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14. ABSTRACT

Breast cancer is a major public health problem in the United States, and the American Cancer Society estimates that 40,000 women died from this disease in 2017. Tumor metastasis is the major cause of mortality in human breast cancer, and effective treatment of metastatic disease will require a better understanding of the signaling mechanisms that drive breast cancer cell invasion (the ability of tumor cells to move away from the primary tumor and into surrounding tissue). Our data suggests that a specific type of phosphoinositide 3-kinase, called PI3Kβ, is strongly implicated in breast cancer metastasis. This proposal examines how PI3Kβ regulates both tumor cells and the immune cells that modulate tumor cell behavior, leading to increased metastasis. Our study could establish PI3Kβ as an important new drug target for the treatment of metastatic disease.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction. The overall focus of this research is to define the mechanisms that drive breast cancer metastasis. The work is based on our preliminary data showing that a single isoform of phosphoinositide 3-kinase, PI3Kβ, is required for two distinct cellular structures: macropinosomes and invadopodia. We proposed the hypothesis that macropinocytosis is required for invadopodia formation, by the targeting of integrins from the dorsal surface of the cell to ventral invadopodia. We further proposed, based on data using primary macrophages from mutant PI3Kβ mice, that loss of PI3Kβ signaling in stromal cells will inhibit metastasis *in vivo.* Thus, these studies will provide new mechanistic insights into tumor cell invasion, and new physiological insights into PI3Kβ signaling in an animal model of breast cancer metastasis.

2. Keywords. Metastasis, phosphoinositide 3-kinase, macropinocytosis, invasion, invadopodia, integrins, mouse models.

3. Accomplishments.

A. Major goals of the project:

a. Define the coupling between macropinosomes and podosomes.

i. Examine invadopodia formation/function under conditions of macropinosome inhibition. Target date: March 2020. Completed date: October 2020.

ii. Analyze the regulation of integrin signaling/trafficking by PI3Kβ. Target date: September 2020. Completed date: December 2020. Hypothesis invalidated.

iii. Regulation of the invadopodia proteome by macropinocytosis
Target date: February 2022. % completed: 10%. Hypothesis invalidated.

b. Test the role of stromal PI3Kβ in tumor growth.

i. Tumor growth in WT and mutant PI3Kβ mice
Target date: March 2021. % completed: 20%.

ii. Tumor metastasis in WT and mutant PI3Kβ mice
Target date: March 2022. % completed: 20%.

iii. Role of PI3Kβ in extravasation
Target date: March 2020. % completed: 75%.

iv. PI3Kβ macrophage-specific mutant mice.
Target date: March 2022. % completed: 0%.

B. What was accomplished.

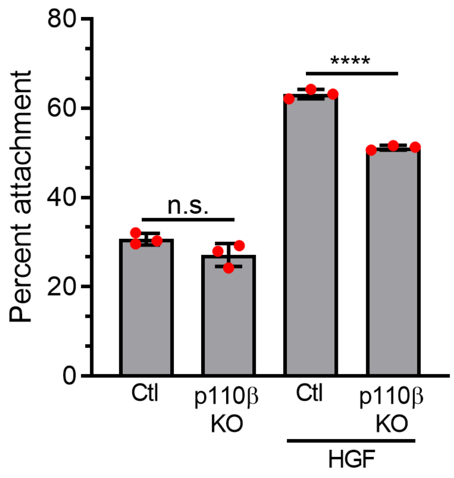

Figure 1. Integrin activation by HGF is inhibited in PI3Kβ KO MDA-MB-231 cells.

