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PRINCIPAL INVESTIGATOR: Alex Toker

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

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14. ABSTRACT We have discovered that the Akt pathway modulates breast cancer cell survival in response to genotoxic agents, and discovered a new substrate of Akt, MERIT40, that is phosphorylated upon exposure of cells to chemotherapeutic drugs. We propose that this represents a major mechanism by which cells exposed to these drugs evade cell death by apoptosis and thus become resistant to the damaging effects of clinically-relevant chemotherapy agents. These findings have important ramifications for the use of chemotherapy drugs in breast cancer patients, and many also suggest that MERIT40 may be used as a clinically relevant biomarker for resistance to doxorubicin.					
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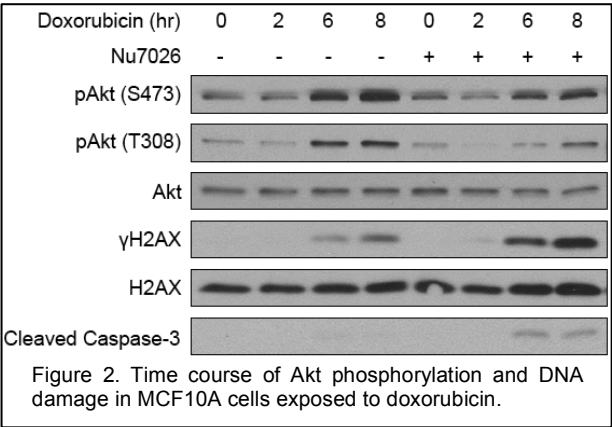
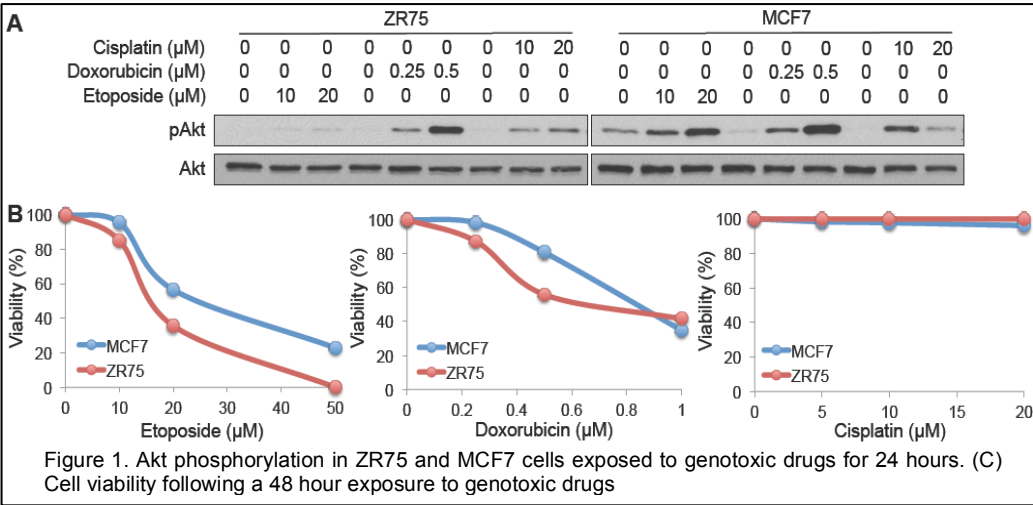
INTRODUCTION

Genotoxic chemotherapy agents are used to treat breast cancer at all stages of the disease. However, the duration of response is frequently limited by chemotherapy resistance mechanisms. Therefore, resistance has a major impact on breast cancer patient survival. Despite the importance of this issue, the molecular mechanisms underlying resistance are poorly understood and strategies to combat chemotherapy resistance are lacking. The phosphoinositide 3-kinase (PI 3-K)/Akt pathway has emerged as a major regulator of numerous cellular phenotypes associated with breast cancer. In this project we hypothesized that a major mechanism of resistance to genotoxic chemotherapy agents is activation of the PI 3-K/Akt signaling cascade. We proposed that *genotoxic drugs induce the activation of Akt to initiate a signaling pathway that renders breast cancer cells resistant to chemotherapy*. This study has two specific aims. In Aim 1 we propose to determine specific contributions of the PI 3-K/Akt pathway in mediating resistance to chemotherapy drugs. In Aim 2 we propose to identify Akt substrates that mediate the response of breast cancer cells to genotoxic chemotherapy agents. Defining the contribution of the PI 3-K/Akt pathway to chemotherapy resistance is of great importance as a significant proportion of breast cancer patients harbor mutations in this critical signaling pathway. An understanding of the mechanisms that contribute to chemotherapy resistance will permit the development of novel strategies to treat breast cancer.

