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*Original Article*

# Type Iγ phosphatidylinositol phosphate kinase dependent cell migration and invasion are dispensable for tumor metastasis

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**Abstract:** Type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) has been associated with poor prognosis in breast cancer patients by promoting metastasis. Among the six alternative-splicing isoforms of PIPKIγ, PIPKIγ_i2 specifically targets to focal adhesions and regulates focal adhesion turnover, thus was proposed responsible for tumor metastasis. In the present study, we specifically depleted PIPKIγ_i2 from mouse triple negative breast cancer (TNBC) 4T1 cells and analyzed their behaviors. As expected, PIPKIγ_i2-depleted 4T1 cells exhibited reduced proliferation, migration, and invasion *in vitro* at a comparable level as pan-PIPKIγ depleted cells. However, PIPKIγ_i2 depletion had no effect on metastasis and progression of 4T1 tumors *in vivo*. PIPKIγ_i2-depleted tumors showed similar levels of growth, hypoxia state, macrophage infiltration, and angiogenesis as parental tumors, although the pan-PIPKIγ depletion led to substantial inhibition on these aspects. Further investigation revealed that depleting PIPKIγ_i2 alone, unlike depleting all PIPKIγ isoforms, had no effect on PD-L1 expression, the status of the epithelial-to-mesenchymal transition, and the anchorage-independent growth of 4T1 cells. In human TNBC MDA-MB-231 cells, we obtained similar results. Thus, while PIPKIγ_i2 indeed is required for cell migration and invasion, these characteristics are not decisive for metastasis. Other PIPKIγ isoform(s) that regulate the expression of key factors to support cell survival under stresses is more critical for the malignant progression of TNBCs.

**Keywords:** PIPKIγ, triple negative breast cancer, cell migration, metastasis, the epithelial-to-mesenchymal transition

## Introduction

Breast cancer is one of the most common malignancies among women worldwide [1]. The processes of invasion and metastasis that cause mortality in patients are distinctive characters in the progression of breast cancer. Therefore, the in-depth understanding of the mechanism underlying metastasis development is critical to define effective preventions and therapies. In addition, new prognostic markers that can predict metastatic potential will be a key for defining therapeutic approaches.

Type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) is one of the major enzymes in cells that generate phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) [2]. PIPKIγ plays a key role in multiple biological processes including cell survival, cell cycle progression, focal adhesion dynamics, vesicular trafficking and actin cytoskeleton reorganization [3] by controlling the spatiotemporal availability of $PI4,5P_2$. Six PIPKIγ alternative splicing isoforms, known as PIPKIγ_i1-PIPKIγ_i6, have been identified in human [4-6]. These isoforms share identical sequences in N terminus and kinase domain. Their unique C-terminal extensions determine their specific subcellular locations and binding partners, which likely contribute to the function specificity of them [6, 7]. PIPKIγ_i2 is the first well-studied PIPKIγ isoform because of its specific localization at focal adhesions. By interacting with talin,

PIPKIγ_i2 generates $PI4,5P_2$ specifically at focal adhesions to promote focal adhesion turnover and cell migration [8-10]. Recent studies show that the upregulation of PIPKIγ correlates with the poor prognosis in breast cancer patients [11]. Increased PIPKIγ activity is observed in hepatocellular carcinoma [12] and PIPKIγ regulates the transcriptional activity of b-catenin downstream of growth factor signaling [13]. Together, these studies indicate that PIPKIγ promotes tumor progression. Our previous studies have demonstrated that depletion of all PIPKIγ isoforms in 4T1 breast cancer cells attenuated tumor growth and metastasis [14]. Yet, whether individual PIPKIγ isoforms make unique contributions to tumor progression is unclear. Because of the known function of PIPKIγ_i2 in focal adhesion turnover and growth factor-mediated directional cell migration [9], we proposed that PIPKIγ_i2 is the major player in the development of metastasis in breast cancer.

Results from our current study demonstrated that PIPKIγ_i2 indeed participates in the proliferation, migration and invasion potential of 4T1 tumor cells *in vitro*, similar to depleting all PIPKIγ isoforms. However, PIPKIγ_i2 depletion exhibited little effect on tumor growth and metastasis *in vivo*. Further investigation revealed the differences between pan-PIPKIγ depleted and PIPKIγ_i2 deleted 4T1 tumors on the EMT status, anchorage-independent growth, and tumor interactions with the host microenvironment. These data indicated that PIPKIγ_i2-dependent cell migration and invasion are not sufficient to support tumor metastasis. It is obvious that other PIPKIγ isoform(s) plays more important role in promoting tumor metastasis and progression by enhancing the EMT and anti-stress survival of breast cancer cells.

## Materials and method

### *Plasmid construct and lentivirus*

Vectors encoding short hairpin RNA (shRNA) sequences were constructed by cloning shRNA oligonucleotides (Invitrogen, Carlsbad, CA) into the pLKO.1 vector (AddGene, Cambridge, MA). The shRNA sequences targeting mouse PIPKIγ isoforms were GAGCGACACATAATTTCTA (PIPKIγ_i2 specific) and GAGAGGATGTGCAGTATGA (pan-PIPKIγ). The sequence of control scrambled shRNA is 5'-GTACCTGTACTTCATGCAG-3'. The shRNA sequences targeting human PIPKIγ isoforms were 5'-GCGTGGTCAAGATGCACCTCAAGTT-3' (pan-PIPKIγ) and 5'-GAGCGACACATAATTTCTA-3' (PIPKIγ_i2 specific). Human siRNA sequences were the same as human shRNA.

Generation of replication-defective infectious viral particles and the transduction of the cells were carried out following the protocol provided by Addgene (Addgene Inc, Cambridge, MA). Briefly, HEK293T cells were transfected with the pLKO.1 plasmid encoding the shRNA, the packaging plasmid pMDG-dR8.91, and the envelope plasmid pCMV-VSV-G at a ratio of 2:1:1 using FuGENE® 6 transfection Reagent (Promega, Madison, WI); Supernatants were collected 72 hrs after transfection and concentrated using Lenti-X™ concentrator (Clotech Laboratories, Inc; Mountain view, CA).

### *Cell cultures and transfection*

Cells were cultured using DMEM-alpha medium supplemented with 10% FBS, penicillin, and streptomycin (Invitrogen, Carlsbad, CA) with 5% $CO_2$ at 37°C. For lentivirus infection, cells were firstly transduced by in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO), then were infected by lentivirus carrying control, PIPKIγ or PIPKIγi2 shRNA for 48 hours. To get stable depletion of PIPKIγ from 4T1 cells, 4T1 cells were selected by 5 μg/ml puromycin 48 hours after infection. For transient siRNA transfection, cells were reverse-transfected by using RNAiMAX according to manufacturer's instruction (Invitrogen). Cells were used for further analyses 48 hours after transfection.

### *Antibodies*

Rabbit monoclonal PIPKIγ antibodies recognizing all isoforms (pan-PIPKIγ) or specifically recognizing PIPKIγ_i2 were generated as described previously [14, 15]. The following antibodies were used for western blot, immunofluorescence and immunohistochemistry. pERK1/2, ERK1/2 and pAKT, AKT antibodies (Cell Signaling, Danvers, MA); β-actin antibody (Sigma, St. Louis, MO); MMP9 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, Carlsbad, CA). PD-L1 anti-

body (clone G9) was generously provided by Dr. Haidong Dong at Mayo Clinic.

*Cell proliferation and anchorage-independent growth*

Cells were seeded into 96-well culture plates at a density of 1000 cells/well. The proliferation was measured by MTT assay at different points. For anchorage-independent growth, we used agarose colony formation assay. Soft agar assays were performed in cell culture medium containing 10% FBS using 6-well plates in triplicate as described [15]. 4T1 cells ($1 \times 10^4$) were mixed with 0.4% agar and plated on a layer of 0.6% agar in 6-well plates. Cells were incubated at 37°C for 8 days. Colonies were stained with MTT and scanned with Gelcount colony counter. Numbers of colonies were quantified using GelCount software. The experiment was repeated three times independently.

