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14. ABSTRACT Resistance to antiestrogens is a serious clinical problem in breast cancer treatment, and a better understanding of the mechanisms of antiestrogen resistance is urgently needed. Our hypothesis, which is supported by our preliminary data, is that the signaling molecule Cas has an important causal role in the development of antiestrogen resistance. As a corollary, understanding of the pathways that Cas activates may identify key regulators of antiestrogen resistance and novel targets for breast cancer treatment, and measurements of Cas signaling levels may provide useful prognostic information for breast cancer patients. Our objective is to test our hypothesis, and to identify the signaling pathways that mediate Cas-induced antiestrogen resistance. Our working model is that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen esistance. Testing this model relies on reciprocal analysis of dominant-negative and constitutively active forms of the various esistance. Testing this model relies on reciprocal analysis of dominant-negative and constitutively active forms of the various genetic and cellular tools described in the report in detail, allowing us to perform rigorous functional studies on the antiestrogen resistance in breast cancer cells during the upcoming year.

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Annual Report for W81XWH-04-1-0523

TITLE: CAS SIGNALING IN BREAST CANCER

1. Introduction

Antiestrogens, especially tamoxifen, have proven to be effective in the treatment of hormone-responsive breast cancer. In metastatic breast cancer, antiestrogens lead to a response in nearly one half of patients with estrogen receptor (ER)-positive primary tumor (1). Resistance to antiestrogens, however, is a serious clinical problem. About 40% of ER-positive tumors fail to respond to antiestrogen therapy, and most, if not all, breast tumor patients that initially respond to antiestrogens will eventually develop resistance (2). Furthermore, there are currently no biomarkers that reliably predict tamoxifen responsiveness in patients with ER-positive tumors. A better understanding of the mechanisms of antiestrogen resistance is therefore urgently needed.

A recent mutagenesis approach has identified three independent loci associated with antiestrogen resistance (3), and the target genes of two of the loci, BCAR1 and BCAR3, have been characterized. Interestingly, sequence analysis of BCAR1 demonstrated it to code for the docking protein p130Cas (Cas), which we and others have previously identified to be a key molecule in intracellular signaling pathways (4). Subsequent studies demonstrated that enhanced activation of Cas signaling can induce antiestrogen resistance, at least in cell culture conditions (5). Recent studies have demonstrated that Cas is likely to have a relevant role also in clinical breast cancer; studies on breast cancer samples have shown that high levels of Cas expression correlate with poor relapse-free and overall survival, and the response to tamoxifen therapy in patients with recurrent disease was found to be reduced in patients with primary tumors that expressed high levels of Cas (6).

Our **hypothesis**, which is supported by our preliminary data, is that Cas has an important causal role in the development of antiestrogen resistance. As a corollary, understanding of the pathways that Cas activates may identify key regulators of antiestrogen resistance and novel clinical targets for breast cancer treatment, and measurements of Cas signaling levels may provide useful prognostic information for breast cancer patients. Our **objective** is to test our hypothesis, and to identify and characterize the signaling pathways that mediate Cas-induced antiestrogen resistance. Our additional objective is to develop novel tools to be used as prognostic reagents for ER-positive patients with intrinsic resistance to tamoxifen.

2. Body of the Report

In order to meet the objectives outlined above, two specific aims were set forth in our grant application. **In the first aim**, our goal is to identify and characterize the signaling pathways that mediate Cas-dependent antiestrogen resistance. Our hypothesis is that the interaction of Cas with two signaling molecules, Crk and BCAR3, is required for Cas-dependent antiestrogen resistance. We further hypothesize that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance; this working model will be tested in Aim 1 during the first two years of the grant funding. If this model proves to be *in*correct, we will utilize a novel function-based screening method to identify Cas-interacting proteins that mediate antiestrogen resistance. Depending on the nature of the interacting molecules, further experiments will be planned to dissect their roles in antiestrogen resistance. **In the second aim**, to be accomplished during the latter part of the grant period, our goal is to identify the tyrosine residues in Cas that become phosphorylated in breast cancer cells. Cas activates signaling pathways by binding to Src homology 2 (SH2)-domain containing

signaling molecules, such as Crk, in a tyrosine phosphorylation-dependent manner. Further, our preliminary studies indicate that hyperphosphorylation of Cas correlates with antiestrogen resistance. Thus, we will employ two types of mass spectrometers in a multi-tiered strategy to systematically map the tyrosine residues in Cas that become phosphorylated in breast cancer cells *in vivo*.