BODY

Aim 1: Determine specific contributions of the PI 3-K/Akt pathway in mediating chemoresistance to genotoxic drugs.

Aim 1, Task 1: Analysis of the ability of genotoxic agents to induce phosphorylation of Akt in breast cell lines. We have demonstrated that genotoxic chemotherapy agents induce Akt phosphorylation in breast cancer cells and non-tumorigenic breast epithelial cells in a time- and concentration-dependent manner. Sublethal concentrations of etoposide, doxorubicin and cisplatin, induce Akt phosphorylation (Figure 1). The extent of Akt phosphorylation differs between each of the drugs with doxorubicin



consistently able to induce the most significant increase in Akt phosphorylation in both tumorigenic and non-tumorigenic breast cell lines. Doxorubicin is frequently used as a single agent therapy for primary and recurrent breast cancer [1], and doxorubicin resistance is frequently observed in the clinic. Akt phosphorylation at Ser473 and Thr308 occurs several hours after drug treatment and is coincident with phosphorylation of histone H2AX (γH2AX), a marker of DNA damage (Figure 2). Akt phosphorylation is sustained for at least 24 hours (Figure 1A). At early time points, Akt phosphorylation is inhibited by the DNA-PK inhibitor Nu7026. DNA-PK has previously been implicated in

mediating Akt phosphorylation at Ser473 in response to DNA damage [2]. Interestingly, disruption of Akt phosphorylation by Nu7026 enhances H2AX phosphorylation and induction of apoptosis, as illustrated by cleavage of caspase-3, in response to doxorubicin treatment (Figure 2). This suggests that Akt phosphorylation contributes to cell survival following DNA damage.

Aim 1, Task 2: Examine the contribution that components of the PI 3-K signaling pathway make to the cellular response to genotoxic stress. We have shown that modulation of PI 3-K/Akt activity alters cellular sensitivity to genotoxic agents. Hyperactivation of the PI 3-K pathway, induced by overexpression of constitutively active PIK3CA H1047R (Figure 3B) or PTEN knockdown (Figure 3C), renders MCF10A cells more resistant to doxorubicin. In contrast, silencing of Akt isoforms sensitizes MCF10A cells to doxorubicin with Akt3 playing a dominant role in regulating cellular viability (Figure 3A). Having observed that Akt3 makes a significant contribution to drug sensitivity we decided to investigate the ability of the MAGI3-Akt3 fusion protein to confer resistance to genotoxic agents. A recent whole-exome and whole-genome sequencing effort to discover mutations and gene rearrangements in human breast cancers identified a translocation event between the *Akt3* gene and the *MAGI3* gene that produces an in-frame fusion protein [3]. This is the first fusion protein to be identified in the PI 3-K/Akt pathway. The translocation event results in significant disruption to the integrity of both Akt3 and MAGI3. We have demonstrated that MAGI3-Akt3 is constitutively phosphorylated in the Akt3 kinase domain in the absence of growth factors and its expression