*In vitro cell migration, wound healing assay and invasion assay*

Migration assay was performed in modified Boyden chamber with 8 μm pore polycarbonate membranes (Neuroprobe, Gaithersburg, MD, USA) as described [14]. Cells were starved in serum free medium overnight and added to upper compartment ($2 \times 10^4$ cells). The lower compartment was filled with medium containing chemotaxis. After 4 h of incubation, cells that had not migrated to the lower chamber were removed with a cotton swab. Migrated cells attached to the underside of the filter were stained with DAPI and counted under microscope. Cell numbers in 5 randomly selected non-overlapping fields on the membrane were averaged to represent the number of migrated cells. For wound healing assay, a wound was created by scratching the confluent cell monolayer with a pipette tip. Phase contrast images of the wound area were acquired with 10 × objective at 0 and 12 h after the cell monolayer was scratched. The width of wound in each picture was determined by ImageJ software.

For invasion assay, cells ($5 \times 10^4$) were seeded in the upper chamber of transwell fitted with Matrigel-coated membrane (8 μm pore size, BD Biosciences, Bedford, MA). After 20-hour incubation at 37°C in 5% $CO_2$, cells on the upper surface of the inner chamber were removed with cotton swabs. Invaded cells that adhered on the lower surface of the membrane were stained and counted. The invasion index was determined according to the manufacturer's instructions.

*Matrix degradation assay*

Coverslips were 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. $1.0 \times 10^5$ cells were plated on the coated coverslips and incubated at 37°C for 6-8 hours. To assess the ability of cells to form 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. ImageJ was used to analyze the data.

*Immunoblotting*

This experiment was performed as previously described [8]. Cell lysates were separated on 10% SDS-polyacrylamide gels by electrophoresis and transferred to PVDF membrane (Millipore Immobilin P, Bedford, MA). After blocking the non-specific binding sites, the membranes were incubated with primary antibodies, following by secondary antibody. The immunoreactive bands were visualized using the chemiluminescence detection system (Promoga, Madison, WI). Membranes were stripped and re-probed with anti-actin antibody for internal loading control.

*Animal studies*

Female BALB/c or NOD SCID mice (6-8 weeks of age) were purchased from Jackson Lab (Bar Harbor, ME) and housed in regular or a specific pathogen-free animal facility, respectively. All experimental procedures were approved by the institutional Animal Care and Use Committee of Mayo Clinic. 4T1 cells ($5 \times 10^5$) were implanted

subcutaneously into the mammary fat pad of female BALB/c mice. MDA-MB-231 cells ($2 \times 10^6$) were implanted subcutaneously into the mammary fat pad of female NOD SCID mice. Tumor volume (V) was measured with calipers every several day and calculated by using the standard formula $V = 0.5 \times AB^2$, where *A* and *B* are the smallest and the largest diameters, respectively. At the endpoint, primary tumors were resected and weighted. Lungs were harvested, and metastatic nodules on the surface of lungs were counted.

*Tumor hypoxia analysis*

Two hours before tumor excision, mice were injected intraperitoneally with a saline solution containing 60 mg/kg Pimonidazole (HypoxyprobeTm-1 plus Kit; Chemicon). Tumors were then harvested and fixed in 10% neutral buffered formalin and embedded in paraffin. The formation of pimonidazole adducts was detected by immunostaining with Hypoxyprobe-1-Mab1 FITC Ab according to the manufacturer's instructions. Pimonidazole-positive hypoxic areas in the whole tumor were analyzed using MetaMorph software.

*Immunohistochemistry and immunofluorescence*

Tissue samples were fixed in 10% formaldehyde and embedded in paraffin. Tissue sections (5 μm) were cut and stained using a standard immunohistochemistry procedure as previously described [14]. Briefly, tissue sections were deparaffinized in xylene and rehydrated through alcohol gradient. Antigen retrieval was performed using a steamer for 30 minutes in 0.1 M citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with hydrogen peroxide (DAKO, Carpenteria, CA, USA) for 10 minutes, and serum free protein (DAKO, Carpenteria, CA, USA) was used to prevent nonspecific protein binding. Sections were then incubated with primary antibodies overnight at 4°C, following by incubation with secondary antibody (DAKO, Carpenteria, CA, USA) for 60 minutes. The sections were developed with DAB Peroxidase Substrate Kit (DAKO, Carpenteria, CA, USA), slightly counterstained with hematoxylin, dehydrated, cleared and mounted with Permount. Immunofluorescence was performed as described above but with fluorescent secondary antibodies and mounting medium with DAPI (Vector labs, Burlingame, CA).

*Statistical analysis*

Data are presented as mean values ± SEM. Statistical analysis was carried out using Student's *t* tests. Two-tailed *P* values < 0.05 were considered statistically significant. Survival of 4T1 bearing mice was estimated by Kaplan-Maier curves; Log-rank test was used to estimate the survival differences.

## Results

*PIPKIγ_i2 is required for cell proliferation and migration in vitro*

To investigate the role of PIPKIγ_i2 in breast cancer progression, we constructed the lentiviral vector-based PIPKIγ_i2 or pan-PIPKIγ shRNA constructs. The corresponding lenriviral particls were used to establish 4T1 cell lines with stable depletion of PIPKIγ_i2 or pan-PIPKIγ. We first confirmed the knockdown efficiency and specificity of these shRNAs by immunoblotting using pan-PIPKIγ or PIPKIγ_i2 specific antibodies. As shown in **Figure 1A**, pan-PIPKIγ shRNA reduced all PIPKIγ proteins including PIPKIγ_i2 by more than 90%; whereas the PIPKIγ_i2 specific shRNA significantly depleted the isoform 2 but only had little effect on other isoforms. By monitoring the amount of live cells using MTT assay, we found that loss of PIPKIγ_i2 moderately decreased the proliferation of 4T1 cells in 2D monolayer culture to a comparable level as the knockdown of pan-PIPKIγ (**Figure 1B**). To elucidate whether PIPKIγ_i2 participates in cell survival/proliferation pathways, we examined whether depletion of PIPKIγ_i2 would affect the activity of AKT and ERK1/2. As shown in **Figure 1C**, AKT activity (measured as the ratio of phosphorylated AKT to total AKT) was significantly reduced to 30% of control in PIPKIγ_i2-depleted cells. The activity of ERK (measured as the ratio of phosphorylated ERK to total ERK) was also decreased to about half of control when PIPKIγ_i2 was knockdown. Notably, cells expressing pan-PIPKIγ shRNAs exhibited similar levels of suppression on AKT activity, suggesting that AKT is the downstream player of pan-PIPKIγ and PIPKIγ-i2 in promoting cell proliferation/survival *in vitro* under 2D culture condition.