During the first and second years of funding, we have focused our efforts on the first aim of the original application, as proposed. **The task** that is to be accomplished as part of this aim is as follows:

Task 1. Test our working model that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 complex to mediate antiestrogen resistance (months 1-24).

a. Generate mammalian expression constructs of activated and dominant-negative forms of BCAR3; we already have constructs for Cas, Crk, Rac and the JNK pathway.

b. Test whether expression of an activated form of Crk and activated form of BCAR3 will rescue the CasΔSD and CasΔCT-phenotypes in antiestrogen resistance, respectively. In these studies, stable MCF-7 cell lines expressing the corresponding constructs will be generated, and cell proliferation in the presence of tamoxifen will be studied.

c. Test whether dominant-negative forms of Crk and BCAR3 block Cas-induced antiestrogen resistance. These studies will be performed as above.

d. Determine whether a dominant-negative form of Rac will block Cas-, Crk- and BCAR3-induced antiestrogen resistance. Also determine whether an activated form of Rac will rescue the defect in and inhibition of antiestrogen resistance by CasΔSD and CasΔCT, as well as by dominant-negative forms of Crk and BCAR3. These studies will be performed as above.

e. Determine what is the role of the JNK-pathway downstream of Rac in Cas-mediated antiestrogen resistance. The effect of overexpression of activated and dominant-negative JNK on Cas-,Crk-, BCAR3- and Rac-induced antiestrogen resistance will be studied.

As outlined in Task 1, our experimental strategy for testing our working model that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance relies on reciprocal testing of dominant-negative and constitutively active forms of the various signaling molecules in this pathway. As such, bulk of our efforts have focused on generating the genetic and cellular tools described above to be able to perform rigorous functional studies on the antiestrogen resistance in breast cancer cells. With the recent generation of the DNA constructs for activated and dominant-negative forms of BCAR3, we now have all the necessary DNA tools available that are needed for these studies. Importantly, we have been able to design a point mutant construct of BCAR3 that specifically disrupts binding of BCAR3 to Cas, as opposed to other proteins utilizing the same protein-protein interaction domain within the BCAR/SHEP proteins. Our recent collaborative paper with Dr. Pasquale's laboratory reported this finding (7). [Other constructs to be used in these studies have been described in our previous studies in refs (8-10)].

As a second step, we are currently generating MCF-7 cell lines expressing physiological or pathophysiological levels of the various constructs described in Task 1. To achieve this objective, we are using DNA constructs generated in a retrovirus background, which allows us to select either clones (to allow selection of a homogenous set of cells) or pools (to eliminate clonal variation) of MCF-7 cells expressing the desired levels of the exogenously delivered

protein(s). We are in the process of selecting several MCF-7 cell lines at the moment. First, we are selecting MCF-7 cell lines overexpressing either Crk or BCAR3 in the CasΔSD or CasΔCT-expressing background, respectively. With these to-be-generated cell lines, we will be able to assess the relative significance of Crk and BCAR3 signaling pathways in Cas-mediated antiestrogen resistance. That is, we will be able to assess as to whether Crk and/or BCAR3 are *sufficient* components in antiestrogen resistance pathways downstream of certain Cas mutants. Second, we are generating cell lines expressing dominant-negative forms of either Crk or BCAR3 in the Cas-overexpressing MCF-7 background. These studies will help us to assess whether Crk and/or BCAR3 are *necessary* components in Cas-mediated antiestrogen resistance pathways. Third, we are generating breast cancer cell lines that overexpress wild-type and activated forms of Crk and BCAR3. Analysis of these cell lines will help us to assess whether Crk and BCAR3 are *necessary* for antiestrogen resistance in breast cancer cell lines *in vitro*. Fourth, cell lines expressing either Cas, Crk, or BCAR3 will be subjected to retroviral infection with a construct expressing a dominant-negative form of Rac. This line of investigation will allow us to assess the putative *necessary* role of the small GTPase Rac in antiestrogen resistance mediated by Cas, Crk and/or BCAR3. Finally, we are generating breast cancer cell lines that express the activated form of Rac in a CasΔSD or CasΔCT-expressing MCF-7 background. This study will allow us to assess whether Rac is a *sufficient* component in Cas-mediated antiestrogen signaling pathways.

We have also taken a slightly different angle to address the question as to how Cas affects cell growth. Thus, we have also employed Cas -/- knock-out cells, and compared their growth profile to cells that have physiological levels of Cas. The advantage of this approach (as opposed to studies described above) is that we will not be expressing exogenous levels of Cas, but rather take the reverse approach and reduce Cas levels by genetic means. While these studies are in early stages, we have uncovered that Cas likely plays a role in cell cycle progression from G1 to S phase of cell cycle. The is visualized in the FACS analysis of cells expressing wild-type Cas at physiological levels ("WT") or cells lacking Cas and instead expressing a control empty vector ("EV") (Fig. 1). Quantification of the FACS analysis is shown in Fig. 2, which demonstrates a delay of bulk of the cells lacking Cas in entering the S phase. While these cells eventually "catch up" (Fig. 3), we expect that enhanced Cas expression in breast cancer cells may lead to antiestrogen resistance by providing a growth advantage in the G1-S transition of cell cycle. Studies are currently in the plans to address this in more detail.

