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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> During the second year of this project, Tasks 1c and 2a have been completed. This includes biochemical and phenotypical characterization of isogenic breast cell lines with heterozygous and homozygous PTEN loss and a library of other cell lines which each represent a distinct mutation of the PI3K/mTOR pathway. Exposure of various isogenic pairs of PTEN knock out cell lines to lithium chloride has also been completed.					
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## **Introduction**

The phosphatidylinositol 3-kinase (PI3K) pathway mediates key cellular functions, including growth, proliferation, survival and angiogenesis. The gene encoding the catalytic domain of PI3K, *PIK3CA*, has been found to be mutated in breast cancers at high frequency. The tumor suppressor PTEN reverses the effects of PI3K by dephosphorylating the same site on membrane phosphatidylinositols that is phosphorylated by PI3K. Genomic analysis of the PTEN gene has identified it as one of the most commonly mutated or deleted tumor suppressors in human malignancies. In breast cancer, genetic alterations of both PTEN alleles are found with a frequency of about 5%, however monoallelic loss of PTEN is observed in as many as 50% of cases, and this can lead to aberrant PTEN signaling, resulting in early metastasis and poor prognosis.

Physiologic models of PTEN loss are needed to test potential anti-cancer therapies in preclinical animal models of breast cancer. Our lab's approach is to exploit somatic cell gene targeting to create paired isogenic cell lines with critical genetic alterations as their only differentiating factor. The changes in downstream signaling pathways can then be reliably detected, and response to new therapeutic agents can be identified. Thus, the ability to create isogenic paired human cell lines enables the evaluation of genetic alterations for predictive biomarkers of response to novel therapies.

Using gene targeting, our laboratory has introduced two common "hotspot" *PIK3CA* mutations into the MCF-10A non-tumorigenic human breast epithelial cell line. (1) Surprisingly, this study led to the discovery that GSK3beta inhibitors including lithium, an FDA approved therapy for bipolar disorders, have selective anti-neoplastic properties against human breast cancer cell lines containing oncogenic *PIK3CA* mutations. These results are now being rapidly translated into a clinical trial to determine if women with breast cancers harboring mutant *PIK3CA* will respond to lithium therapy. Because of the known opposing interactions between PI3K and PTEN, we hope to build upon this work and study the sensitivity to lithium in breast cancer cells with PTEN loss using preclinical models. If successful, we will rapidly translate these findings to an early phase clinical trial studying the safety and efficacy of oral lithium treatment in patients with breast carcinoma and examining their breast cancers for PTEN loss. We hope to provide the rationale for the use of lithium as a targeted anti-cancer treatment for breast cancer patients whose tumors harbor mutations/loss of PTEN.

## **Body**

### **Statement of work**

*Task 1. Creation of isogenic breast cell lines with heterozygous and homozygous PTEN loss. (months 1-18)*

1a. Create targeting vectors (months 1-3)

1b. Infect and screen breast epithelial and cancer cell lines (months 3-12)

1c. Biochemical and phenotypical characterization (months 12-18)

*Task 2. PTEN loss sensitizes cells to lithium in vitro. (months 18-30)*

2a. Expose various isogenic pairs of PTEN knock out cell lines to lithium chloride (months 18-24)

2b. Biochemical and phenotypical characterization of response to lithium chloride (months 24-30)

*Task 3. PTEN loss sensitizes cells to lithium in vivo. (months 24-36)*

3a. Establish xenografts of isogenic pairs of breast cancer cell lines in nude mice (months 24-30)

3b. Treatment studies using oral and intraperitoneal administration of lithium (months 28-36)

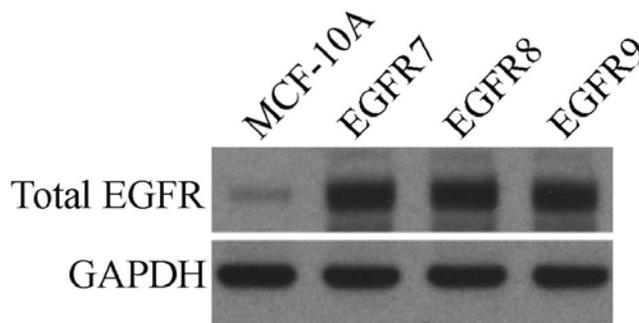
### **Task 1a. Create targeting vectors (months 1-3)**