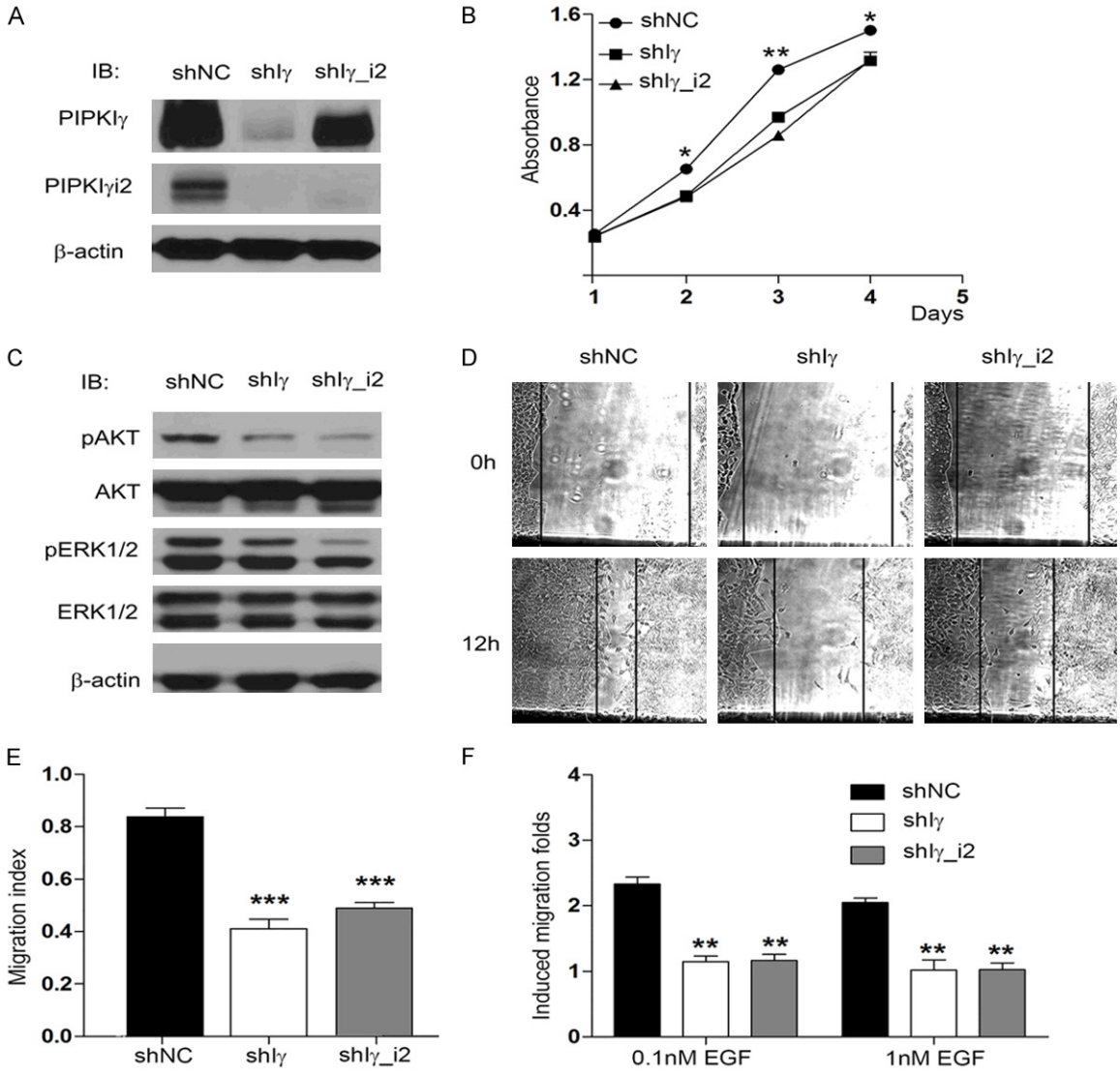


**Figure 1.** Knockdown of pan-PIPKIγ and PIPKg_i2 reduce the proliferation and migration of 4T1 cells *in vitro* to a similar level. (A) The expression of all PIPKIγ isoforms or PIPKIγ_i2 alone was silenced by stably expressing short hairpin RNAs (shRNAs) targeting a common mRNA sequence shared by all PIPKIγ isoforms (shIγ) or a PIPKIγ_i2 specific sequence (shIγ_i2), respectively. Cells stably expressing a non-specific shRNA (shNC) were used as control. The knockdown efficiency of both shRNAs was confirmed by immunoblotting using indicated antibodies. (B) MTT assay was applied to 4T1 cells expressing shNC, shIγ or shIγ_i2 in 96-well plates. Triplicates were set for each time point. (C) 4T1 cells expressing shNC, shIγ or shIγ_i2 were subjected to immunoblotting to determine the levels of phosphorylated AKT (pAKT), total AKT, phosphorylated ERK1/2 (pERK1/2), and total ERK1/2 proteins. b-actin was blotted in same cell lysates for loading control. (D) 4T1 cells expressing shNC, shIγ or shIγ_i2 were subjected to wound healing assay. Representative images were taken at 0 and 12 hours after the wound was created. (E) Motility of cells descripbed in (D) was calculated by measuring the width of the wound. Migration Index = ($width_{0h}$-$width_{12h}$)/$width_{0h}$. (F) EGF-induced directional migration was measured using Boyden chamber. Cells migrated to the reverse side of the membrane were stained with DAPI, counted under microscope, and normalized against non-treated samples. Duplicates were used for each condition. (E and F) results from three independent experiments were statistically analyzed and plotted. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Consistantly with the role of PIPKIγ_i2 in focal adhesion assembly [8], we have shown previously that PIPKIγ_i2 is required for cell migration of human breast cancer MDA-MB-231 cells [9]. We thus examined the effect of PIPKIγ_i2 on mobility of 4T1 cells using *in vitro* cell migration assays. Results from wound-healing assay suggested that the migratory capacity of PIPKIγ_i2-depleted 4T1 cells was significantly reduced (**Figure 1D**). The quantification of the width of remaining wound area showed a 50% decrease in PIPKIγ_i2-depleted 4T1 cells com-

pared to control cells, similar as pan-PIPKIγ depleted cells (**Figure 1E**). Moreover, expression of PIPKIγ correlated positively with epidermal growth factor receptor (EGFR) expression in breast cancer patients [11]. In the context that EGFR signaling plays critical role in breast cancer progression partially by promoting migration and dissemination of tumor cells, we next examined the directional cell migration induced by EGF using modified Boyden chamber. As expected, loss of PIPKIγ_i2 led to a significant decrease of the EGF-induced directional migration in 4T1 cells, similar as the pan-PIPKIγ knockdown (**Figure 1F**). Together, our results suggested that PIPKIγ_i2 indeed is vital for 4T1 cells to exert the capacities of migration and might play an important role in breast cancer metastasis.

*Depletion of PIPKIγ_i2 suppressed the invasion potential of 4T1 cells in vitro*

The ability of cells to invade into the surrounding environment is a critical step for local dissemination of tumor cells, the early step of metastasis [16, 17]. To determine the effect of PIPKIγ_i2 depletion on cell invasion, we employed the *in vitro* invasion assay using the Matrigel-coated Transwells. As shown in **Figure 2A**, the invasive potential of 4T1 cells was significantly weakened by both pan-PIPKIγ depletion and PIPKIγ_i2 depletion to a comparable extent (**Figure 2A**). MMP9, a matrix metalloproteinase (MMP) that contributes to tumour invasion, is highly expressed in human cancers including breast cancer [18]. A direct correlation between MMP9 expression and breast cancer progression has been established [19]. We found that PIPKIγ_i2 depletion and pan-PIPKIγ depletion both dramatically repressed MMP9 levels in 4T1 cells (**Figure 2B**), suggesting that PIPKIγ_i2 may be required for tumor cell invasion by supporting the MMP9-dependent matrix degradation. Although normal 4T1 cells exhibited rather mild matrix degradation, PIPKIγ_i2-depleted 4T1 cells lost the ability to degrade the gelatine matrix in the same time frame (**Figure 2C**). The percentage of cells capable of degrading matrix and the area of degradation were reduced to a comparable level in pan-PIPKIγ and PIPKIγ_i2 depleted cells. Additionally, the average number of fluorescence-negative speckles created by each degradation-performing cell decreased in both pan-PIPKIγ and PIPKIγ_i2 depleted cells (**Figure 2D**), which coincided with the decreased degradation area per cell of these two groups. Our results suggest that depletion of PIPKIγ_i2 yielded similar effect on suppressing the invasion potential of 4T1 cells as pan-PIPKIγ depletion, indicating that PIPKIγ_i2 may play a more important role among all PIPKIγ isoforms in promoting cancer metastasis.

