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Award Number: W81XWH-08-1-0319

TITLE: XBP1, Unfolded Protein Response, and Endocrine Responsiveness

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CONTRACTING ORGANIZATION:

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Washington, DC 20007, USA

REPORT DATE: May 2010

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

XX Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2010	Annual	15 APR 2009 - 14 APR 2010

4. TITLE AND SUBTITLE
XBP1, Unfolded Protein Response, and Endocrine Responsiveness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0319

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert Clarke

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Almost 50% of all ER+ breast tumors will not respond to endocrine therapy. Resistance to endocrine therapy remains a significant clinical problem and advanced ER+ breast cancer is largely an incurable disease. Endocrine manipulation in sensitive cells can result in the induction of cell death through autophagy and/or apoptosis. We have recently obtained data implicating the unfolded protein response (UPR) as induced by the splicing of X-box binding protein-1 (XBP1) in the regulation of endocrine responsiveness in breast cancer cells. UPR is a key component of the endoplasmic reticulum stress response and has not previously been implicated in endocrine responsiveness. We hypothesize that XBP1(S) is a key regulator of breast cancer cell fate, acting through its regulation of UPR, BCL2, and BCL2:BECN1 heterodimers, and their subsequent effects on autophagy and apoptosis. We will determine how XBP1(S) affects cell fate, evaluating the role of an induction of UPR that activates a prosurvival autophagy. In endocrine sensitive cells, autophagy should persist and become a cell death mechanism that can also initiate apoptosis. In resistant cells, basal autophagy should represent a survival mechanism to deal with the loss of autocrine and other growth factor signaling that accompanies endocrine therapy. We will explore the mechanistic role of XBP1(S) and its integrated signaling through UPR and BCL2 to regulate cell fate in both endocrine sensitive and resistant cells.

15. SUBJECT TERMS
Antiestrogens, aromatase inhibitors, BCL2 family, bioinformatics, drug resistance, endoplasmic reticulum, Faslodex, Fulvestrant, gene networks, mitochondria, molecular signaling, Tamoxifen, unfolded protein response.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	8	**19b. TELEPHONE NUMBER** *(include area code)*

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1. Crawford et al., . *PLoS One.* 5:e8604, 2010.

INTRODUCTION

In 2008, over 40,000 American women will die of breast cancer [1]. In the same period, there will be over 178,000 newly diagnosed cases of invasive breast cancer, almost 70% of which will be estrogen receptor-α positive (ER+) [2,3]. However, 50% of all ER+ breast tumors will not respond to endocrine therapy [4]. Tamoxifen produces an overall 26% proportional reduction in mortality [5] but many ER+ tumors that show an initial response to tamoxifen eventually recur [4]. Resistance to endocrine therapy remains a significant clinical problem and advanced ER+ breast cancer is largely an incurable disease. Endocrine manipulation in sensitive cells can result in the induction of cell death through autophagy and/or apoptosis. However, the control of these processes, and an understanding of how the dual nature of autophagy is regulated in breast cancer cells (autophagy can be prodeath or prosurvival), is largely unknown. We have recently obtained data implicating the unfolded protein response (UPR) as induced by the splicing of X-box binding protein-1 (XBP1) in the regulation of endocrine responsiveness in breast cancer cells. UPR is a key component of the endoplasmic reticulum stress response and has not previously been implicated in endocrine responsiveness.

We propose that XBP1 uses a specific cellular stress response mechanism (the unfolded protein response), members of the BCL2 gene family, and two other genes, *i.e.*, beclin 1 (BECN1) and MYC to mediate this control of cell fate. The choice to live or die is a critical decision for a breast cancer cell, and a greater understanding of how this choice is regulated is needed. This IDEA award would allow us to explore, in a timely and effective manner, these very recent observations that have lead directly to the construction of our novel hypothesis.

The proposed research could lead to better approaches to predict an individual patient's responsiveness to endocrine therapies and to the development of new strategies to improve the efficacy of endocrine therapies and increase overall survival. For example, measuring the coexpression of activated XBP1 and its key downstream targets that regulate cell survival could be used to more accurately predict the sensitivity of a tumor to endocrine therapy. Inhibiting the activation of XBP1 could either prevent the development of resistance or restore sensitivity.