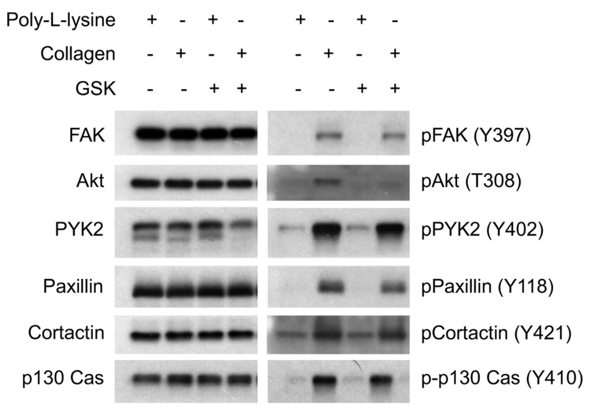

Figure 2. MDA-231 cells were treated with DMSO or the PI3Kβ-selective inhibitor GSK2636771, then plated on collagen I for 20 min.

a. PI3Kb in integrin signaling. Our published data show that PI3Kβ is required for integrin-dependent cellular responses in tumor cells. We have found that HGF stimulates inside-out integrin activation in MDA-MB-231 cells, as measured by an increase in adhesion to fibronectin (Fig. 1). While this response is reduced in PI3Kβ KO MDA-MB-231 cells, the reduction is fairly small. We also measured signaling responses to integrin activation by measuring the phosphorylation of known integrin effectors (1-6) after plating MDA-MB-231 cells on collagen I. As shown in Fig. 2, integrin-stimulated kinase signaling to FAK, Pyk2, paxillin, cortactin and p130Cas were unaffected by inhibition of PI3Kβ with the selective inhibitor GSK2636771; Akt phosphorylation was partially inhibited as expected (7). These data are not consistent with a major role for PI3Kβ in integrin signaling in breast cancer cells, and we have focused on Aim 2 instead.

b. Animal studies. All laboratories at the Albert Einstein College of Medicine, with the exception of laboratories directly involved in Covid-19 research, were closed between mid-March and June 1, 2020. Laboratories are still limited to 50 % of full occupancy. In addition, due to staffing problems in the animal facility, investigators were asked to cull back colonies to maintenance levels during the shutdown. This essentially ended our breeding of homozygous mutant PI3K mice for over 3 months. While we have built back the colonies to their former level, we have now encountered a major problem with the frequency of homozygous mutant PI3Kβ mice, which remains well below predicted Mendelian ratios. We are also frequently losing litters from het/het crosses, for unclear reasons. Introduction of "fresh" mice from Jackson labs into our heterozygous population has not improved the situation. Thus, our ability to produce homozygous mutant PI3K mice has been significantly below expectations.

An additional problem has been the use of E0771 cells to produce primary tumors and spontaneous metastases. After orthotopic injection of E0771 cells into the mammary fat pad of C57Bl/6 mice, tumor take is seldom better than 50%, and tumor growth is extremely variable. Even with large tumors, we find that spontaneous metastasis to the lung is low. After a major effort to improve the system by variations in injection site, tumor cell number, and presence of Matrigel, we have decided to switch to a different syngeneic cell line, AT3 cells. We have recently obtained a metastatic clone of GFP-labeled AT3 cells from the Hudson lab at Georgetown, and we are testing their metastatic potential in our mice. Given the difficulties in completing these experiments in global PI3Kβ mutant mice, we have held off on producing the macrophage-specific knock-in mice.

c. Interactions between breast cancer cells and stromal cells - macrophages. Given the problems with generating mutant mice *in vivo* for experiments, we have in the interim focused on *in vitro* analysis of the role of PI3Kβ in tumor cell-stromal cell interactions. We have made a number of exciting discoveries.

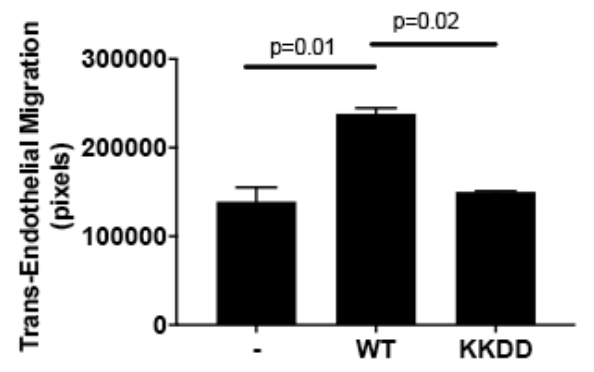

Figure 3. Macrophage-stimulated transendothelial migration of MDA-MB-231 cells is inhibited by mutation of PI3Kβ.