*PIPKIγ_i2 depletion is not sufficient to suppress tumor growth and metastasis in vivo*

We reported previously that depletion of all PIPKIγ isoforms improved the survival of 4T1 tumor bearing mice by decreasing the tumor growth and lung metastasis [14]. As summarized above, our results from *in vitro* studies suggested that PIPKIγ_i2 likely is the PIPKIγ isoform that supports tumor progression including growth and metastasis. Thus, we further explored whether PIPKIγ_i2 depletion in 4T1 tumor cells affects tumor progression *in vivo*. Similar as we have reported [14], pan-PIPKIγ depleted 4T1 tumors grew significantly slower than control tumors in BALB/c mice (**Figure 3A** and **3B**). Animals bearing pan-PIPKIγ depleted 4T1 tumors suffered substantially less lung metastasis (**Figure 3C**) and had significantly better survival (**Figure 3D**). Unexpectedly, PIPKIγ-i2 depleted 4T1 tumors showed similar *in situ* growth (**Figure 3A** and **3B**) and lung metastasis (**Figure 3C**) comparing to the control tumors. Moreover, PIPKIγ_i2 depletion did not improve the survival rate of tumor bearing mice as the pan-PIPKIγ depletion did (**Figure 3E**). These data indicated that PIPKIγ_i2 alone is not sufficient to support the tumor growth and lung metastasis in mice. This is inconsistent with the results we obtained from *in vitro* studies using cells cultured as 2D monolayer.

It has been well documented that tumor cells receive completely different stimuli *in vivo* comparing to *in vitro* 2D culturing condition by interacting with the surrounding microenvironment. Not only these *in vivo* signals from variant types of host cells in the tumor microenvironment influence tumor progression, but also tumor cells communicate with and manipulate surrounding host cells to build a tumor-friendly microenvironment [20-23]. In the past couple of decades, roles of hypoxia [20], tumor-associated macrophages [21], and tumor-induced angiogenesis [21-23] in promoting metastasis and therapy resistance of cancers have been

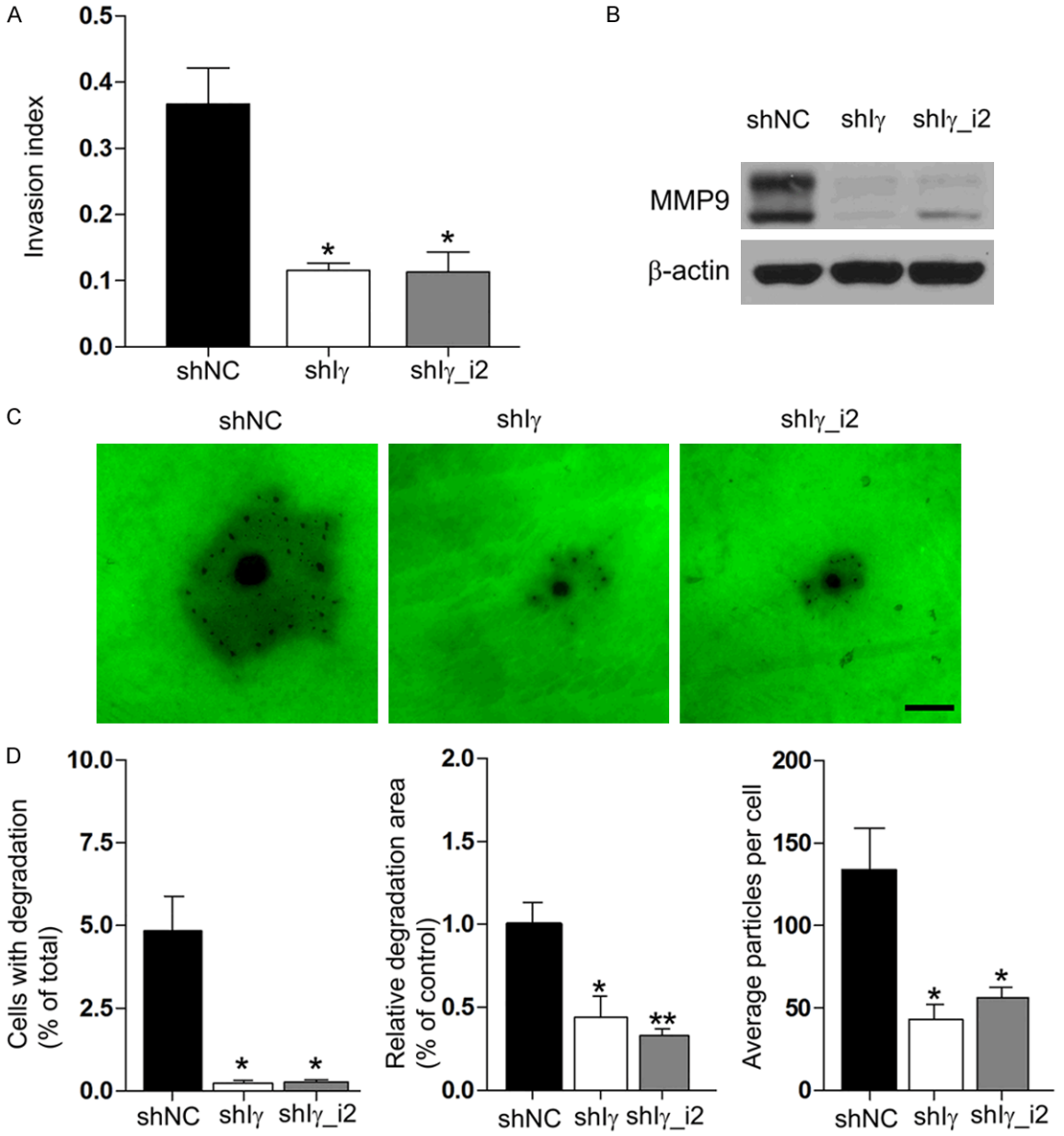


**Figure 2.** Loss of PIPKIγ_i2 alone exhibited similar effect on inhibiting the *in vitro* invasion of 4T1 cells as depletion of all PIPKIγ isoforms. (A) 4T1 cells stably expressing control shRNA (shNC), pan-PIPKIγ shRNA (shIγ), or PIPKIγ_i2 shRNA (shIγ_i2) were subjected to *in vitro* invasion assay using Matrigel-coated Transwell invasion chamber. Duplicates were applied to each experimental sampel and the invasion index was calculated following the manufacturer's introduction. (B) MMP9 expression in 4T1 cells described in (A) was analyzed by immunoblotting using indicated antibodies. (C) Representative images of Matrix degradation performed by each group of 4T1 cell. (D) The percentage of cells showing matrix degradation (% of total counted cells), relative area of degraded matrix on slide (% of control group), and particles per degraded cell were quantified under microscope (total counted cells = 200). (A and D) results from three independent experiments were statistically analyzed and plotted. $*P < 0.05$; $**P < 0.01$.

widely appreciated. To gain insights into the different effects of pan-PIPKIγ depletion and PIPKIγ_i2 depletion on 4T1 tumor progression, we analyzed the hypoxia levels, tumor-associated macrophages, and angiogenesis in control, PIPKIγ_i2-depleted, or pan-PIPKIγ-depleted 4T1 tumors. By examining pimonidazole adduct formation, we found that depletion of PIPKIγ_i2 could not reduce the hypoxia levels in tumors as pan-PIPKIγ depletion could do (**Figure 4A**),