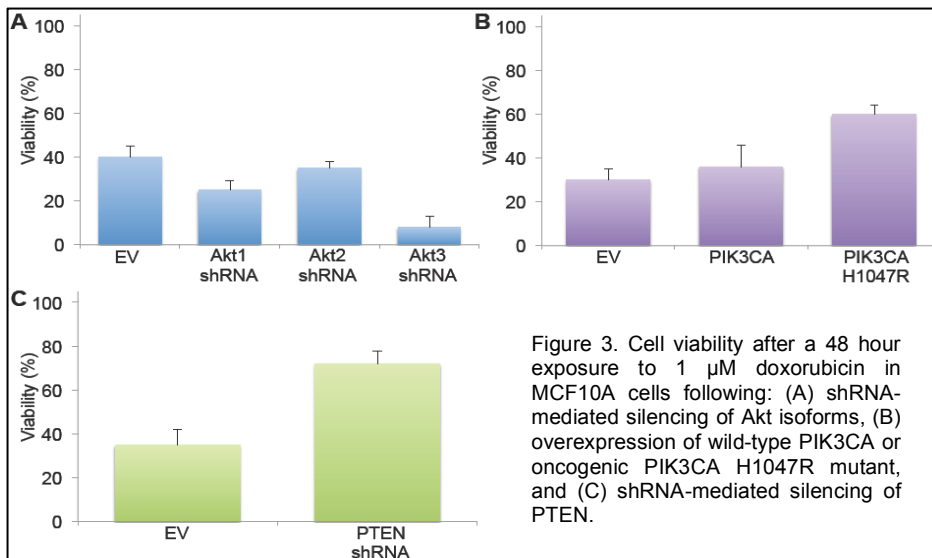


Figure 3. Cell viability after a 48 hour exposure to 1 μ M doxorubicin in MCF10A cells following: (A) shRNA-mediated silencing of Akt isoforms, (B) overexpression of wild-type PIK3CA or oncogenic PIK3CA H1047R mutant, and (C) shRNA-mediated silencing of PTEN.

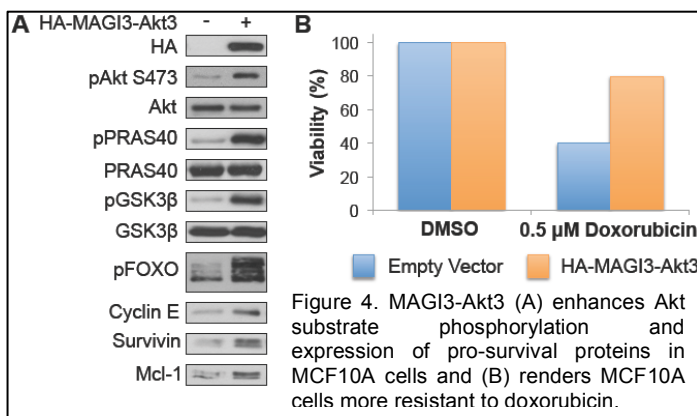


Figure 4. MAGI3-Akt3 (A) enhances Akt substrate phosphorylation and expression of pro-survival proteins in MCF10A cells and (B) renders MCF10A cells more resistant to doxorubicin.

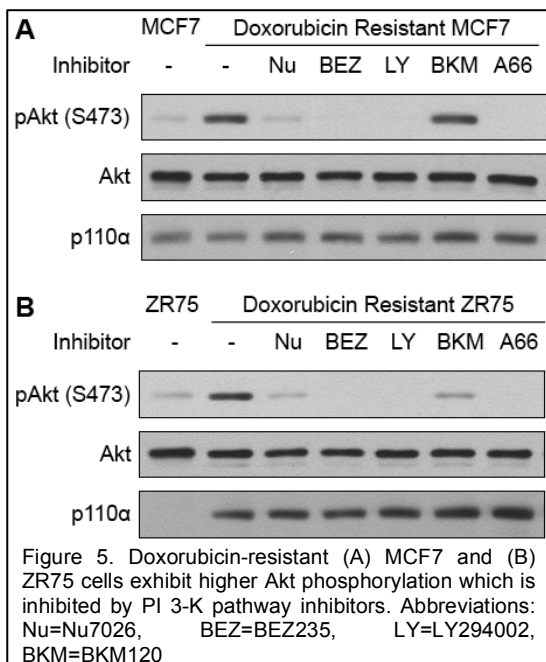


Figure 5. Doxorubicin-resistant (A) MCF7 and (B) ZR75 cells exhibit higher Akt phosphorylation which is inhibited by PI 3-K pathway inhibitors. Abbreviations: Nu=Nu7026, BEZ=BEZ235, LY=LY294002, BKM=BKM120

enhances Akt substrate phosphorylation [3] (Figure 4A). MAGI3-Akt3 also elevates the expression of pro-survival proteins including survivin and Mcl-1 (Figure 4A). We have preliminary data suggesting that MAGI3-Akt3 renders MCF10A cells more resistant to doxorubicin (Figure 4B). We are currently extending these preliminary findings to determine the contribution of MAGI3-Akt3 to chemotherapy resistance, which will provide insight into the contribution of Akt3 signaling to

chemotherapy resistance. Studies are also in progress to determine if wild-type alleles of PIK3CA and PTEN can rescue defects in the PI 3-K pathway that are commonly found in breast cancer cells and re-sensitize cells to chemotherapy agents.