The grant was awarded on June 1st 2010. Dr. Higgins graduated from her Oncology Fellowship Program in Johns Hopkins Hospital on June 30th and accepted a position as a faculty member with the Breast Cancer Program at Massachusetts General Hospital on July 1 2010. A request to transfer the grant to support Dr. Higgins as she continued this work was submitted and processed in June 2010 in anticipation of this move. Johns Hopkins University has since relinquished all interest in this grant and it was transferred to Massachusetts General Hospital in September 2010. Dr. Higgins provided a detailed progress report between June 1<sup>st</sup> 2010 and Sept 30<sup>th</sup> 2010 and a further annual report to cover the period from Sept 30<sup>th</sup> 2010 to June 30<sup>th</sup> 2011. This report covers the period from July 1<sup>st</sup> 2011 – June 30<sup>th</sup> 2012.

Dr. Higgins prepared and designed the planned experiments to employ gene targeting in the two breast cancer cell lines that are wild type for both *PIK3CA* and *PTEN*: HCC712 and HCC1187, which are estrogen receptor positive and negative, respectively. Targeting vectors have already been created by a former Park lab mentee (2) and were used for knocking out the *PTEN* gene in these cells.

### Task 1b. Infect and screen breast epithelial and cancer cell lines (months 3-12)

Unfortunately despite several months of screening infected cells, we were unable to produce HCC712 and HCC1187 cell lines with knocked out *PTEN*. We hypothesize that this is due to the high level of genetic instability within these malignant cell lines. However, Dr. Higgins designed an alternative strategy to comprehensively study the distinct effects of several key alterations of the PI3K pathway using a library of cell lines previously created by members of the Park laboratory harboring either a *PIK3CA* mutation, (1) an *AKT1* mutation, (3) or loss of *PTEN* (2). Additionally Dr. Higgins and her team stably transduced Epidermal Growth Factor Receptor (EGFR) in MCF10A human breast epithelial cells using the retroviral expression vector pFBneo, which was a kind gift from Dr. Anil K. Rustgi (University of Pennsylvania). Retrovirus containing the coding sequence for EGFR was generated using Fugene6 (Roche Diagnostics, Indianapolis, IN) per the manufacturer's protocol in HEK-293T cells. Purified retrovirus was then used to infect MCF-10A cells following the manufacturer's protocol. Stable transformants were selected using 180  $\mu\text{g}/\text{mL}$  G418 (Invitrogen, Carlsbad, CA). EGFR expression was confirmed by western blot using antibodies against total EGFR protein. (Figure 1) Parental MCF-10A cells were also stably transduced in parallel with an empty retroviral expression vector pFBneo (named Empty Vector or EV) and selected in the same manner to serve as controls for all experiments.



**Figure 1:** Overexpression of Epidermal Growth Factor Receptor in MCF-10A human breast epithelial cells. Western blot demonstrating levels of total EGFR in parental MCF-10A, and three stably transduced EGFR overexpressing clones, EGFR7, EGFR8, EGFR9. GAPDH is shown as a loading control.