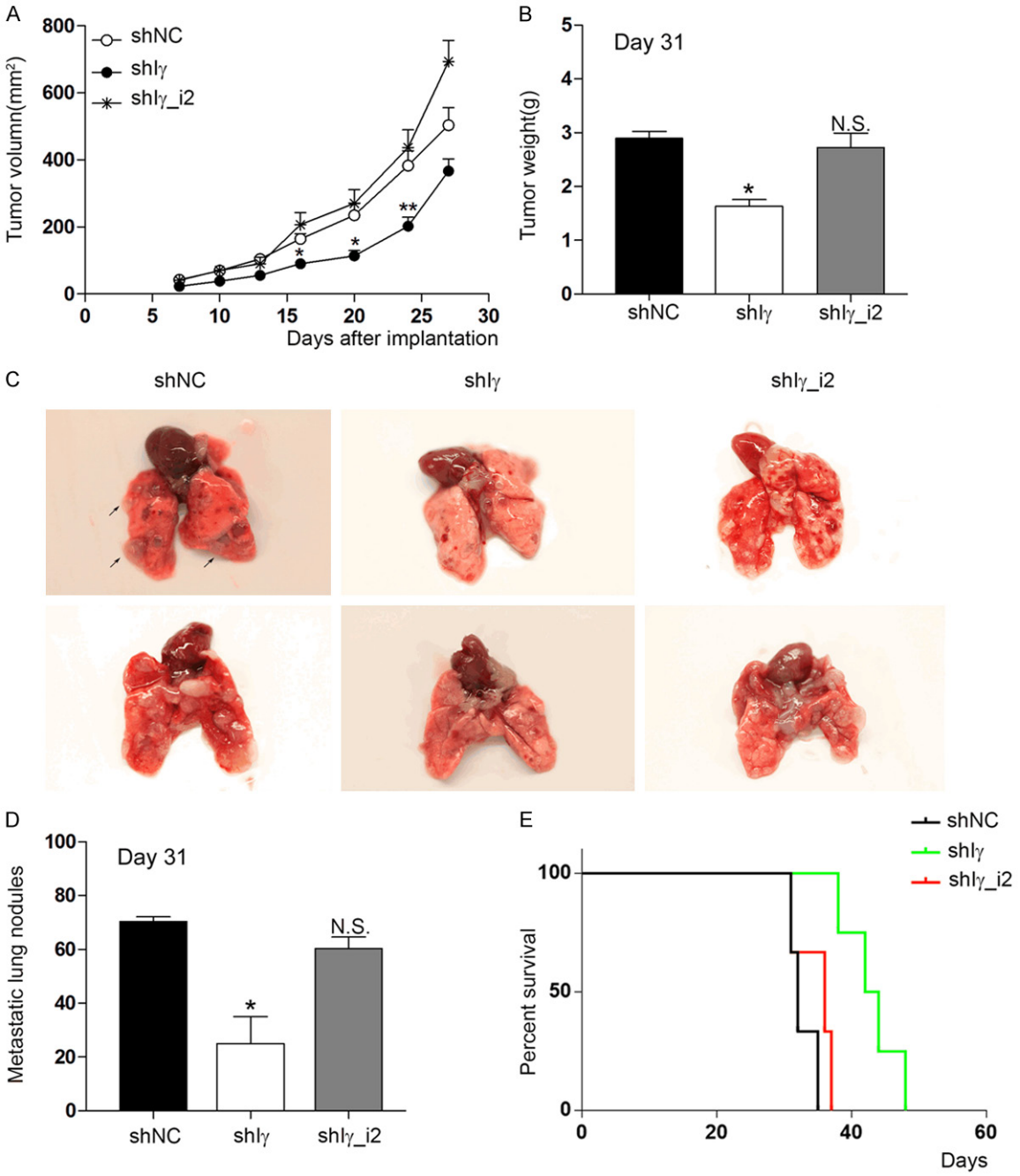


**Figure 3.** Depletion of PIPKIγ_i2 alone failed to suppress the growth and lung metastasis of 4T1 tumors. (A) Growth curves of tumors in BALB/c mice inoculated with control (shNC), pan-PIPKIγ depleted (shIγ), or PIPKIγ_i2-depleted (shIγ_i2) 4T1 cells. Indicated 4T1 cell lines were injected into mammary fat pad of BABL/c mice. Tumor volume was measured with calipers and calculated by using the standard formula. (B) On day 31 after injection, mice were terminated and tumors were excised and weighted. (C) Comparison of surface nodules on gross lungs from control, PIPKIγ or PIPKIγi2-depleted 4T1 bearing mice on day-31 after implantation. (D) The lung metastasis of each group described in (C) was quantified by manually counting nodules visible on the lung surface. (E) Survival (Kaplan-Meier) curve of BALB/c mice inoculated with indicated 4T1 tumor cells (n = 6/group). Log-rank text: $P = 0.0101$ (shNC vs. shIγ); $P = 0.2116$ (shNC vs. shIγ_i2). (A, B, and D) 6 mice/group. $^*P < 0.05$; $^{**}P < 0.01$; N.S., no significant difference.

suggesting that mice bearing pan-PIPKIγ depleted tumors suffered less from hypoxia-dependent angiogenesis and metastasis [24]. Tumor-associated active macrophages marked