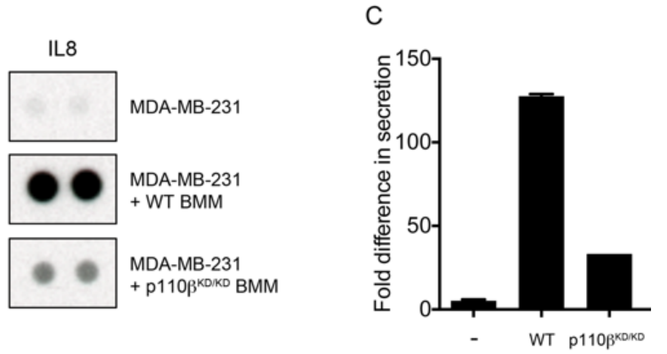

Fig. 4. WT or mutant PI3Kβ BMMS were cocultured for xx h. The conditioned media was analyzed using R&D antibody arrays.

i. PI3Kβ regulates macrophage-stimulated tumor cell transendothelial migration (TEM). Tumor cell intravasation and extravasation requires the movement across endothelial layers. Using a previously published assay that mimics tumor cell extravasation (8), we compared MDA-MB-231 TEM in the absence or presence of BMMs. Whereas wild type BMMs markedly increased TEM, PI3Kβ mutant BMMs had little effect on MDA-MB-231 TEM (Fig. 3).

ii. The production of IL8 by breast cancer cells promotes the recruitment of neutrophils and macrophages and promotes metastasis to the lung (9,10). Moreover, IL8 binding to the CXCR1 receptor promotes breast cancer stem cell formation (11). We find that production of IL8 in MDA-MB-231 cells is activated by co-culture with BMMs, but is markedly suppressed in experiments with mutant PI3Kβ BMMs (Fig. 4).

iii. We measured the ability of BMMs to stimulate invadopodia formation in MDA-MB-231 cells. Whereas WT BMMs stimulated gelatin degradation in control MDA-MB-231 cells, this response was blocked in p110β KO MDA-MB-231 cells (Fig. 5, left). Similarly, macrophage induction of gelatin degradation by control MDA-MB-231 cells was markedly reduced with BMMs from mutant PI3Kβ mice (Fig. 5, right). Thus, PI3Kβ signaling in both tumor cells and macrophages is required for invadopodia function.

iv. Tumor cell intravasation in primary breast tumors occurs via a multicellular assembly, the Tumor Microenvironment of Metastasis Doorway (TMEM doorway). The structure consists of a Tie2+ macrophage, a angiopoietin+ macrophage, and a tumor cell (12). Release of angiopoietin from the endothelial cell stimulates Tie2-dependent release of VEGF from the macrophage, which increases vascular permeability and allows for episodic increases in tumor cell intravasation. We have made the striking observation that mutant PI3Kβ macrophages are defective for release of VEGF; we show this using both antibody arrays and ELISA (Fig. 6). The loss of VEGF secretion by macrophages would seriously disrupt TMEM doorway functions, providing an additional mechanism for PI3Kβ signaling during metastasis. These studies suggest that measurements of TMEM-doorway function in mutant PI3Kβ mice (when available) will be an important new avenue of investigation.

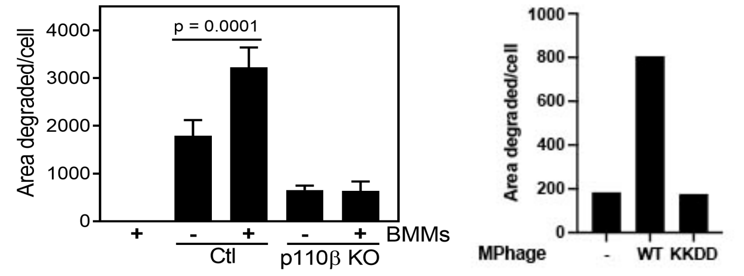

Fig. 5. BMM-stimulated gelatin degradation in MDA-MB-231 cells. A. Control tumor cells or CRISPR p110β knockout tumor cells with WT macrophages. B. Macrophages expressing WT or mutant PI3Kβ BMMs with WT MDA-MB-231 cells.