Aim 1, Task 3: Examine combination therapy with DNA damaging agents and chemical inhibitors of the PI 3-K/Akt pathway as an approach to kill breast cancer cells. We have established chemotherapy resistant breast cancer cell lines. The resistant lines were generated

exposing cells to low dose doxorubicin for four months. Basal Akt phosphorylation is significantly higher in the resistant lines (Figure 5). Akt phosphorylation in the resistant lines can be inhibited by PI 3-K inhibitors, suggesting that prolonged exposure to genotoxic agents induces feedback activation of the PI 3-K pathway (Figure 5). Interestingly, we observed a significant increase in the expression of the catalytic subunit of PI 3-K (p110 α) in doxorubicin-resistant ZR75 breast cancer cells. We are continuing to investigate the role of feedback activation of the PI 3-K pathway in mediating chemotherapy resistance in these cell lines. We will explore the idea that downregulation of PI 3-K pathway activity, with shRNA or inhibitors, could resensitize these cell lines to chemotherapy agents. We are also initiating studies to examine the cytotoxicity of PI 3-K/Akt pathway inhibitors alone or in combination with genotoxic chemotherapy agents toward non-tumorigenic and tumorigenic breast cell lines.

Aim 1, Task 4: In vivo assays to determine Akt signaling specificity. We have developed cell lines in which we can

reproducibly silence Akt isoform expression by addition of doxycycline. This system will be discussed in Aim 2. These cell lines will be used to perform xenograft experiments to examine the contribution of Akt1, Akt2 and Akt3 to the antitumor activity of doxorubicin.

Aim 2: Identify isoform-specific substrates that mediate the response of breast cancer cells to DNA damaging chemotherapies.

Cell line	PI 3-K pathway mutation	Akt1	Akt2	Akt3
MDA-MB-231	KRas (G13D)	+	+	+
MDA-MB-468	PTEN mutant	+	+	+
ZR-75-1	PTEN missense	+	+	-
T47D	PIK3CA (H1047R)	+	+	-
MCF7	PIK3CA (E545K)	+	+	-
MCF10A	-	+	+	+

Table 1. Breast cell lines utilized for inducible knockdown of Akt isoforms.

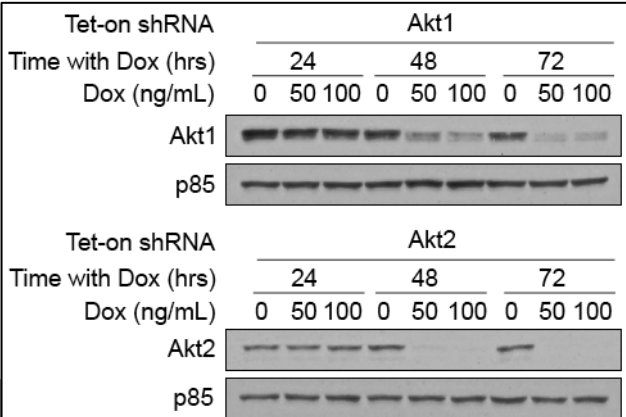


Figure 6. Time-course and concentration-dependence of Akt isoform depletion following doxycycline treatment in T47D breast cancer cells using tet-inducible shRNA constructs.

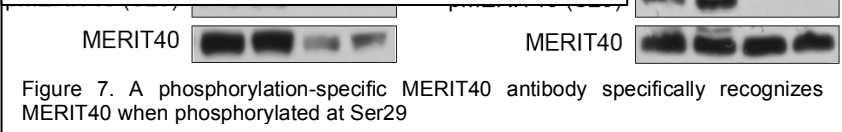


Figure 7. A phosphorylation-specific MERIT40 antibody specifically recognizes MERIT40 when phosphorylated at Ser29

Aim 2, Task 1: Silencing Akt isoforms in breast cell lines. We have developed highly specific inducible (Tet-on) shRNA constructs to silence Akt1, Akt2 and Akt3. These constructs have been introduced into a variety of breast cancer cell lines and non-tumorigenic MCF10A breast epithelial cells (Table 1). The breast cancer cell lines have been chosen as they are known to contain hyperactivating mutations in the PI 3-K pathway. We have generated stable cell lines and confirmed that doxycycline treatment can specifically silence Akt isoforms (Figure 6).