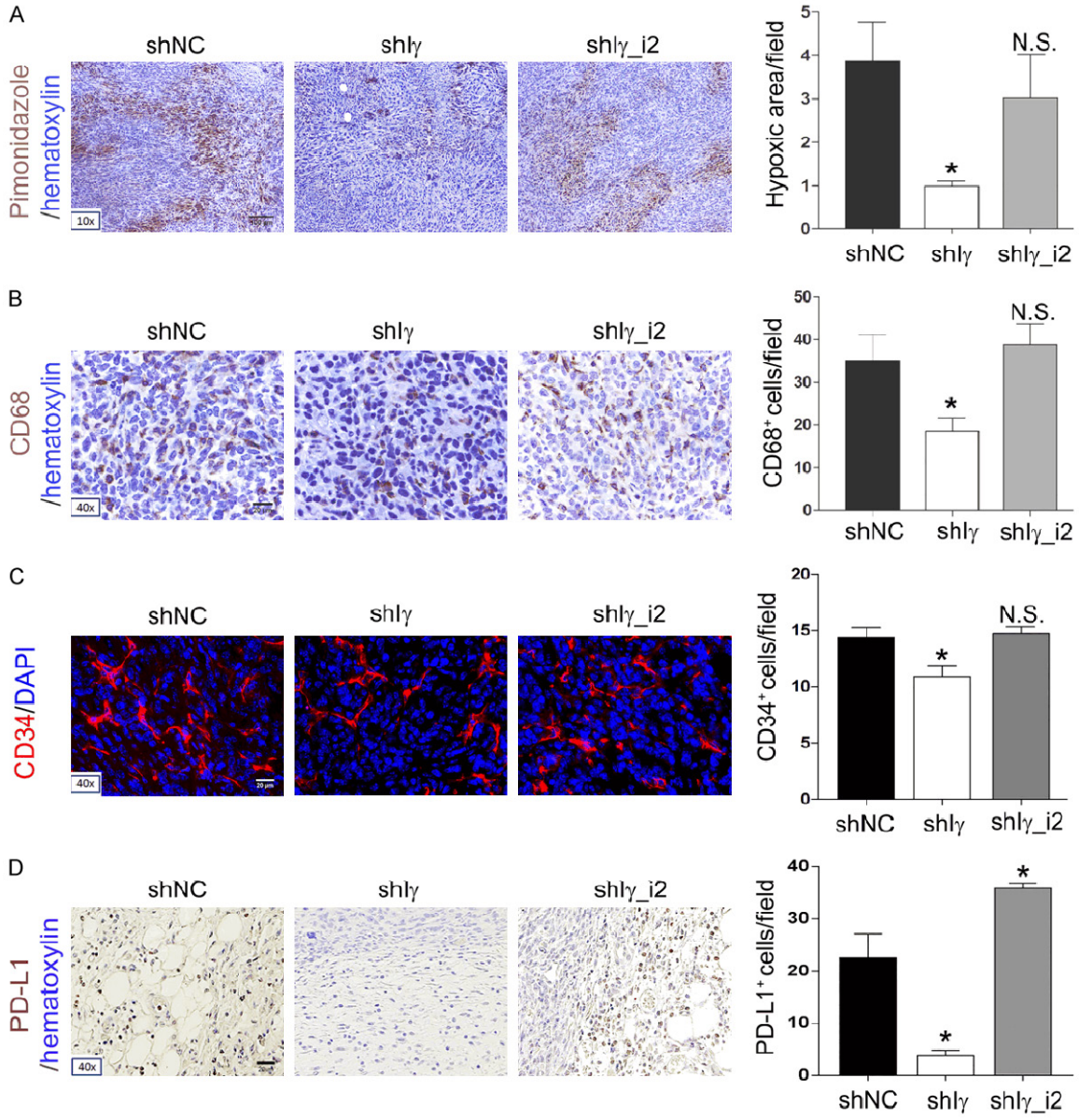


**Figure 4.** Suppressing PIPKIγ_i2 expression appeared no effect on tumor interaction with the host microenvironment. 4T1 tumor cells stably expressing control shRNA (shNC), pan-PIPKIγ shRNA (shIγ), or PIPKIγ_i2 specific shRNA (shIγ_i2) were inoculated subcutaneously into the mammary fat pat of BALB/c mice (n = 6/group). On Day-31 post inoculation, animals were terminated and tumors were collected and fixed by formalin for tissue processing. A. Hypoxia levels were measured by examining pimonidazole adducts formation. Left, representative images. Right, Quantitative analysis of the percent hypoxic area in the whole tumor section. Nuclear counterstain: hematoxylin stain. B. The infiltrated macrophages in tumors were detected by immunohistochemistry staining for CD68. Left, representative images. Right, Average number of CD68-positive cells in each field. C. The microvessel density in tumors was assessed using the marker of endothelial cells CD34. Left, representative images. Right, Average number of CD34-positive cells in each field. D. PD-L1 positive cells were stained by IHC in in tumors . Left, representative images. Right, Average number of PD-L1 positive cells in each field. A-D. 5 fields, 2 slides per tumor, and 3 mice per experimental group were quantified, statistically analyzed and plotted. *$P < 0.05$; N.S., no significant difference.

by CD68 are significantly less in pan-PIPKIγ depleted tumors than in control tumors; however, PIPKIγ_i2-depleted tumors associated with much more CD68-positive cells at a level comparable to control tumors (**Figure 4B**). Macrophage infiltration has been correlated with increased tumor-associated angiogenesis and poor prognosis [21]. Another important

impact resulted from tumor-associated macrophages is their ability to induce PD-L1 expression in tumor and inflammatory cells. Corresponding to the decreased macrophage infiltration, PD-L1 expression in tumor-associated microenvironment was also lower in pan-PIPKIγ-depleted tumors compared to control and PIPKIγ_i2-depleted tumors (**Figure 4D**). In the context that PD-L1 via activating PD-1 suppresses T cell activation and inhibits anti-tumor immunity [26], our result suggest that 4T1 tumor cells with all PIPKIγ isoforms depleted will be much more prone to be eliminated by the host immune system, however depleting PIPKIγ_i2 alone had no such an effect. To test the angiogenesis level in tumors, we employed CD34 as a marker, because CD34 is a cell surface sialomucin expressed by epithelial, vascular, and stromal cells that concern hematopoietic reconstitution and angiogenesis [25]. When we evaluated CD34-positive cells in 4T1 tumors obtained from all three experimental groups, no difference was observed between the control and PIPKIγ_i2-depleted tumors (**Figure 4C**). However, pan-PIPKIγ-depleted tumors exhibited significantly less CD34 expression comparing to control tumors (**Figure 4C**). Based on these data, we reason that depletion of all PIPKIγ isoforms has much more significant effect on interrupting the ability of tumor cells to acclimate to and amend the host microenvironment. Depletion of PIPKIγ_i2 alone is not sufficient to achieve this.

*Loss of all PIPKIγ isoforms but not PIPKIγ_i2 alone partially reverses the EMT*

To investigate the molecular base underlying the progression difference between PIPKIγ_i2 depleted 4T1 tumor and pan-PIPKIγ depleted 4T1 tumor, we investigated and compared more signaling pathways in these cells. As shown in **Figure 5**, the EMT status of pan-PIPKIγ depleted 4T1 cells was different from the parental and PIPKIγ_i2 depleted cells. Comparing to 4T1 cells expressing the negative control shRNAs, pan-PIPKIγ depleted cells showed significantly upregulated E-cadherin and ZO-1, two typical epithelial markers, and downregulated vimentin and Snail, two typical mesenchymal markers (**Figure 5A**). However, PIPKIγ_i2 depleted cells exhibited similar levels of E-cadherin, ZO-1 and Snail, and slightly increased vimentin comparing to control cells (**Figure 5A**). These data indicates that loss of all PIPKIγ isoforms at least partially reversed the EMT, but depletion of PIPKIγ_i2 alone had no such an effect. The EMT is a cancer-promoting transdifferentiation programme driven by EMT transcription factors (EMT-TFs) such as Snail, ZEB and Twist proteins, which suppress the expression of epithelial proteins and induce the expression of mesenchymal proteins [27]. Our results clearly suggest that the pan-PIPKIγ depleted 4T1 tumor cells possessed less EMT characteristics and were more epithelial-like comparing to control tumor cells; whereas cells with only PIPKIγ_i2 depleted had no such a change of de-malignancy. Moreover, we found that pan-PIPKIγ depleted 4T1 cells formed much less colonies in soft agar comparing to control or PIPKIγ_i2 depleted cells (**Figure 5B**). This suggests that pan-PIPKIγ depletion made 4T1 cells less capable for anchorage-independent growth. Cells undergoing EMT are more de-differentiated and are more resistant to environmental stress [27]. In this context, the decreased anchorage-independent growth of pan-PIPKIγ depleted cells plausibly reflects the consequence of decreased Snail and reversed EMT.

Breast cancers, including TNBCs, are highly heterogeneous. To determine whether the different effects of pan-PIPKIγ depletion and PIPKIγ_i2 depletion on the *in vitro* and *in vivo* behaviors of 4T1 tumors are cell type specific, we examined human TNBC cell line MDA-MB-231 cells, which is one of the most aggressive human TNBC cell lines. Similar as in 4T1 cells (**Figure 1B**), depletion of pan-PIPKIγ and PIPKIγ_i2 both significantly inhibited cell growth when determined by using MTT assay (**Figure 6A**). When we determined the cell mobility, PIPKIγ_i2-depleted MDA-MD-231 cells migrated notably slower than the control cells, similar as pan-PIPKIγ depleted cells (**Figure 6B**). These results suggest a conclusion as we obtained in 4T1 cells that PIPKIγ_i2 is the major isoform of PIPKIγ in promoting cell adhesion and migration *in vitro*. We also reason that the *in vitro* survival and/or proliferation of MDA-MB-231 cells under the 2-D culture condition likely is more dependent upon the integrin-mediated adhesion to the extracellular matrix, thus loss of PIPKIγ_i2 or all PIPKIγ isoforms both interrupted cell survival drastically (**Figure 6A**). Similar as in 4T1 cells, the protein level of