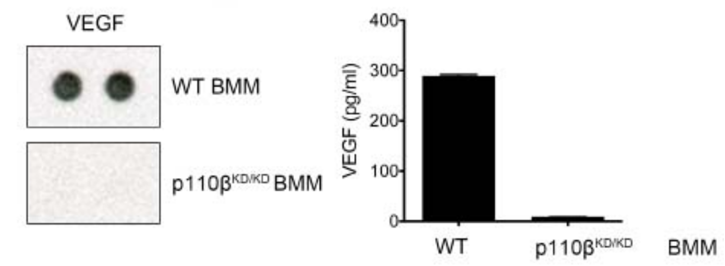

Fig. 6. VEGF secretion from WT or mutant PI3Kβ macrophages was measured by antibody array (left) and ELISA (right).

b. Interactions between breast cancer cells and stromal cells - platelets. Platelet binding to circulating tumor cells is necessary for metastatic dissemination (13-15). Potential mechanisms include protection from immune surveillance (16), induction of tumor cell EMT (17), and recruitment of neutrophils to the metastatic site (18). Platelets inhibit NK cell activity towards tumor cells *in vitro* (19,20), either by physically shielding the tumor cells or preventing the induction of inhibitory responses in NK cells (16,21,22). Importantly, platelet PI3Kβ is required for signaling by the GPVI adhesion receptor (23,24), which binds to galectin-3 on breast cancer cells (25), and for integrin αIIbβ3 activation (26,27), which is essential for thrombus formation (15). In addition, PI3Kβ may play a role in tumor cell responses to platelet binding. The adhesion GPCR CD97 (ADGRE5) in tumor cells binds to platelets (28); CD97 dimerizes with and activates the LPA receptor, which is an upstream activator of PI3Kβ (8). Thus, in addition to its role in tumor cell and macrophage invasion, PI3Kβ is likely to be important for pro-metastatic interactions between platelets and CTCs.

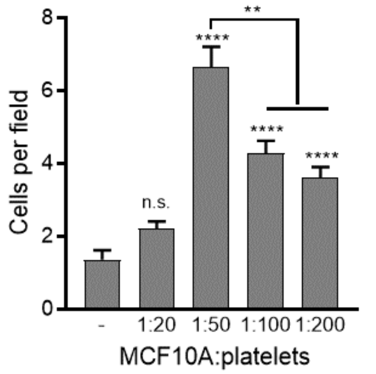

Figure 7. MCF10A cells were incubated with human platelets for 40h, then assayed for invasion towards serum in a Matrigel invasion assay. Data reflects 15 fields/conditions. ** p < .01; **** = p < .0001.

We have made progress in exploring the role of PI3Kβ in platelet-tumor cell interactions. Previous studies have shown that incubation of platelets with tumor cells for 40h enhances the invasion of the tumor cells in Matrigel invasion assays. To optimize this assay for MCF10A breast epithelial cells, we incubated the tumor cells with freshly isolated human platelets at varying rations for 40h, washed to remove the platelets, and then measured tumor cell invasion toward serum in an Matrigel invasion assay. As shown in Figure 7, invasion was increased by platelets in a dose-dependent manner, with a 6-fold increase in invasion with platelets in 50-fold excess over tumor cells.

