysis was performed using Mouse Ref8 Gene Expression BeadChip (Illumina). Gene expression data was normalized using faster cyclic loess and processed with the Ingenuity Pathway Analysis (IPA) program. The expression level Z-scores were mapped to colors from red (z = 1, above mean) to green (z = −1, below mean)

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. PIPKIγ is highly phosphorylated in breast invasive ductal carcinomas
A, phospho-PIPKIγ antibody (pY-PIPKIγ) specifically recognizes phosphorylated Y639 in PIPKIγ. Flag-tagged wild-type (WT) or Y639F hPIPKIγ was expressed in and immunoprecipitated from 293T cells with or without 10 ng/ml EGF stimulation for 5 min. The precipitates were analyzed by immunoblotting using indicated antibodies. B, 4T1 cells were treated with 10 ng/ml EGF or HGF for the indicated time, then cell lysates were analyzed by immunoblotting using indicated antibodies. C, representative images of pY-PIPKIγ staining on benign tissue or invasive dual carcinoma (IDC). H&E, hematoxylin and
eosin. Scale bar, 100 µm. D, levels of pY-PIPKIγ in IDC correlate with tumor grades. Top table summarized the staining intensity of anti-pY-PIPKIγ in IDC and results were plotted and correlated with IDC grade (bottom). Pearson's Chi-squared test, \( p < 0.001 \).
Figure 2. Loss of PIPKIγ attenuates the growth and metastasis of 4T1 breast tumors

A, PIPKIγ was depleted in 4T1 cells by lentivirus-mediated stable expression of mPIPKIγ-shRNA1. B, growth curves of tumors in mice inoculated with control or PIPKIγ-depleted 4T1 cells. **, P < 0.01; ***, P < 0.001. C, the average weight of tumors from control or PIPKIγ-depletion group at day-35 were plotted (left). Representative images of primary tumor in control and PIPKIγ-depletion groups were shown (right). **, p < 0.01. D, frequency of metastasis in distant organs of mice inoculated with control (shCtrl) or PIPKIγ-depleted (shPIPKIγ) 4T1 cells was determined at day-14 post inoculation by PET-CT. E,
representative PET-CT images of lung metastasis was shown at day 28 after inoculation. Green circles marked the tumor signal in lung of control or PIPKIγ-depleted groups. F, top, images of lung surface nodules from control or PIPKIγ-depletion groups at day-35; bottom, tumor nodules in the lung were quantified and plotted. ***, \( p < 0.001 \). G, survival (Kaplan-Meier) curve of mice inoculated with control or PIPKIγ-depleted 4T1 cells. B–G, \( n = 10–12 \) mice/group. Log-rank text, \( p < 0.0001 \).
Figure 3. PIPKIγ depletion mediated by a distinct mouse PIPKIγ shRNA also suppresses the progression of 4T1 breast cancer in mice
A, PIPKIγ was eliminated in 4T1 cells by expressing mPIPKIγ-shRNA2. B, levels of phosphorylated-ERK1/2 and total ERK1/2 in 4T1 cells expressing control (Ctrl) or mPIPKIγ-sh2 were determined by immunoblotting. C, growth curves of tumors in mice inoculated with 4T1 cells expressing control or mPIPKIγ-sh2 (n = 9–10/group). D, the dorsal and ventral images of lung from mice inoculated with control or PIPKIγ-depleted 4T1 cells at day-34 post inoculation. Arrows, tumor nodules. E, depletion of PIPKIγ delayed lung metastasis of 4T1 cells independent of primary tumor size. Left two bars, mice inoculated with 4T1 cells expressing control or mPIPKIγ-sh2 (n = 9–10/group).
inoculated with control or PIPKIγ-depleted 4T1 cells were sacrificed when tumors in the control group reached 1500 mm$^3$, then lung surface nodules were quantified and plotted. Right bar, quantification of lung surface nodules in three mice inoculated with PIPKIγ-depleted cells when their tumor volume reached 1500 mm$^3$. *, p < 0.05. F, hematoxylin and eosin staining of lung tissue from the control and PIPKIγ-depletion group when the primary tumor size was 1500 mm$^3$. Arrows, prominent tumors in lung tissues. Scale bar, 500 µm.
Figure 4. PIPKιγ depletion inhibits metastasis of 4T1 breast tumors independent of primary tumor growth

