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14. ABSTRACT The level of PIP3 is tightly regulated by the activities of two opposing enzymes, phosphatidylinositol 3-kinase (PI3K) and Phosphatase and tensin homolog (PTEN), acting as "on/off" switches. We hypothesized that PI3K activity is tolerated within a relatively narrow window in cells - "too much of PIP3 is just as lethal as too little", thus PIK3CA/PTEN double mutants may elevate PIP3 to a lethal level. To test this hypothesis, we determined the effect of PTEN inactivation in human mammary epithelial cells carrying activated alleles of PIK3CA. We also generated a Tet-regulated transgenic mouse mammary tumor model expressing oncogenic PIK3CA and produced mammary tumor induced by mammary gland specific loss of PTEN. We are now ready to test our hypothesis in vivo with concurrent activation of PIK3CA and inactivation of PTEN.					
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Final Report for Award Number W81XWH-06-1-0341

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Introduction

The lipid phosphatidylinositol 3,4,5-trisphosphate (PIP₃) is a critical second messenger in cell signal transduction. The level of PIP₃ is tightly regulated by the activities of two opposing enzymes, phosphatidylinositol 3-kinase (PI3K) and Phosphatase and tensin homolog (PTEN), acting as “on/off” switches. We hypothesize that PI3K activity is tolerated within a relatively narrow window in cells - “too much of PIP₃ is just as lethal as too little”. The abnormal elevation of PIP₃ levels has been frequently found in human cancers bearing somatic activating mutations in the *PIK3CA* gene or loss of PTEN function (Bachman et al., 2004). Interestingly, while both *PIK3CA* and *PTEN* mutations occur so frequently in cancers, *PIK3CA* mutations and *PTEN* loss are almost mutually exclusive (Broderick et al., 2004; Byun et al., 2003; Saal et al., 2005). Since the two genes act as “on/off” switches on PI3K signaling, the reciprocal nature of *PIK3CA* mutations and *PTEN* inactivation indicate that while either *PIK3CA* activation or *PTEN* loss of function results in an elevation of PIP₃ sufficient for oncogenesis, the *PIK3CA*/PTEN double mutants may elevate PIP₃ to a lethal level. To test this hypothesis, we want to inactivate *PTEN* in cells expressing activating mutants of *PIK3CA*. We will also test our idea by simultaneous activation of PI3K and inactivation of *PTEN* in an animal breast tumor model.

Aim 1. To determine the effect of *PTEN* inactivation in human mammary epithelial cells (HMECs) expressing activated alleles of *PIK3CA*.

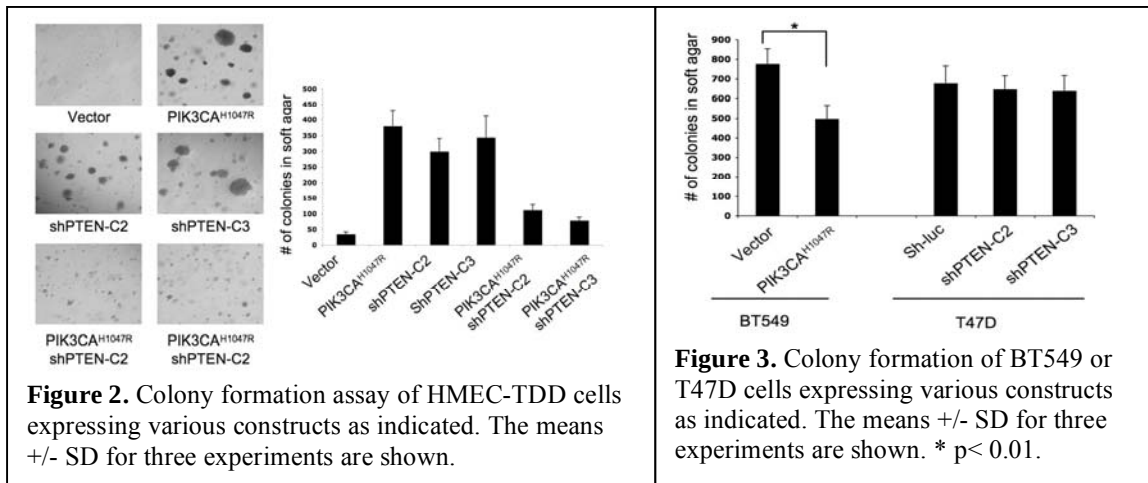
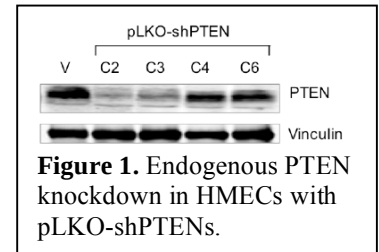
Aim 2. To determine the effect of concurrent inactivation of *PTEN* and activation of *PIK3CA* in an animal breast tumor model.