We next tested the role of PI3Kβ in platelet-induced MCF10A invasion. Platelets from WT or mutant PI3Kβ mice (KKDD) were incubated for 40h with MCF10A cells. WT platelets robustly stimulated MCF10A invasion, and this response was inhibited by 50% with mutant PI3Kβ platelets (Figure 8A). In an alternative approach, we treated isolated human platelets without or with wortmannin, a pan-PI3K inhibitor that covalently modifies the catalytic subunit of PI3Ks; given that PI3Kβ is the predominant PI3K in platelets, this assay reflects the role of platelet PI3Kβ. The wortmannin-treated platelets were then incubated with MCF10A cells for 40h, followed by the Matrigel invasion assay. Whereas control platelets increased MCF10A invasion, this was significantly inhibited by wortmannin pre-treatment (Figure 8B). Taken together, these data suggest that PI3Kβ is an important regulator of platelet-tumor cell interactions.

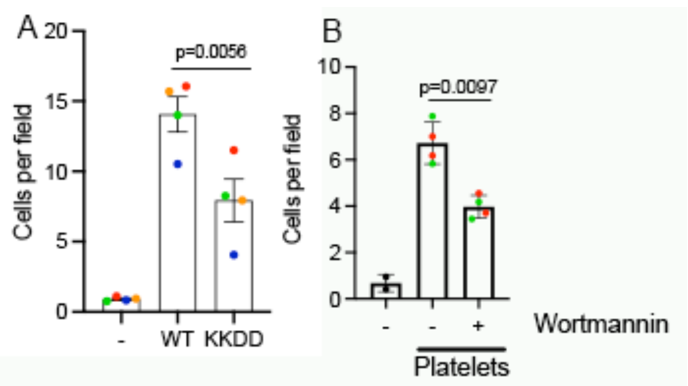

Figure 8. A. Platelets were incubated for 40h with MCF10A cells without or with 500 nM TGX221. Invasion of MCF10A was assayed as in Figure 1. B. Platelets were pre-incubated with wortmannin, washed, and incubated with MCF10A cells for 40 h. Invasion of MCF10A was assayed as in A.

C. Opportunities for Training/Professional Development.

Nothing to report.

D. How were results disseminated.

Nothing to report.

E. Plans during the next reporting period.

a. We will test the GFP-AT3 tumor cells for tumor growth and spontaneous metastasis in our C57Bl/6 mice. If metastasis is robust, we will continue with the analysis of spontaneous metastasis in WT versus mutant PI3Kβ animals.

b. We will analyze tumor-associated immune cells in primary tumors and metastases from AT3-injected mice.

c. Given the changes in VEGF secretion in mutant PI3Kβ macrophages, and the reduction in IL8 induction we see during tumor cell incubations with mutant macrophages, we think it will be important to systematically analyze the altered secretome of PI3Kβ mutant macrophages. We are working with our mass spectrometry facility to identify the secretome of both mouse macrophages and human tumor cells upon co-incubation, using density labeling to distinguish proteins from each cell type.

4. Impact.

A. Impact on the principal discipline. While the animal studies have so far been limited in numbers, our data shows a reduction in metastasis in mice expressing mutant PI3Kβ. Moreover, our new cell-based studies provide even stronger mechanistic data implicating PI3Kβ in breast cancer metastasis. Thus, our data could serve as preclinical evidence for the utility of PI3Kβ inhibition in the treatment or prevention of breast cancer metastasis.

B. Impact on other disciplines.

Nothing to report.

C. Impact on technology transfer.

Nothing to report.

D. Impact on society.

Nothing to report.

5. Changes/problems.

A. Changes in approach. None.

B. Problems/delays. As reported above, we were severely impacted by the effects of the COVID shutdown on our mouse breeding program. This has been compounded by difficulties in breeding the homozygous mutant PI3Kβ mice since the fall. These problems have hindered our ability to generate mice for the metastasis experiments.

C. Changes that had an impact on expenditures. Due to the reduced frequency of the mutant PI3Kβ genotype, mouse breeding costs will be higher than expected.

D. Changes in humans/animals/biohazards. None.

6. Products.

A. Publications.
Nothing to report.

B. Websites.
Nothing to report.

C. Technologies.
Nothing to report.

D. Inventions.
Nothing to report.

E. Other products.
Nothing to report.

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