Aim 2, Task 2: Identify Akt substrates downstream of exposure to genotoxic agents A major goal of this task was to evaluate MERIT40 as a novel Akt substrate contributing to the cellular response to genotoxic chemotherapy treatment. MERIT40 is a component of the nuclear BRCA1 A complex which contains Abraxas, Rap80, BRCC36, BRE and the tumor suppressor protein BRCA1 [4-6]. MERIT40 also participates in a cytoplasmic complex with ABRO1, BRCC36 and BRE

[7]. Phospho-proteomic studies have demonstrated phosphorylation of MERIT40 at Ser29 in a consensus sequence that conforms to the Akt phosphorylation motif (RxRxxS/T). Using a combination of biochemical and molecular genetic approaches, we have identified MERIT40 as a novel Akt

substrate. We have collaborated with Cell Signaling Technology to develop an antibody that specifically recognizes MERIT40 when phosphorylated at Ser29. Using a combination of site-directed mutagenesis and shRNA approaches we have confirmed that the phospho-MERIT40 antibody specifically recognizes MERIT40 when phosphorylated at Ser29 (Figure 7). We have also developed an antibody that recognizes both phosphorylated and unphosphorylated MERIT40. MERIT40 phosphorylation is induced when cells are exposed to genotoxic chemotherapy agents like doxorubicin (Figure 8A). Furthermore, doxorubicin-induced MERIT40 phosphorylation is coincident

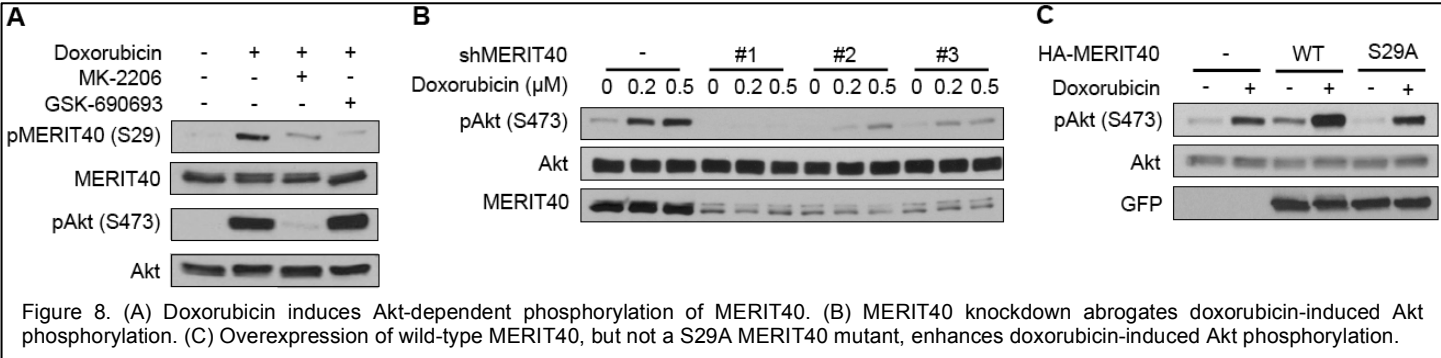


Figure 8. (A) Doxorubicin induces Akt-dependent phosphorylation of MERIT40. (B) MERIT40 knockdown abrogates doxorubicin-induced Akt phosphorylation. (C) Overexpression of wild-type MERIT40, but not a S29A MERIT40 mutant, enhances doxorubicin-induced Akt phosphorylation.

with Akt activation and can be blocked by the Akt inhibitors MK-2206 and GSK-690693 (Figure 8A). Interestingly, knockdown of MERIT40 completely abrogates Akt phosphorylation induced by doxorubicin (Figure 8B). In addition, overexpression of wild-type MERIT40, but not a Ser29Ala MERIT40 mutant enhances doxorubicin-induced Akt phosphorylation (Figure 8C). This suggests that phosphorylated MERIT40 participates in the regulation of Akt activity.

MERIT40 has recently been shown to interact with the poly-(ADP-ribose) polymerase Tankyrase (TNKS) [8]. We have shown that mutation of Ser29 disrupts the interaction between MERIT40 and TNKS (Figure 9A). Furthermore, knockdown of TNKS prevents doxorubicin-induced Akt phosphorylation in a manner analogous to knockdown of MERIT40 (Figure 8B versus 9B). TNKS has been shown to regulate pro-survival signaling pathways [9, 10] and we are currently exploring the possibility that the phosphorylation-dependent interaction between MERIT40 and TNKS protects cells from the cytotoxicity of genotoxic chemotherapy agents via modulation of Akt activity. We have shown that knockdown of MERIT40 using specific shRNA constructs can sensitize breast cancer cells to doxorubicin (Figure 4A). We will examine the ability of wild-type MERIT40 and a MERIT40 Ser29Ala mutant to rescue cell viability in response to genotoxic drug exposure. In addition to investigating MERIT40 as a novel Akt substrate we are currently in the process of initiating phosphoproteomic studies to identify additional novel Akt substrates.