BODY

Overview: The main objective of our study is to show how XBP1(S)-UPR mechanistically affects endocrine responsiveness. We propose that XBP1(S)-regulation of UPR activates prosurvival autophagy in endocrine resistant cells but can switch to prodeath autophagy leading to an autophagic and/or apoptotic cell death in sensitive cells. In endocrine resistant cells, **we propose** that XBP1(S) maintains a high level of *prosurvival* autophagy (UPR), while also increasing BCL2 expression that feeds back to limit *prodeath* autophagy (BCL2:BECN1) and/or apoptosis (BCL2).

We hypothesize that XBP1(S) is a key regulator of breast cancer cell fate, acting through its regulation of UPR, BCL2, and BCL2:BECN1 heterodimers, and their subsequent effects on autophagy and apoptosis. Specifically, we hypothesize that XBP1(S) uses UPR (proautophagy) and BCL2 (antiapoptosis) and BCL2:BECN1 interactions (antiautophagy) to regulate the balance between autophagy and apoptosis and to determine breast cancer cell fate in response to antiestrogens and aromatase inhibitors (which we will model with estrogen deprivation).

Specific Aims We will use a series of human breast cancer cell lines/variants and apply established and state-of-the art methods to address our specific aims. We will explore the mechanistic role of XBP1(S) and its integrated signaling through UPR and BCL2 to regulate cell fate in both endocrine sensitive and resistant cells.

AIM 1: We will determine how XBP1(S) affects cell fate, evaluating the role of an induction of UPR that activates a prosurvival autophagy. In endocrine sensitive cells, autophagy should persist and become a cell

death mechanism that can also initiate apoptosis. In resistant cells, basal autophagy should represent a survival mechanism to deal with the loss of autocrine and other growth factor signaling that accompanies endocrine therapy, with the switch to prodeath signaling being concurrently suppressed.

AIM 2: We will determine how XBP1(S) signals (*e.g.*, through BCL2 and BECN1) to affect endocrine responsiveness and cell survival. We will then use these data to build an interactive *in silico* model of how this signaling operates (how the nodes are connected and function) in the context of endocrine responsiveness.

KEY RESEARCH ACCOMPLISHMENTS

- From the tasks carried out in Year 2 of this grant, we were able to emphasize the central role for autophagy in antiestrogen resistance and the important roles played by BCL2 and potentially also a role for the up-regulation of myc in these resistant cells.
- We showed that combination therapy that includes an antiestrogen and a BCL2 inhibitor can significantly re-sensitize antiestrogen resistant breast cancer cells.
- Published one peer-reviewed article and two meeting abstracts (see Reportable Outcomes).

Progress on our Statement of Work

Months 13-23:

Aim 1 - BCL2 proteins integrate central functions in determining ICI responsiveness likely by regulating functional autophagy to dictate the balance between apoptotic and necrotic cell death. Endogenous of BCL2 in antiestrogen-sensitive LCC1 breast cancer cells are lower than in antiestrogen-resistant LCC9 cells. While levels of BCL2 decrease in LCC1 cells, levels of BCL2 LCC9 cells remains unchanged following treatment with ICI 182,780 for 24 or 72 h (**Figure 1**). Inhibiting BCL2 with a small-molecule inhibitor, YC137, re-sensitized LCC9 cells to ICI 182,780 (**Figure 2**). Cell death in LCC9 cells that are re-sensitized to ICI 182,780 by co-treatment with YC137 occur via autophagy induced necrosis (**Figures 3 and 4**).

