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AD \_\_\_\_\_

Award Number: DAMD17-97-1-7224

TITLE: Characterization of Breast Cancer Susceptibility Gene  
BRCA2

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REPORT DATE: September 1999

TYPE OF REPORT: Annual

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

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<b>1. AGENCY USE ONLY (Leave blank)</b>	<b>2. REPORT DATE</b> September 1999	<b>3. REPORT TYPE AND DATES COVERED</b> Annual : (16 Aug 98 -15 Aug 99)
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<b>4. TITLE AND SUBTITLE</b> Characterization of Breast Cancer Susceptibility Gene BRCA2	<b>5. FUNDING NUMBERS</b> DAMD17-97-1-7224
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<b>6. AUTHOR(S)</b> Edward Lobenhofer ,Ph.D. Dr. Jeff Marks	
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<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Duke University Medical Center Durham, North Carolina 27710  <b>E-MAIL:</b> Ek12@acpub.duke.edu	<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>
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<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>
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**13. ABSTRACT (Maximum 200 Words)**  
 Estrogen promotes proliferation in the MCF-7 breast cancer cell line via high levels of estrogen receptor. The primary mode of estrogen action has been considered to be through the transcriptional activation of gene containing estrogen response elements including the immediate early genes c-myc and fos. Recent reports have indicated that estrogen is capable of activating the MAPK cytoplasmic signaling cascade. In this study, specific small molecule inhibitors of MAPK (PD098059) and PI3-K (LY294002) activity were used to determine the influence of these cascades on estrogen-mediated mitogenesis. Both compounds decreased the fraction of cells entering DNA synthesis after treatment with 17β-estradiol. While a small decrease was noted in estrogen stimulated transcriptional activity, these drugs did not inhibit expression of myc or fos. However, both drugs did prevent the accumulation of cyclin D1, cdk2 activation, and hyperphosphorylation of the retinoblastoma protein indicating that the block occurred at, or prior to, this point in the cell cycle. Interestingly, the downstream targets of these kinase cascades, Erk1, Erk2, and PKB, were not activated over basal levels in response to estrogen treatment. These studies indicate that estrogen initiates mitogenesis by inducing the transcription of immediate early genes, but cytoplasmic signaling pathways play an important role in the control of subsequent events in the cell cycle.

<b>14. SUBJECT TERMS</b> Estrogen, Proliferation, Breast Cancer, MAPK, PI3-K, Predoc Award	<b>15. NUMBER OF PAGES</b> 11
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	<b>16. PRICE CODE</b>
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<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Limited
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## Introduction/Summary of Last Year's Report

Last year's annual report detailed the completion of Specific Aim #1 (*develop key reagents in order to characterize the BRCA2 gene and protein*) as well as a substantial portion of Specific Aim #2 (*characterize the BRCA2 protein*). Pitfalls were also detailed explaining the refocusing of my research efforts from the hereditary/genetic basis for breast cancer to a concentration on the molecular mechanisms controlling estrogen's ability to function as a mitogen.

Estrogen acts to promote DNA synthesis in the MCF-7 human breast cancer cell line through high levels of estrogen receptor (ER). The primary mode of estrogen action has been considered to be through transcriptional activation of genes containing estrogen response elements including the immediate early genes *c-myc* and *c-fos* (1-3). After immediate early gene synthesis, the cell cycle converges on the expression and activity of the cyclins and the cyclin-dependent kinases (CDK), particularly the D-type cyclins. Cyclin D1 levels increase in response to mitogen stimulation, resulting in complex formation with either CDK4 or CDK6 (4, 5). This holoenzyme serves two functions: 1) sequestration of CDK inhibitors (such as  $p27^{Kip1}$  and  $p21^{Cip1}$ ) from cyclin E, facilitating the formation of active cyclin E-CDK2 complexes, and 2) phosphorylation of the retinoblastoma (Rb) protein, which is also phosphorylated by cyclin E-CDK2 (6-8).