A, representative bioluminescent images of tumor-bearing mice before and after tumor resection. B, images of lung metastasis in mice in control or PIPKιγ-depletion group were shown at day-7 after tumor resection. C, survival curve of mice inoculated with control or PIPKιγ-depleted 4T1 cells after tumor resection. Log-rank text, $p < 0.05$. D, $5\times10^5$ control or PIPKιγ-depleted 4T1 cells were directly injected into tail vein of BALB/c mice. Representative images of lung metastasis in mice from day 3, 6, 10, and 18 after injection.
are shown. E, survival curve of mice inoculated with control or PIPKIγ-depleted 4T1 cells after injection. Log-rank test, \( p < 0.01 \). A–E, \( n = 6–9 \) mice/group.
Figure 5. Expression of exogenous PIPKιγ rescues the impaired tumor progression caused by depletion of endogenous PIPKιγ

A, The re-expression of hPIPΚιγ-WT, -KD or -Y639F and the knockdown of endogenous mPIPΚιγ in 4T1 cells were analyzed by immunoblotting using indicated antibodies. These eight lines were implanted in mice (n = 5–9/line), and the tumor growth curves were determined in B. *, p < 0.05; **, p < 0.01. C, representative bioluminescent images of the indicated groups were shown at day-32 after inoculation. D, frequency of lung metastasis in mice of the indicated groups at day-32 was summarized.
Figure 6. PIPKIγ depletion inhibits the proliferation, migration and invasion of 4T1 cells in vitro

A, 4T1 cells were infected with lentivirus carrying control or mPIPKIγ-shRNA1 for 48 hrs; cell proliferation was determined by MTT assay at different time points. B, images of the wound on the monolayer of control and PIPKIγ-depleted cells at 0 and 12 hrs after wound creation (top). Bottom panel, quantification of wound width at 12 hrs. C, migration of cells with or without PIPKIγ depletion was measured using Boyden chamber in the presence of indicated amount of EGF. D, migration of eight 4T1 cell lines (described in Fig. 5) responding to 1 nM EGF was determined. E, invasion assay was performed using Matrigel
invasion chamber using indicated cells. Invasion index was calculated according to the manufacturer’s instructions. A–E, *, p < 0.05; **, p < 0.01; ***, p < 0.001. F, conditioned culture medium and cell lysates from control or PIPKIγ-depleted 4T1 cells were collected and analyzed by immunoblotting to determine MMP9 level.
Figure 7. Loss of PIPKιγ weakens the ability of 4T1 breast tumors to manipulate the microenvironment

A, gene expression of control or PIPKιγ-depleted tumors was analyzed by microarray. B, infiltrated macrophages in tumor tissue sections from control or PIPKιγ-depletion groups were identified by CD68 staining (left). Right, quantification of CD68 positive cells in control or PIPKιγ-depleted tumors. C, the microvessel density in tumor sections from control and PIPKιγ-depleted tumors was evaluated using the endothelial cell marker CD34 (left). Right, quantification of CD34 positive cells in indicated tumors. D, immunofluorescent microscopy images visualize cytokeratin 8 (CK8, red) positive epithelial
cells and α-smooth muscle actin (SMA, green) positive mesenchymal cells in indicated tumors. DAPI staining represented nuclei (left). Right, quantification of cytokeratin 8 or α-SMA positive cells in indicated tumors. B–D, *, p < 0.05; **, p < 0.01. Scale bar, 20 µm.