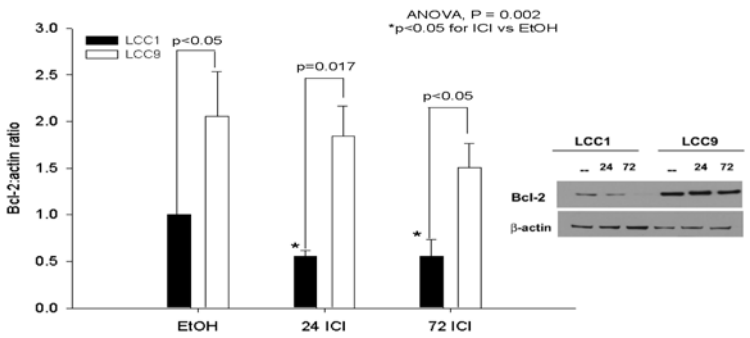

Figure 1: Increased level of BCL2 in antiestrogen resistant cells. Western blot analysis of total cell lysates following treatment with ICI 182,780 for indicated amount of time in LCC1 versus LCC9 cells (Crawford et al., 2010).

Aim 2- Inhibition of endogenous MYC re-sensitizes LCC9 endocrine resistant cells to antiestrogens. Co-treatment with MYC inhibitor, 10058-C4 (25 μM), and ICI 182,780 (100 nM) of LCC9 cells for 7 days synergistically inhibit cell proliferation (RI=1.51) (**Figure 5**). Next, we will determine whether co-treatment of 10058-F4 and ICI 182,780 in resistant cells induce cell death via apoptosis or autophagy-induced necrosis.

We have had a few technical difficulties in transfecting XBP1 cDNA into LCC1 and LCC9 cells but we are in the process of solving those issues at the moment. We plan to determine the time-dependent induction of autophagy (by looking at ATG5 induction) or apoptosis (BID induction) in LCC1 or LCC9 cells overexpressing XBP1(S).

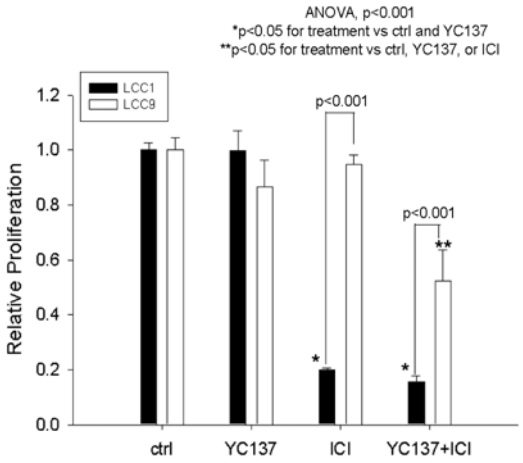

Figure 2: Inhibition of BCL2 increases sensitivity to ICI 182,780 in LCC9 cells. Cell proliferation was measured after 7 days of treatment (Crawford et al., 2010).

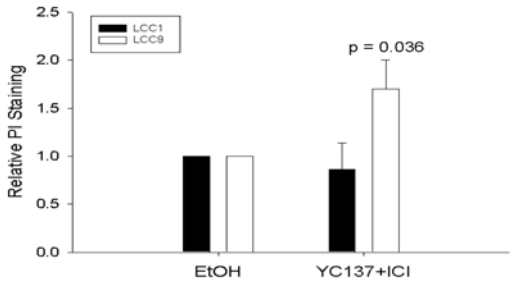

Figure 3: Increases levels of necrosis (indicated by increase in propidium iodide [PI] staining) in LCC9 cells after co-treatment with YC137 and ICI 182,780 (48 h) (Crawford et al., 2010).

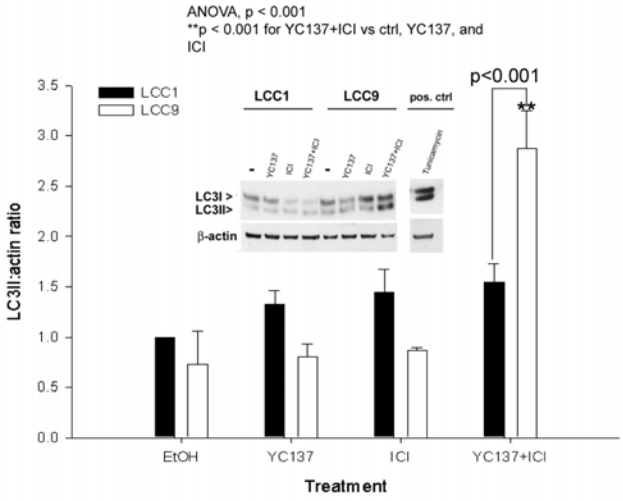

Figure 4: Increased level of autophagy (as detected by increased levels of LC3II) following co-treatmet with YC137 and ICI 182,70 in LCC9 cells.