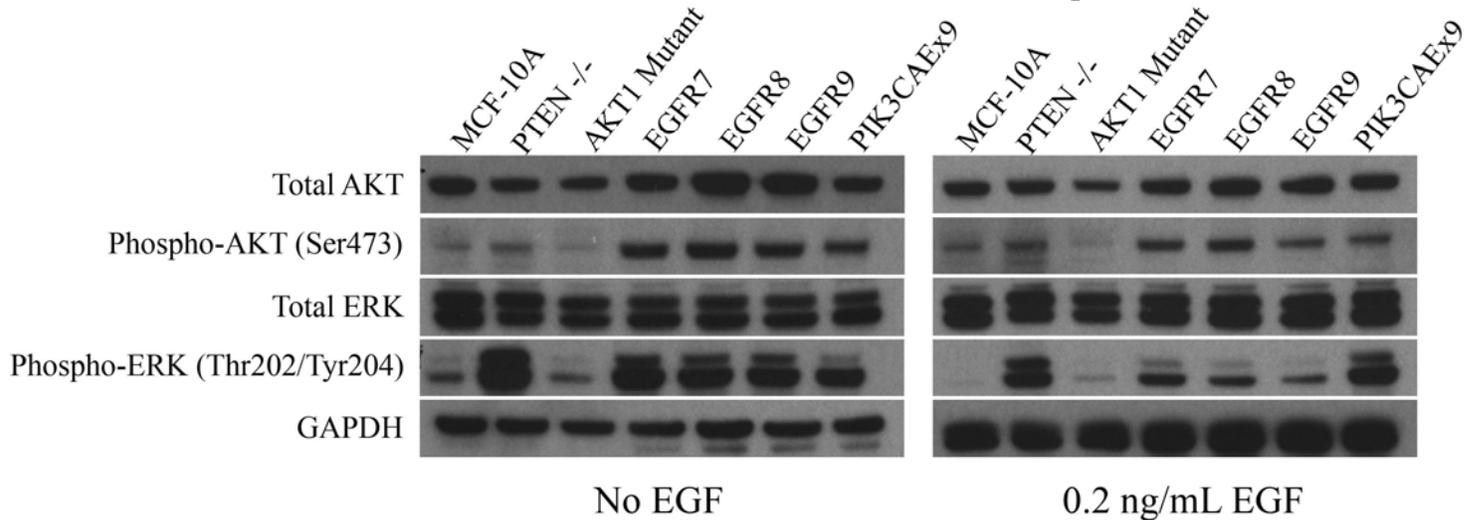
We plan on using alternative strategies to pursue gene targeting of *PTEN* in the breast cancer cell lines and can also resort to the use of RNA interference if necessary.

### Task 1c. Biochemical and phenotypical characterization (months 12-18)

Using the library of cell lines detailed above, extensive immunoblotting experiments were performed to biochemically characterize parent MCF10A cells in comparison with cell lines harboring a *PIK3CA* mutation, an *AKT1* mutation, loss of *PTEN* or overexpression of EGFR. We performed western blot analyses to determine the degree of MAPK and PI3K pathway activation by comparing relative levels of phosphorylated and total Akt and Erk in the absence of exogenous EGF and in the presence of physiologic concentrations of EGF.

Lysates for cells grown in each experimental condition have been prepared as previously described. (4) Western blotting was performed using the NuPage XCell SureLock electrophoresis system (Invitrogen, Carlsbad, CA) and PVDF membranes (Invitrogen, Carlsbad, CA). Primary antibodies were added overnight at 4 °C, while secondary antibodies, conjugated with horseradish peroxidase were added for 1 hr at RT. Antibodies used in this study were anti-EGFR rabbit antibody (2232; Cell Signaling Technology),

anti-phospho EGFR (Tyr 1173) rabbit anti-body (4407L; Cell Signaling Technology), anti-AKT rabbit antibody (9272; Cell Signaling Technology), anti-phospho AKT (Ser 473) rabbit antibody (9271; Cell Signaling Technology), anti-p42/p44 MAP kinase rabbit antibody (9102; Cell Signaling Technology), anti-phospho p42/p44 MAP kinase (Thr-202/Tyr-204) mouse antibody (9106; Cell Signaling Technology), anti-cyclin D1 rabbit antibody (2922; Cell Signaling Technology), anti-GSK3 $\beta$  rabbit antibody (9315; Cell Signaling Technology), anti-phospho GSK3 $\beta$  rabbit antibody (9336S; Cell Signaling Technology), and anti-GAPDH mouse antibody (6C5) (ab8245; Abcam). Blots were exposed to Kodak XAR film using chemiluminescence for detection (Perkin Elmer). All experiments were performed at least 3 times. Results of biochemical characterization of the various cell lines are shown in Figure 2.