Figure 1:

Absence of p130Cas leads to a delay in the cell cycle progression

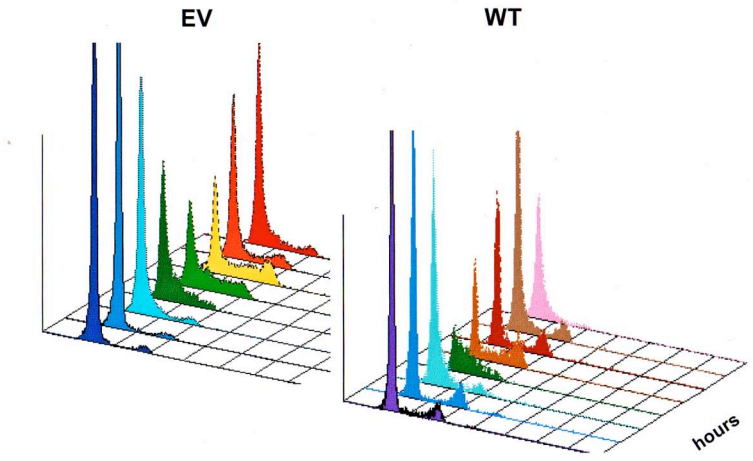


Figure 2:

Quantification of Cell Cycle Progression in EV and WT Cells

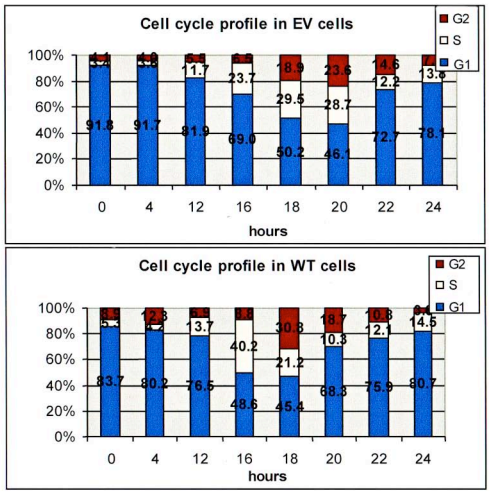
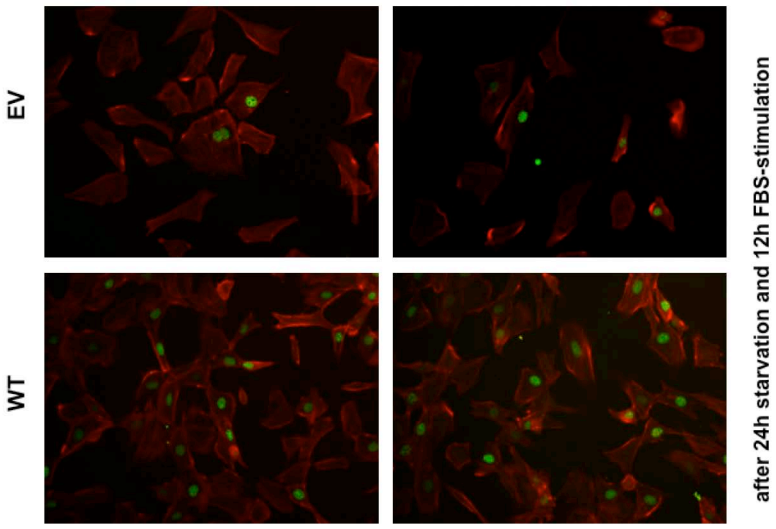


Figure 3:

Delay in Cell Cycle Progression correlated with different kinetics of BrdU Incorporation



In conclusion, we are well on schedule in accomplishing our proposed studies within the Task 1, and have expanded our studies "downstream" to attempt to understand the effect of Cas at the level of cell cycle, as well. Overall, the results obtained so far will form a strong basis for additional studies during the final year of DoD funding.

3. Key Research Accomplishments

1. Generation of all of the DNA constructs needed to accomplish studies outlined in Aim 1 of the original application.

2. By virtue of generating the requisite DNA constructs (see above), we are well on schedule to generate the proposed breast cancer cell lines in Task 1 in order to rigorously examine the working model of this grant application; that is, that the Rac-JNK

pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance in breast cancer.

3. As an alternative and complementary strategy, we are employing cells that are genetically engineered to lack the gene coding for Cas, and studying them in classical cell cycle studies to uncover the effect of Cas on cell proliferation.

4. Reportable Outcomes

1. Generation of molecularly-defined key DNA constructs and breast cancer cell lines to examine antiestrogen resistance *in vitro*.

2. Generation of molecular defined cell lines that are genetically deficient of Cas.

5. Conclusions

Our preliminary data presented in the original grant application supports the role of the docking protein Cas in antiestrogen resistance. During the first two years of funding, we have accomplished the bulk of the goals outlined in Aim 1; in these studies, the intracellular signaling pathways downstream of Cas mediating antiestrogen resistance will be interrogated in detail at the molecular level. Immediate next studies will take advantage of the newly generated tools described above, in studies also outlined above. No changes have been made, nor proposed, to the technical design to accomplish the original goals of the original application. Importantly, we have expanded our goals to have cell cycle studies as they relate to G1-S transition as an additional read-out. We expect these studies to significantly complement and strengthen the originally proposed studies. For details, see above.

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7. Appendix

None.