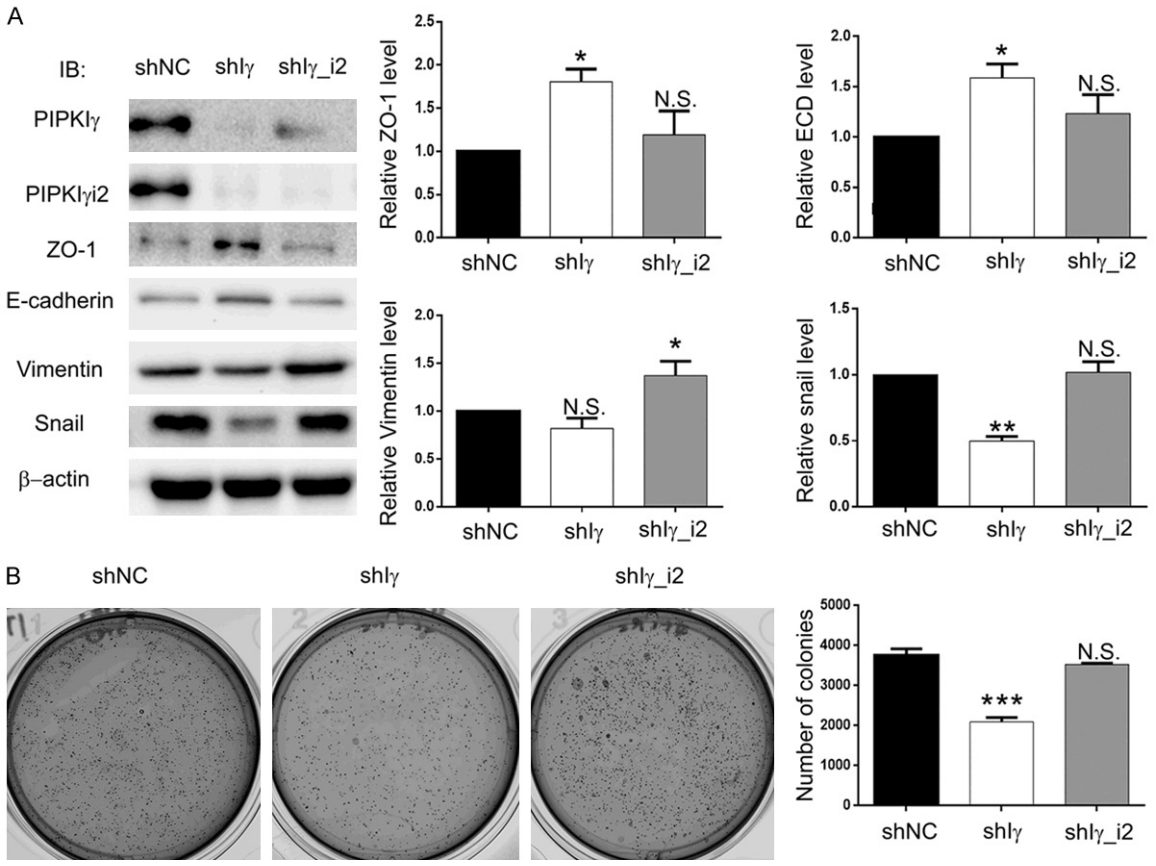


**Figure 5.** Depletion of pan-PIPKIγ, but not PIPKIγ_i2 alone, inhibited the EMT. A. Protein levels of indicated EMT markers were determined by immunoblotting in control (shNC), pan-PIPKIγ (shIγ) or PIPKIγ_i2-depleted (shIγ_i2) 4T1 cells. ECD, E-cadherin. Levels of indicated proteins were then determined by measuring the corresponding band using ImageJ. Results from three independent experiments were statistically analyzed and plotted. B. $1 \times 10^4$ cells of each indicated group were subjected to soft agar colony formation assay. Left, Representative images were required on day 8 by Gelcount software. Results from three independent experiments were statistically analzyed and plotted. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; N.S., no significant difference.

Snail in pan-PIPKIγ depleted MDA-MB-231 cells was significantly decreased comparing to the control cells; whereas the specific depletion of PIPKIγ_i2 had no such an effect (**Figure 6C**). To determine how depletion of all PIPKIγ isoforms or only the isoform 2 would affect the *in vivo* progression of MDA-MB-231 tumors, we established MDA-MB-231 cell lines stably expressing pan-PIPKIγ or PIPKIγ_i2-specific shRNA using lentivirus. Similar as the transient knockdown-mediated by siRNA, shRNA targeting pan-PIPKIγ, but not PIPKIγ_i2, reduced the protein level of Snail, although both efficiently eliminated the expression of their own targets (**Figure 6D**). More interestingly, the *in situ* growth of pan-PIPKIγ depleted MBA-MB-231 tumors in NOD SCID mice was markedly slower than the control tumors; however the PIPKIγ_i2 depleted MBA-MB-231 tumors grew at a comparable rate as the controls (**Figure 6E**). Results from these studies follow the same trend as we observed in 4T1 tumors, i.e. PIPKIγ_i2 governs the cell mobility and contributes to cell survival under 2D *in vitro* culture condition, but has no effect on the *in vivo* tumor growth and metastasis. On the other hand, our results implicated another PIPKIγ isoform(s) that participates in the regulation of Snail protein levels, the EMT, and consequently the *in vivo* progression of TNBC tumors.

## Discussion

PIPKIγ involves in various biological functions through controlling the generation of PtdIns(4,5)$P_2$. It has been reported that the expression of PIPKIγ is inversely correlated with the survival of breast cancer patients [11]. Our previous studies have demonstrated PIPKIγ knockdown inhibited primary tumor growth and metastasis

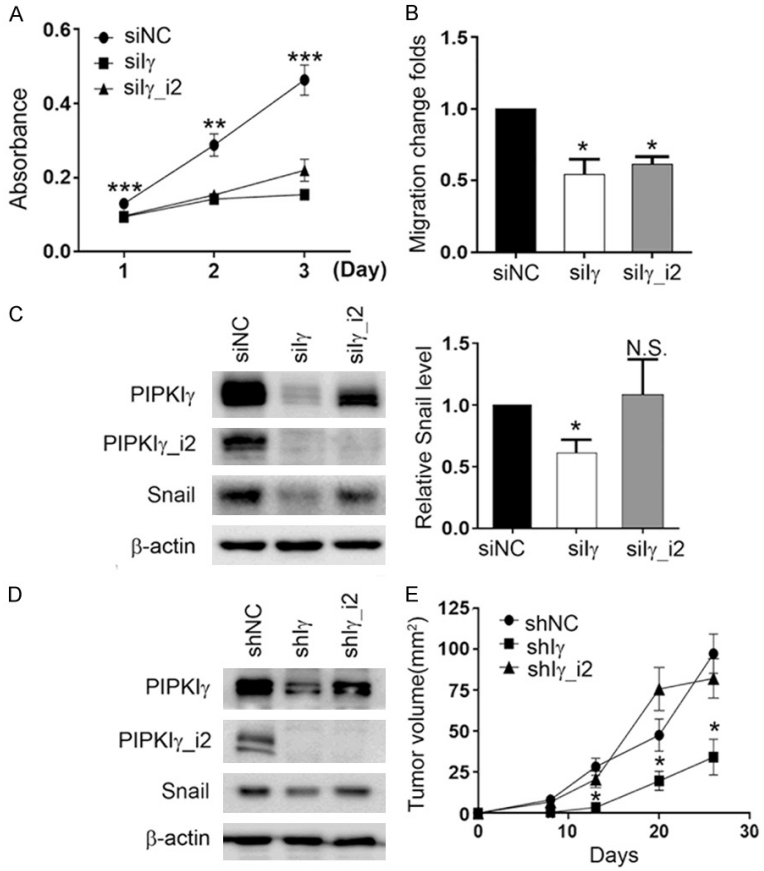


**Figure 6.** PIPKIγ_i2 deficiency inhibits the *in vitro* but not *in vivo* aggressiveness of MDA-MB-231 tumors. (A-C) MDA-MB-231 cells were transfected with siNC, siγ or siγ_i2 for 48 hours, and them subjected to MTT assay to determine cell survival/proliferation (A), to Boyden Chamber assay to determine FBS (10%)-induced directional cell migration (B), or immunoblotting to analyze Snail protein levels (C). For Boyden Chamber *in vitro* cell migration assay (B), results were normalized against the migration index of control cells. Results from three indicated experiments were quantified, statistically analyzed, and plotted. (D and E) MDA-MB-231 cells stably expressing the control (shNC), pan-PIPKIγ (shIγ), or PIPKIγ_i2 (shIγ_i2) shRNAs were established using lentivirus. (D) Depletion of pan-PIPKIγ or PIPKIγ_i2 were confirmed by immunoblotting. Snail protein levels were examined as well. (E) Cells from each of these MDA-MB-231 cell lines ($2 \times 10^6$ cells/ mouse) were injected into mammary fat pad of in NOD SCID mice (n = 6). Tumor volume was measured with calipers weekly and calculated by using the standard formula to establish the growth curve of *in situ* tumors. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; N.S., no significant difference.

of the 4T1 triple negative breast cancer, however, the underlying molecular mechanism is not clear. Moreover, which of the six alternative splicing isoforms of PIPKIγ plays the major role in promoting cancer metastasis and progression is unknown. We reported previously that PIPKIγ_i2 is the only isoform of PIPKIγ that specifically targets to focal adhesions and regulates focal adhesion turnover [8]. PIPKIγ_i2 is required for the directional cell migration and invasion, which have always been considered essential for the local dissemination of tumor cells, therefore are necessary for metastasis to occur. In this context, we proposed that PIPKIγ_i2 is the PIPKIγ isoform that makes the major contribution to metastasis formation by promoting cell migration and invasion. To test this hypothesis, we determined the role of PIPKIγ_i2 in the progression of breast cancer *in vitro* and *in vivo*.