Body

During the past year, we made good progress toward both Aims. We obtained preliminary data from our cell culture work that support our hypothesis that too much PIP3 may inhibit growth of tumor cells. We finally overcome the hurdle of developing mouse mammary gland tumor model induced by loss of PTEN in mammary glands. We are now ready to make the final compound mice for concurrent activation of PIK3CA and inactivation of PTEN to test our hypothesis in vivo. Details of the studies and results are as follows:

On Aim1: Inactivation of *PTEN* in mammary epithelial cells carrying *PIK3CA* mutation

As reported last year, we identified two working lentiviral shRNAs against PTEN, pLKO-shPTENs. These two shPTENs were able to knockdown ~90% of endogenous PTEN in HMECs when they are stably expressed in HMECs (**Figure 1**). Notably, knockdown PTEN substituted PIK3CA^{H1047R} to induce robust colony formation of HMECs expressing hTERT and p53DD (HMEC-TDD) in soft agar (**Figure 2**). Moreover, introduction of shPTENs into HMECs-TDD in the presence of PIK3CA^{H1047R} significantly suppressed colony-forming ability of these cells in anchorage independent (AI) growth assay (**Figure 2**), consistent with our hypothesis that too much PI3K activity resulting from simultaneous activation of PIK3CA and loss of PTEN may be lethal to cancer cells.



To further test this hypothesis in breast cancer cell lines, we introduced PIK3CA^{H1047R} into BT549 harboring PTEN loss, and shPTEN into T47D carrying a PIK3CA mutation. Whereas expression of PIK3CA^{H1047R} in BT549 cells significantly reduced colony formation efficiency of BT549 cells, knockdown of PTEN in T47D cells had no effect on AI-growth of these cells in soft agar (**Figure 3**). Interestingly, however, T47D cells have very low levels of phospho-AKT, indicating that these cells may not have hyper elevated PIP3 level (data not shown). We are carrying out the same assay on more cancer cell lines featuring PTEN loss or PIK3CA mutational activation to more vigorously test our hypothesis.

On Aim 2: Simultaneous inactivation of PTEN and activation of PIK3CA in a mouse mammary tumor model

Activation of PIK3CA-H1047R induce mammary tumorigenesis:

To explore the functional role of the oncogenic allele of *PIK3CA* in a dynamic process of breast cancer development in vivo, we recently generated a transgenic mouse strain in FVB/N background carrying the most frequently mutated allele of human *PIK3CA*, H1047R, under the control of a tet-operator regulated promoter, coupled via an IRES element to Luciferase for in vivo imaging (referred to hereafter as iPIK3CA). By breeding the iPIK3CA transgene with an MMTV-rtTA (MTB) mouse line in an FVB/N background obtained from Dr. Chodosh's laboratory (Gunther et al., 2002), we have obtained bi-transgenic mice (iPIK3CA/MTB) that conditionally express PIK3CA-H1047R in the mammary epithelium of transgenic mice in the presence of doxycycline (**Figure 4**). In addition, we have followed the induction of transgene expression and luciferase activity by quantitative real-time PCR and luciferase activity analysis, respectively, in mammary tissues harvested from female iPIK3CA/MTB mice maintained on doxycycline for 4 days and found them to occur in register (data not shown).