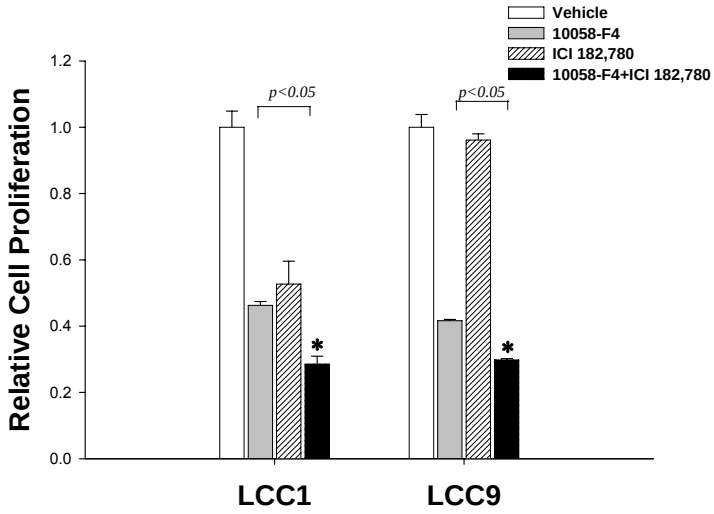

Figure 5: Co-treatment with MYC inhibitor, (25 μM) 10058-F4, and ICI 182,780 (100 nM) for 7 days synergistically inhibits cell proliferation in LCC9 cells. *p<0.05* versus 10058-F4 without ICI 182,780, RI=1.51 for LCC9 cells. RI values were obtained by calculating the expected cell survival (S_{exp}; the product of survival obtained with drug A alone and the survival obtained with drug B alone) and dividing S_{exp} by the observed cell survival in the presence of both drugs (S_{obs}). S_{exp}/S_{obs} > 1.0 indicates a synergistic interaction.

REPORTABLE OUTCOMES

Papers and Meeting Reports*

- Crawford et al., . *PLoS One.* 5:e8604, 2010.

- AACR 2010 abstract #2919: XBP1 and the unfolded protein response in antiestrogen resistance in breast cancer. Ayesha N. Shajahan, Rebecca B. Riggins, Alan Zwart, F. Edward Hickman, Robert Clarke.

- 4601: XBP1 regulated function of c-MYC and BCL2 in antiestrogen resistance in breast cancer Lauren M. McDaniel, Ayesha N. Shajahan, Robert Clarke.

*We include in the appendix reprints of the paper that is already published and for which we have proofs or reprints. We do not list here or include in the appendices any published abstracts, but can do so if requested.

CONCLUSIONS

We have made very good progress on the proposed work in our second year. The data strongly support our data on a central role for autophagy and UPR, linking in BCL2 and potentially also a role for the up-regulation of myc in resistant cells. The data suggest that resistant cells are more reliant upon myc for cell survival. Less clear is how to use the data in sensitive cells, since inhibition of myc appears to be beneficial. If these data are validated further, determining how to use myc inhibitors will be challenging because most breast cancers are heterogeneous and will contain both resistant and sensitive cells. Further studies are required to appreciate fully the clinical relevance of these data.

The data with BCL2 are very interesting and support the design of additional studies to combine antiestrogens and BCL2 family inhibitors. We will begin to address this *in vivo* in year 3 (and probably into a no-cost extension – we have only now obtained approval to initial the animal studies due to delays here at Georgetown). The potential that these small molecule inhibitors may be candidates for consideration as new drug therapies is exciting because some are now entering clinical trials in other cancers. We hope to be able to eventually use the data from this grant (the data we have and those we will obtain as we complete the studies) to design clinical trials to directly test this hypothesis and potentially improve endocrine therapies for women with breast cancer.

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