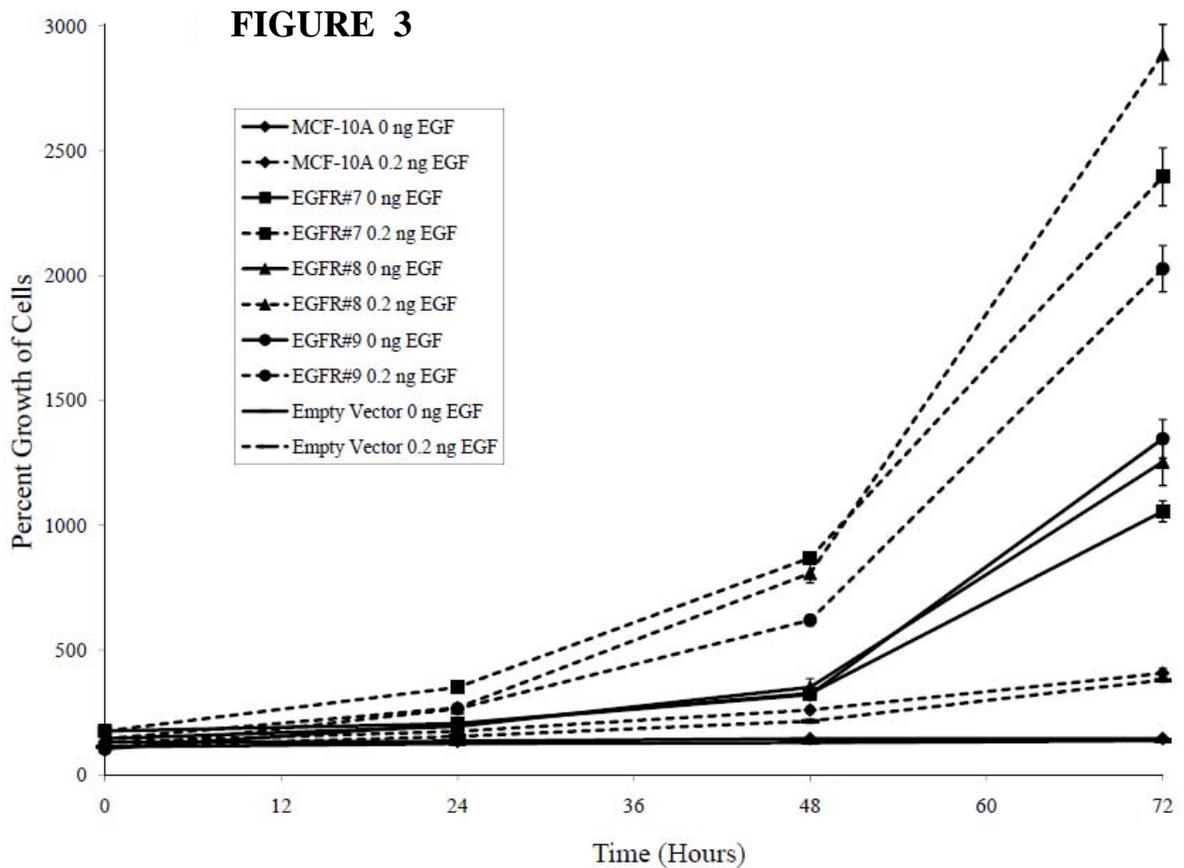
**Figure 2:**

Alterations in the PI3K pathway activate multiple oncogenic pathways to varying degrees. Western blot demonstrating levels of total AKT, phosphorylated AKT(Ser473), total ERK and phosphorylated ERK (Thr202/Tyr204) in parental MCF-10A, PTEN<sup>-/-</sup>, AKT1 mutant (E17K), EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones) and PIK3CAEx9 (E545K) cell lines in the absence of EGF (left panel) or presence of 0.2 ng/ml EGF (right panel). GAPDH is shown as a loading control. Results are representative of multiple independent experiments.