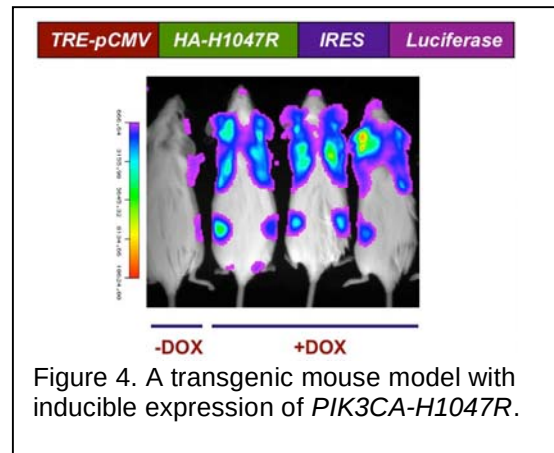


Figure 4. A transgenic mouse model with inducible expression of *PIK3CA*-H1047R.

These iPIK3CA/MTB mice developed hyperplastic lesions with 100% penetrance when they were treated for doxycycline (2 mg/ml) for 1-2 weeks, as determined by examination of carmine-stained whole mounts and histological analysis (**Figure 5**). In contrast, mammary glands from control MTB animals treated with doxycycline, and iPIK3CA/MTB maintained in the absence of doxycycline, did not display any morphological abnormalities (data not shown). When induced for six months, approximately 50% of the bi-transgenic mice developed multi-focal mammary tumors. Again, no single tumor was observed in control mice over the same period of time. Notably, the tumors developed upon PIK3CA-H1047R expression displayed heterogeneous phenotypes, and this heterogeneity was present even within a single mammary gland harboring multiple tumors (**Figure 6**). These results suggest that oncogenic activation of PIK3CA is able to induce mammary tumor initiation but not sufficient for tumor progression. Other genetic alternations are likely required to cooperate with PIK3CA activation for tumor progression. Since secondary genetic events acquired during tumor progression may influence the dependency of a specific oncogene, we are now determining whether the tumors that have been induced for a longer time and have reached more advanced stages remain dependent upon oncogenic PIK3CA activation for maintenance of their transformed state.