Our results from *in vitro* studies revealed that depletion of PIPKIγ_i2 alone exhibited similar effect as depletion of all PIPKIγ isoforms on a series of cellular events involved into tumor progression. These include cell proliferation, migration, matrix degradation, and invasion. Moreover, matrix metalloproteases (MMPs) mediate extracellular matrix (ECM) degradation that is critical for tumor invasion and metastasis [28, 29]. Among MMPs, MMP-9 is often observed to be upregulated in aggressive malignancies [18, 19]. We found that MMP9 was markedly decreased in both pan-PIPKIγ and PIPKIγ_i2 depleted cells to a similar extent. Together, our results suggest that PIPKIγ_i2, as we expected, likely is the major isoform of PIPKIγ in regulating metastasis-related cellular events. In addition, depletion of PIPKIγ_i2 alone showed the similar inhibition of Akt and Erk1/2 activity as depletion of all PIPKIγ isoforms. The PI3k/Akt and MAPK/ERK1/2 pathways play important roles in regulating cell proliferation and survival [30]. Consistently, pan-PIPKIγ depletion and PIPKIγ_i2 depletion both inhibited cell proliferation and survival of 4T1 cells.

However, we surprisingly found out that depletion of PIPKIγ_i2 alone had no effect on the progression of 4T1 tumor in BALB/c mice.

PIPKIγ_i2 depleted 4T1 tumors grew *in situ* and spread to lung in a completely comparable rate as the wild type tumors. This is totally in an opposite to the effects of pan-PIPKIγ depletion, which efficiently blocked tumor growth and metastasis. When we inoculated 4T1 tumor cells via the tail vein to bypass the early local dissemination steps, depletion of PIPKIγ_i2 still showed no effect on lung metastasis (Data not shown), although depletion of all PIPKIγ isoforms indeed significantly inhibited metastasis under this condition. These results clearly demonstrated that PIPKIγ_i2 does not play an essential, decisive role in the formation of metastasis, although it is an important regulator for cell migration and invasion. This suggests that the abilities of cells to migrate and invade on 2-D surface are not relevant, or at least not decisive, to the formation of metastasis, although the level of PIPKIγ_i2 depletion we achieved likely was not be low enough to entirely eliminate the migration or invasion. Nevertheless, our results suggest that other isoforms of PIPKIγ govern more important cellular events that make essential contribution to the development of metastasis of 4T1 tumors.

To elucidate the impact of PIPKIγ_i2 depletion in progression of 4T1 tumors, we examined multiple characteristics of PIPKIγ_i2 depleted 4T1 tumors and compared with control and pan-PIPKIγ depleted tumors. These include the features that highly correlate with malignancy and poor prognosis, such as tumor hypoxia level, tumor-infiltrated macrophage, and tumor-associated angiogenesis. These are events happening in tumor-associated microenvironment, because the abilities of tumor cells to use and alter the host cells/tissues to benefit themselves are critical for tumor progression. Hypoxia enhances the epithelial to mesenchymal transition (EMT) in tumor cells by inducing the expression of EMT associated-transcription factors [31, 32]. Cancer cells undergoing EMT not only become more potentiated to acquire invasive properties, but also more capable of remodeling microenvironment to a favorable status for cancer metastasis [27]. Among cell types associated with tumor microenvironment, macrophages are most influential for tumor progression. Clinical studies showed that increased $CD68^+$ macrophage index is associated with high vascularity and nodal metastasis, as well as reduced recurrence-free and overall survival in human breast cancer [33]. Macrophage-secreted cytokines can upregulate PD-L1 expression in tumor cells and inflammatory cells in tumor-associated environment, which will suppress T-cell activation and favor immune escape [26, 34]. PD-L1 expression on host cells can also affect response rate of PD-L1 immunotherapy [35], which indirectly indicates PD-L1 on host cells might contribute to tumoral immune escape. In addition to hypoxia and macrophages, we also determined the density of newly formed microvessels in tumors. Angiogenesis is important in invasive tumor growth and metastasis and is a major prognostic factor in various forms of cancer, including invasive carcinoma of the breast [36]. Results from these analyses clearly showed that PIPKIγ_i2-depleted 4T1 tumors exhibited the same levels of hypoxia, macrophage infiltration, and microvessel density as wild type tumors, although depletion of all PIPKIγ isoforms resulted in significant reduction of all these aspects. As expected, PD-L1 expression in tumor microenvironment was also decreased in pan-PIPKIγ depleted tumors but not in PIPKIγ_i2 depleted or wild type tumors. These data are consistently with the outcome of tumor progression of each experimental group, and indicates that depletion of PIPKIγ_i2 alone is not sufficient to eliminate the aggressiveness of 4T1 tumors; however, depletion of all PIPKIγ isoforms can.

Accumulating evidence suggests that the EMT programme promotes the de-differentiation of tumor cells. This enhances the diversity of tumor evolution in response to extracellular stimuli and endorses tumor cells with higher potential of survival against variant environmental stresses, including anchorage-independent survival in blood stream, survival in strange microenvironments (distant organ), survival under hypoxia, and survival after radiotherapy and chemotherapy [27, 31, 32]. These characteristics increase tumor metastasis and resistance to treatments. To find out the molecular mechanism underlying our observation that depletion of all PIPKIγ isoforms can, yet depletion of PIPKIγ_i2 alone cannot, suppress tumor metastasis and progression, we determined the EMT status of 4T1 cells treated with pan-PIPKIγ or PIPKIγ_i2 shRNAs. Interestingly, we found that cells with all PIPKIγ isoforms depleted showed substantial decreases of

mesenchymal markers including the key EMT transcription factor Snail, and correspondingly upregulated epithelial markers. Based on these results, we reason that one PIPKIγ isoform is important for 4T1 cells to maintain a status more closely to the mesenchymal end on the epithelial-to-mesenchymal status spectrum. This makes 4T1 cells more flexible and adapt to the microenvironment more quickly, which is consistent with our results that pan-PIPKIγ depleted 4T1 cells exhibited less survival when cultured in anchorage-independent condition. However, depletion of PIPKIγ_i2 had no such an effect on EMT status of 4T1 cells. Similar results were observed in human TNBC cell line MDA-MB-231 cells, further supporting this conclusion. These results provide an explanation on the molecular level to our observation that PIPKIγ_i2 depletion could not suppress tumor metastasis and progression, whereas pan-PIPKIγ depletion could.

In summary, results from our current studies support two important conclusions. First, the abilities of tumor cells to proliferate, migrate, and invade on 2-D matrix under the *in vitro* culture condition is not necessarily relevant to their malignancy and aggressiveness *in vivo*. The ability to survive against variant environmental stresses is more critical for the formation of metastasis, which is likely dependent on and promoted by the EMT programme. Second, however, PIPKIγ_i2 as the PIPKIγ isoform specifically targeting to focal adhesions is only important for cell mobility, but not survival. These data support that PIPKIγ_i2 need to cooperate other isoforms to exert the inhibition effect on tumor growth and metastasis. Further study should be conducted to determine which isoform of PIPKIγ regulates the EMT, especially the expression of the EMT transcription factor Snail, the underlying molecular mechanisms, and the subsequent implication in cancer metastasis and progression.

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## Disclosure of conflict of interest

None.

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