### Cell Proliferation Assays

Cells were prepared by seeding each cell line in DMEM:F12 medium without phenol red, supplemented with 1% charcoal dextran-treated fetal bovine serum (Hyclone), 10  $\mu$ g/mL insulin, 0.5  $\mu$ g/mL hydrocortisone, 0.1  $\mu$ g/mL cholera toxin at a density of 100,000 cells per 25 cm<sup>2</sup>. Medium was changed to either EGF-free or 0.2 ng/mL EGF-containing medium in the absence and presence of 10 mM LiCl on days 1 and 4 as indicated. Cells were counted and evaluated for viability on days 1 and 6 using a Vi-CELL Cell Viability Analyzer (Beckman Coulter). All assays and growth conditions were performed in triplicate and repeated at least 3 times.

**Figure 3 -** EGFR overexpression confers EGF independent growth to MCF-10A cells. Cell proliferation assays were performed as, using parental MCF-10A cells, as well as clones stably overexpressing an EGFR transgene and a control clone of MCF-10A cells stably transduced with an empty retroviral expression vector (Empty Vector). Cells were grown in the absence and presence of 0.2 ng/mL EGF (solid or dashed lines, respectively). Data points show percent growth relative to day 0 for each cell line at the displayed time. Bars represent standard error of the mean from triplicate samples. Results are representative of three independent experiments.  $p < 0.001$  for all EGFR clones compared to parental MCF-10A and Empty Vector cells grown in the absence of EGF or in 0.2 ng/ml EGF.



**Task 2. PTEN loss sensitizes cells to lithium in vitro. (months 18-30)**

2a .Expose various isogenic pairs of PTEN knock out cell lines to lithium chloride (months 18-24)

Cell proliferation assays were performed as detailed above. Standard error of the mean (SEM) was calculated for each proliferation assay. Statistical analyses were performed using a two-tailed Student's *t*-test and a one-way ANOVA across cell lines, which were calculated using Microsoft Excel and ezANOVA. A *P* value less than 0.05 was considered statistically significant.

Using identical conditions to our previous work, Dr. Higgins found that treatment with LiCl significantly inhibited the growth of cells that overexpressed EGFR, similar to the response seen with the PIK3CA knock in cell line (Fig. 4). These effects were also observed at physiologic concentrations of EGF (Fig. 4A vs. 4B).