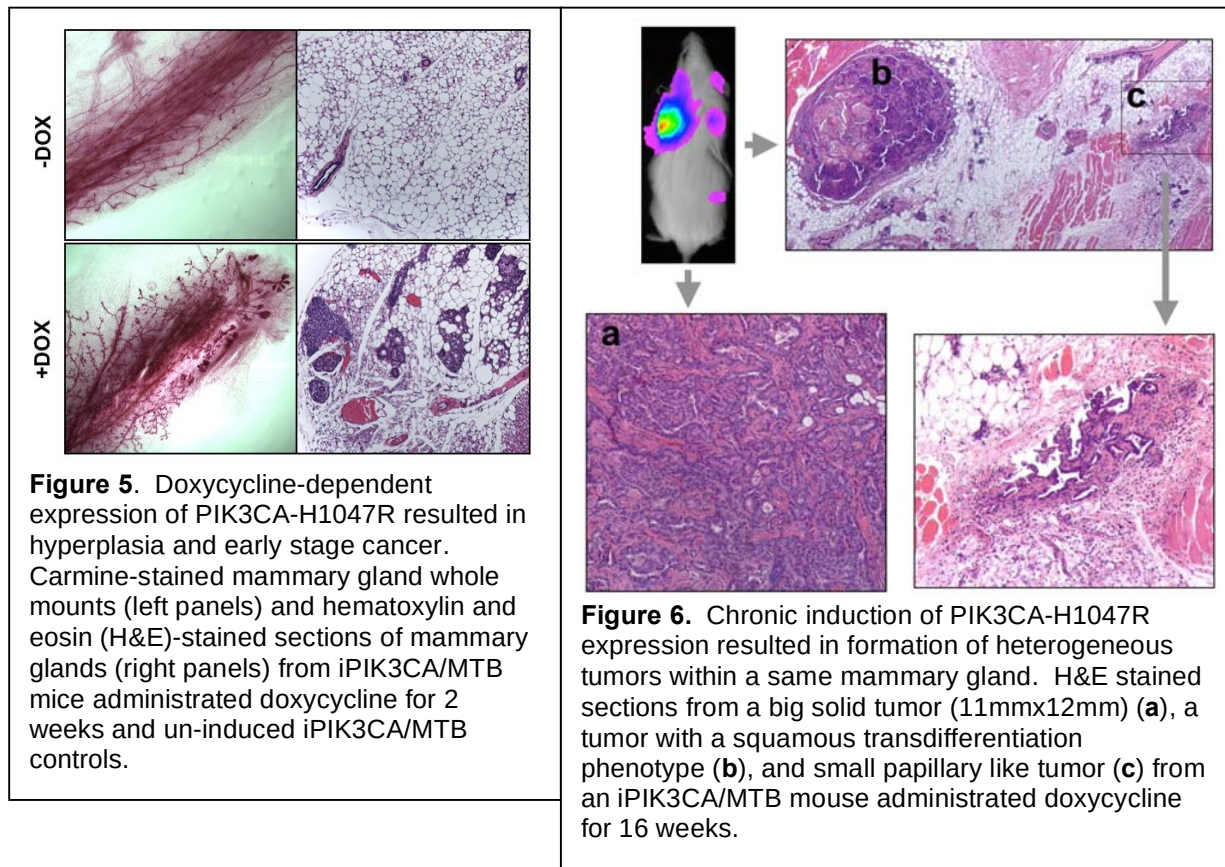


Figure 5. Doxycycline-dependent expression of PIK3CA-H1047R resulted in hyperplasia and early stage cancer. Carmine-stained mammary gland whole mounts (left panels) and hematoxylin and eosin (H&E)-stained sections of mammary glands (right panels) from iPIK3CA/MTB mice administrated doxycycline for 2 weeks and un-induced iPIK3CA/MTB controls.

Figure 6. Chronic induction of PIK3CA-H1047R expression resulted in formation of heterogeneous tumors within a same mammary gland. H&E stained sections from a big solid tumor (11mmx12mm) (a), a tumor with a squamous transdifferentiation phenotype (b), and small papillary like tumor (c) from an iPIK3CA/MTB mouse administrated doxycycline for 16 weeks.

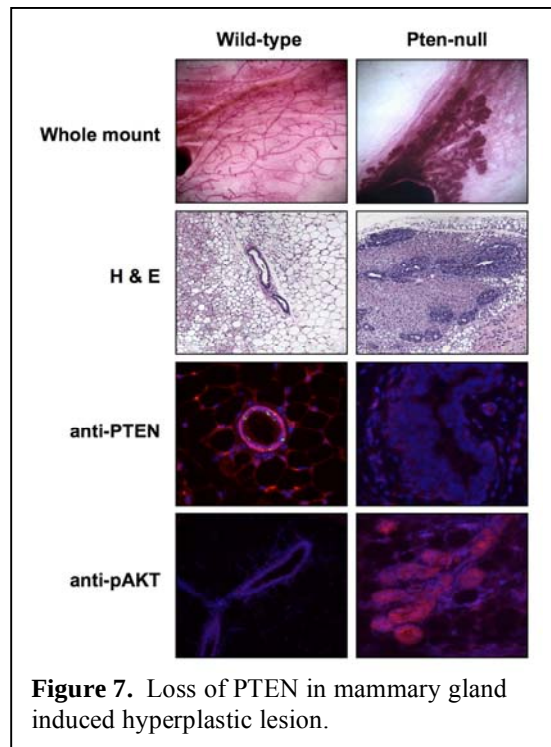
Re-establishing mammary tumorigenesis induced by PTEN loss:

As we reported last year, mice carrying MMTV-Cre; PTEN^{lox/lox} in BL6 background developed early onset of massive lymphomas, precluded further analysis of breast tumorigenesis. To rule out the potential interference from mouse background, we backcrossed these mice to FVB for 6 generations. These MMTV-Cre; PTEN^{lox/lox} mice on FVB background also developed severe lymphoma within 2-3 months.

In parallel, we used transgenic mouse carrying WAP-rtTA-Cre developed by Utomo *et al.* (Utomo *et al.*, 1999) that integrates tetracycline-controlled gene expression and Cre-mediated gene deletion for our study. We are happy to see that female WAP-rtTA-Cre; PTEN^{lox/lox} mice developed hyperplastic lesion with two weeks of doxycycline treatment (**Figure 7**). Loss of PTEN and elevation of AKT phosphorylation in these hyperproliferating cells were confirmed with immunofluorescence analyses on paraffin sections of mammary glands isolated from compound mice (**Figure 7**).

We are now in the position to make the final compound mice for concurrent activation of PIK3CA and inactivation PTEN specifically in mammary gland using WAP-rtTA-Cre. Importantly, using a single WAP-rtTA-Cre line to replace both MMTV-Cre and MMTV-rtTA lines to generate our final compound mice, namely iPIK3CA^{H1047R};PTEN^{lox/lox};WAP-rtTA-Cre, instead of iPIK3CA^{H1047R};PTEN^{lox/lox};MMTV-rtTA;MMTV-Cre,

will significantly reduce our crossing time and efforts. In addition, the integrated expression of rtTA and Cre under a single WAP promoter will eliminate the concerns of the mosaic expression rtTA and Cre from two separate MMTV promoters, allowing us to better examine the simultaneous inactivation of PTEN and activation of PIK3CA in mammary gland tumorigenesis.



Key Research Accomplishments

1. We show that loss of PTEN can cooperate with p53 loss to induce hTERT immortalized HMECs to form colonies in soft agar.
2. We show that HMECs carrying concurrent activation of PIK3CA and loss of PTEN are not able to grow anchorage independently.
3. We generated a bi-transgenic mouse, $iPIK3CA^{H1047R};MTB$, and found that mammary gland specific expression of $PIK3CA^{H1047R}$ induced heterogeneous mammary gland tumor formation.
4. We generated compound $PTEN^{flox/flox}/WAP-rtTA-Cre$ to specifically inactivate PTEN in mammary gland. These female compound mice developed hyperplastic lesion upon doxycycline treatment.

Reportable Outcomes

The support from the Department of Defense Idea Award has helped me to start my independent research program and provided research opportunities for two postdoctoral fellows, Hailing Cheng and Theresa Wang, to pursue their careers in cancer research. Other outcomes, such as cell lines and animal models, are listed under “key research accomplishments”

Conclusion

The research described here is relevant to the pathogenesis and a potential novel therapy for breast cancers. The *PIK3CA* is the most commonly mutated oncogene in breast cancer and loss of the tumor suppressor, PTEN, occurs frequently in patients suffering from this disease. Our newly generated oncogenic *PIK3CA* transgenic animal model will allow us to determine the

oncogenic role of *PIK3CA* in tumor initiation, progression, maintenance and metastasis etc. It should also significantly facilitate preclinical testing for the development of PI3K inhibitors for targeted therapy. Our final goal of simultaneous inactivation of *PTEN* and activation of *PIK3CA* will not only provide a new perspective on the relationship of the two key oncogene and tumor suppressor, *PIK3CA* and *PTEN*, and the signaling pathway under their control in cell regulation and oncogenic transformation, but also a potential novel therapy to all patients plagued with the common tumorigenic mutations.

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