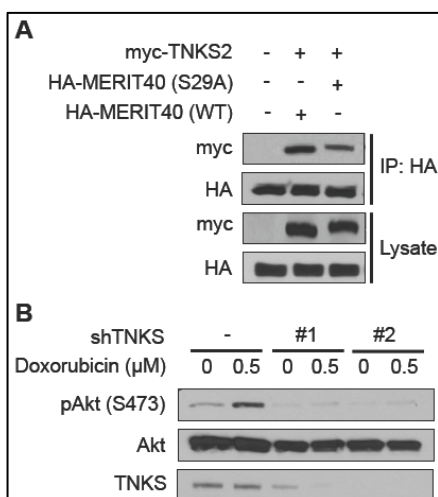


Figure 9. (A) MERIT40 interacts with TNKS in a phosphorylation-dependent

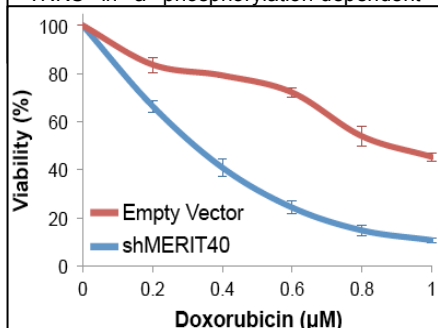


Figure ?. MERIT40 knockdown sensitizes breast cancer cells to doxorubicin.

Aim 2, Task 3: Validate candidates as substrates of the PI 3-K pathway in breast cancer cells. The timeframe for this task is months 12-18.

Aim 2, Task 4: Explore the functional consequences of candidate substrate phosphorylation by Akt in the response of breast cancer cells to DNA damaging chemotherapy. The timeframe for this task is months 18-24.

Aim 2, Task 5: Examine candidate substrate phosphorylation in human breast tissue (non-tumor and tumor) microarrays. The timeframe for this task is months 18-24.

KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated that:

1. genotoxic chemotherapy agents induce Akt phosphorylation.
2. inhibition of Akt enhances DNA damage and induction of apoptosis following exposure to genotoxic drugs, suggesting that Akt phosphorylation contributes to cell survival.
3. hyperactivation of the PI 3-K pathway renders cells more resistant to genotoxic chemotherapy drugs.
4. silencing of Akt isoforms sensitizes cells to genotoxic chemotherapy drugs. In particular, Akt3 may play a dominant role in regulating cellular sensitivity to these drugs.
5. the MAGI3-Akt3 fusion protein renders cells more resistant to genotoxic drugs.
6. chemotherapy-resistant breast cancer cells exhibit enhanced basal Akt phosphorylation suggesting that prolonged exposure to genotoxic drugs induces feedback activation of PI 3-K/Akt signaling.
7. we have developed highly specific inducible shRNA constructs to silence Akt1, Akt2 and Akt3.
8. we have developed an antibody that specifically recognizes MERIT40 when phosphorylated at Ser29 by Akt.
9. MERIT40 phosphorylation is induced when cells are exposed to genotoxic drugs.
10. knockdown of MERIT40 abrogates Akt phosphorylation induced by genotoxic drugs, suggesting that MERIT40 contributes to the regulation of Akt.
11. MERIT40 interacts with tankyrase in a phosphorylation-dependent manner.
12. knockdown of MERIT40 sensitizes breast cancer cells to genotoxic chemotherapy drugs.

REPORTABLE OUTCOMES

The research findings described in this report and supported by this grant have been presented at the following symposia:

1. Alex Toker and Kristin Brown, Keystone Symposia on PI 3-Kinase and Interplay with Other Signaling Pathways in Keystone, Colorado, February 24-28 2013.
Dr. Kristin Brown was awarded a Keystone Symposia Future of Science Fund scholarship based on her presentation of the work above.
2. Alex Toker and Kristin Brown, 2013 Annual AACR (American Association for Cancer Research) Meeting in Washington, DC, April 6-10, 2013. Dr. Kristin Brown was awarded a 2013 Women in Cancer Research Scholar Award.

CONCLUSION

We have discovered that the Akt pathway modulates breast cancer cell survival in response to genotoxic agents, and discovered a new substrate of Akt, MERIT40, that is phosphorylated upon exposure of cells to chemotherapeutic drugs. We propose that this represents a major mechanism by which cells exposed to these drugs evade cell death by apoptosis and thus become resistant to the damaging effects of clinically-relevant chemotherapy agents.

These findings have important ramifications for the use of chemotherapy drugs in breast cancer patients, and many also suggest that MERIT40 may be used as a clinically relevant biomarker for resistance to doxorubicin.

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