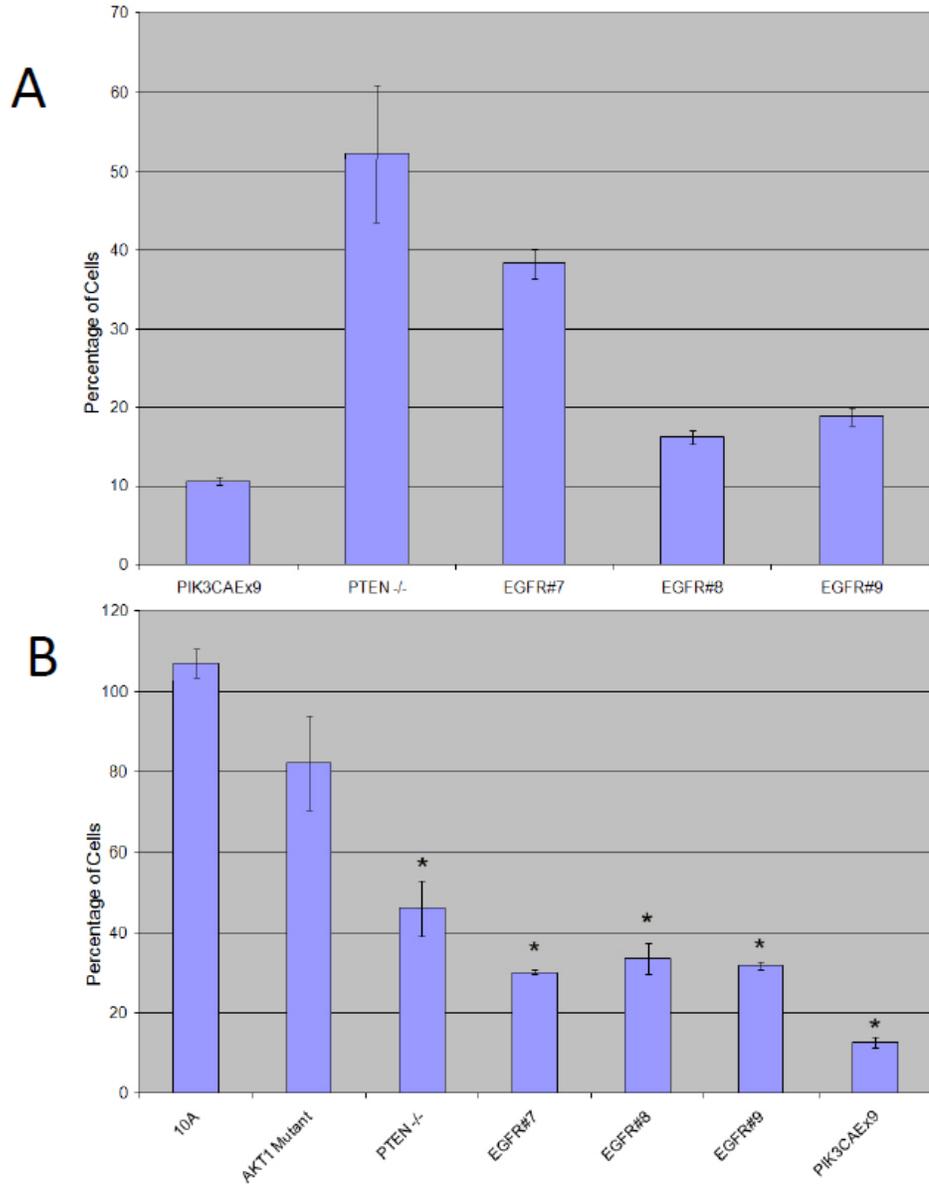
Using a pair-wise comparison one-way ANOVA across cell lines, we found a statistically significant decrease in the proliferation of PTEN<sup>-/-</sup>, EGFR#7, EGFR#8, EGFR#9, and PIK3CAEx9 as compared with parental MCF-10A cells (*p* < 0.05). In contrast, the parental MCF-10A and AKT1 E17K cell lines were not significantly inhibited by LiCl when cultured in 0.2 ng/ml EGF (*p* > 0.05). As stated above, the effect of LiCl in parental MCF-10A and AKT1 E17K cells could not be ascertained in the absence of EGF as these cells do not proliferate under these conditions. Interestingly, the PTEN<sup>-/-</sup> cell line demonstrated intermediate sensitivity to LiCl, when compared to the response seen with PIK3CA knock in and EGFR overexpressing cell lines. Thus, although the biochemical pathways activated by mutant PIK3CA, Pten loss and EGFR overexpression appear similar, they do not uniformly predict for sensitivity to lithium treatment.

**Figure 4**

**Lithium chloride inhibits the growth of EGFR overexpressing and PIK3CAEx9 mutant cells but not MCF-10A parental or AKT1 mutant cell lines.** Cell proliferation and drug treatment assays were

performed with parental MCF-10A, *PTEN*<sup>-/-</sup>, *AKT1* mutant (E17K), EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones) and *PIK3CAEx9* (E545K) cell lines grown in (A) the absence of EGF or (B) presence of 0.2 ng/ml EGF. Note that MCF-10A and *AKT1* mutant cells could not be included in (A) since these cells do not proliferate in the absence of EGF. Bars represent the percentage of cell proliferation in 10 mM lithium chloride *relative* to cells grown in control medium (without lithium) after 6 days in culture. Error bars represent the standard error of the mean from triplicate samples. Results are representative of three independent experiments. \*  $p < 0.003$  compared to parental MCF-10A cells.

Figure 4



To uncover the potential reasons for the differential responses to lithium seen in our panel of cell lines, Dr. Higgins will perform western blotting to elucidate any biochemical changes in the MAPK or PI3K pathways elicited by lithium exposure. (Task 2b)

*Tasks 1a, 1b and 1c have been completed.*

*Task 2a has been completed, Task 2b is ongoing and will be completed by month 30*

*Tasks 3a-b will be completed by month 36*

## Key research accomplishments

1. Overexpression of Epidermal Growth Factor Receptor in MCF-10A human breast epithelial cells
2. Collation of a library of cell lines each representative of distinct genetic alterations of the PI3K/AKT/mTOR pathway
3. Biochemical and phenotypical characterization of the above cell lines is complete
4. Phenotypical characterization of the above cell lines' response to lithium chloride is complete. Biochemical characterization of the lithium chloride response is ongoing.

## Reportable Outcomes

The results to date were presented at the Department of Defense Era of Hope Conference, Florida, August 2011 as a poster presentation.

## Conclusions

*PIK3CA* mutations, EGFR overexpression, Pten loss and *AKT1* E17K mutations have different biochemical signaling profiles and respond differently to pharmacologic treatment with Lithium Chloride.

Although *PIK3CA* mutations, Pten loss and *AKT1* E17K mutations were originally thought to be functionally equivalent based upon their mutual exclusivity in human cancers (5, 6), recent studies have now demonstrated differences in pathway activation among these genetic alterations as well as rare cancers that have lost Pten and concurrently harbor an activating *PIK3CA* mutation (7, 8). In order to perform comparative analyses between *PIK3CA* knock in, *PTEN* knock out, *AKT1* E17K knock in and EGFR overexpressing cells in the same MCF-10A background, we first performed western blot analyses to determine the degree of MAPK and PI3K pathway activation by comparing relative levels of phosphorylated and total Akt and Erk in the absence of exogenous EGF and in the presence of physiologic concentrations of EGF (Figure 2). Representative clones for *PIK3CA* exon 9 knock in, *PTEN* knock out and *AKT1* E17K knock in were used as these cell lines have all previously been described to be indistinct from their clonal sibs (1-3). Because EGFR overexpressing clones have not yet been characterized, all three clones were used for these studies. Previously, we have described that *AKT1* E17K knock in cells do not proliferate in the absence of EGF and concordantly, they display minimal phosphorylation of Akt and Erk (3). As seen in Fig. 2, *AKT1* E17K cells did not demonstrate any significant activation of the PI3K or MAPK pathways relative to parental MCF-10A cells, as shown by the minimal phosphorylation of Akt and Erk under EGF free and physiologic concentrations of EGF (0.2 ng/ml). It should be noted that a slight under loading of the *AKT1* E17K sample accounts for the slight decrease of all proteins in the absence of EGF (Fig.2, left panel). However, in the presence of EGF, there was a slight but reproducible increase in ERK phosphorylation in *AKT1* E17K cells relative to parental MCF-10A cells (Fig.2, right panel). The reason for this is unclear, but reaffirms the notion that signaling via EGF/EGFR can lead to unexpected and varying responses depending on the genetic alterations present within a given cell. In contrast, phosphorylated Akt was increased in the *PTEN* <sup>-/-</sup> cell line, but this was not as pronounced as in the three EGFR overexpressing clones, or the *PIK3CA* knock in cell line in conditions without exogenous EGF (Fig. 2, left panel), though was comparable to these cell lines in conditions with 0.2 ng/ml EGF (Fig. 2, right panel).

EGFR overexpressing clones, *PIK3CA* knock in cells and *PTEN* <sup>-/-</sup> cells also demonstrated activation of the MAPK pathway as displayed by the increased levels of phosphorylated Erk relative to total Erk both in the absence and presence of 0.2 ng/mL EGF (Fig. 2). Interestingly, in the absence of EGF the *PTEN* <sup>-/-</sup> cell lines exhibited a pronounced increase in Erk phosphorylation compared to the EGFR overexpressing cell lines or the *PIK3CA* knock in cell line (Fig.2, left panel). However, in the presence of EGF, Erk phosphorylation in EGFR overexpressing clones was decreased relative to *PTEN* <sup>-/-</sup> cells and *PIK3CA* knock in cells (Fig.2, right panel). The cause for these differences are unknown, but these results are consistent with our previous observations in *PTEN* <sup>-/-</sup> cell lines showing that the presence or absence of EGF as well as duration of exposure to this growth factor can influence the level of Erk phosphorylation (2). Thus, our biochemical analyses reaffirm that the presence of an *AKT1* E17K mutation alone does not confer significant oncogenic pathway signaling in human breast epithelial cells. In contrast, the presence of

a *PIK3CA* oncogenic mutation, the loss of Pten, or overexpression of EGFR does indeed result in Akt and Erk phosphorylation in a manner similar to that found in breast cancer cells. However, the level and pattern of activation seen in these pathways is distinctly different between these three sets of cell lines, as evidenced by the varying levels of phosphorylation seen under conditions with and without exogenous EGF. This further underscores the previously unrecognized complexity of crosstalk that occurs between these important pathways.

Because the mechanism of action of lithium has not been fully elucidated, we wanted to assess whether EGFR overexpression, Pten loss or the *AKT1* E17K mutation could also predict for lithium sensitivity. There were two main reasons for formally testing this hypothesis. First, because one of lithium's targets has been suggested to be GSK3 $\beta$  which is modulated by Akt and Erk activation, we hypothesized that EGFR overexpression and Pten loss via gene targeting would predict for lithium sensitivity based upon our previous data that *PTEN* knock out leads to increased Akt and Erk activation (2) and our own data presented in this study that EGFR overexpression also activates both the MAPK and PI3K pathways (Fig.2). Second, despite the minimal pathway signaling seen with *AKT1* E17K knock in cells (3), it was still formally possible that this mutation could activate other pathways that would lead to lithium sensitivity. There are in fact, examples where the same drug/compound can be extremely effective in various cancers with very different somatic alterations such as the case with *BCR/ABL* translocations in chronic myelogenous leukemia and certain *C-KIT* mutations in gastrointestinal stromal tumors both predicting for response to the small molecule inhibitor imatinib (9). In addition, Wang et al. recently demonstrated that MLL leukemias are also sensitive to lithium treatment (10).

We previously described the selective anti-neoplastic properties of lithium in vitro and in vivo using human breast and colon cancer cell lines that harbor activating mutations in *PIK3CA* (1). A standard 10 mM concentration of lithium chloride (LiCl) was used in vitro based upon our initial tests (1) and the doses previously reported in studies examining the effects of lithium in various in vitro systems and their correlation to in vivo models (10-14). It should be noted that although therapeutic lithium serum levels are 0.8 – 1.2 mEq/L, wide variations between serum lithium levels and intracellular concentrations of lithium have been reported (15).

We hypothesized based on the biochemical results shown in Fig. 2, that EGFR overexpression and *PTEN* loss, but not the *AKT1* E17K mutation would also predict for sensitivity to lithium. We therefore performed multiple growth assays with and without EGF to compare the growth inhibitory effects of lithium in our panel of cell lines. As stated previously, the rationale for testing lithium toxicity under varying EGF conditions stems from our previous observations that differences in EGF concentration can have a profound effect on the downstream signaling cascades imparted by genetic mutations and their relative resistance and sensitivity to drugs such as rapamycin (1). In addition, parental MCF-10A, *AKT1* E17K and empty vector control cells do not proliferate without EGF, but do proliferate with 0.2 ng/ml EGF and therefore can be used as isogenic counterparts when cultured with EGF. Importantly, we have previously described that parental MCF-10A cells and control cells proliferate at approximately an equal rate in 0.2 ng/ml EGF as mutant *PIK3CA* knock in cells in the absence of EGF, yet the former cell lines are resistant to lithium while the mutant *PIK3CA* clones were uniformly sensitive to lithium under these conditions. This strongly suggests that the effects of lithium are not simply due to increased cell proliferation. Using identical conditions to our previous work, we found that treatment with LiCl significantly inhibited the growth of cells that overexpressed EGFR, similar to the response seen with the *PIK3CA* knock in cell line (Fig. 4). These effects were also observed at physiologic concentrations of EGF (Fig. 4A vs. 4B).

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## Appendices

None at this time