The activities of estrogen have generally been thought to occur primarily in the nucleus via interaction with its cognate nuclear receptor. Several recent reports have indicated that estrogen, acting through the estrogen receptor, is capable of inducing the mitogen-activated protein kinase (MAPK) cytoplasmic signaling cascade (9-13). Additional studies have shown that estrogen stimulates the production of phosphatidylinositol (PI) and the activation of PI kinases (14). A primary downstream target of the PI pathway is protein kinase B (PKB/Akt), which has been implicated in cyclin D1 protein stabilization.

To examine the roles of both the MAPK pathway as well as the PI3-K (phosphatidylinositol 3-kinase) cascade, small molecule inhibitors were utilized. Last year, data was presented showing that  $50\mu\text{M}$  PD098059, a MEK1 inhibitor (immediately upstream of MAPK), and  $50\mu\text{M}$  LY294002, an inhibitor of PI3-K activity, prevented cell cycle progression of MCF-7 cells in response to estrogen stimulation. Further studies with these compounds suggested that PI3-K and MAPK activity were not required for transcriptional regulation of immediate early gene synthesis, but were essential for the cyclin D1 protein accumulation. These studies lead to the conclusion that estrogen initiates mitogenesis by inducing the transcription of immediate early genes, but cytoplasmic signaling cascades play an important role in the control of subsequent events in the cell cycle.

## Additional Studies in the Role of Cytoplasmic Signaling Pathways in Estrogen-Induced Mitogenesis

### Drug Dosage/Toxicity

As reported last year, the concentrations of inhibitory molecules being employed in these studies were neither cytotoxic nor pro-apoptotic to MCF-7 cells after 24 hours. However, dose response curves demonstrated that  $5\mu\text{M}$  LY294002 was as potent as the  $50\mu\text{M}$  concentration, and, for this reason, experiments were repeated with this dose. Furthermore, the commercial availability of U0126, an inhibitor of active and inactive MEK1 as well as MEK2, provided another means to insure that observations were specific for inhibition of the MAPK cascade rather than nonspecific effects of the drugs. Titration experiments determined that  $25\mu\text{M}$  of U0126 was a potent inhibitor of estrogen-induced mitogenesis without cytotoxic or apoptotic effects.

An additional control for drug specificity was performed by examining the "relative health" of MCF-7 cells after exposure to the small molecule inhibitors. MCF-7 cells were treated for 24 hours in the presence of the PD098059, LY294002, U0126, or ICI 182,780 (an ER antagonist) at which time the cells were washed three times and stimulated with estrogen. Cell cycle progression occurred in all samples, except those treated with the estrogen antagonist, suggesting that the drugs impacting signaling cascades

were acting specifically on their reported pathways and that the inhibition was not irreversible. One reported mechanism for ICI 182,780 function is degradation of the ER (15). Thus, a failure for estrogen to elicit a mitogenic response in cells treated with ICI is not surprising since accumulation of ER protein must occur prior to the cells being receptive to estrogen stimulation.

### **Estrogen-Mediated Transcription in the Presence of MEK1/2 and PI3-K Inhibitors**

The most well understood mode of estrogen action is the transcriptional activation of genes containing estrogen response elements (EREs). Among the genes containing putative EREs are several key cell cycle regulatory genes, *c-myc*, *c-fos*, and cyclin D1 (1, 3, 16). If the PI3-K and MAPK inhibitors affected transcriptional regulation by the estrogen receptor, they could stop cell cycle progression by preventing the induction of these genes. To determine if the effects of PD098059, U0126, and LY294002 on estrogen-stimulated mitogenesis were due to interference with ER-mediated transcriptional activity, we initially measured estrogen-induced expression from functional EREs. MCF-7 cells were transfected with a luciferase gene under the control of either a naturally occurring (vitellogenin, TK-ERE) or artificial estrogen-responsive promoter (3X-ERE) and cell extracts were harvested 24 hours later. In the absence of drugs, estrogen induced a five- to six-fold increase in luciferase activity as opposed to near basal levels seen in the presence of the ER antagonist, ICI 182,780. The MEK1 and PI3-K inhibitors decreased transcription by approximately 50% while inhibition of the protein kinase A (PKA) or protein kinase C (PKC) cascades had no impact on ER-mediated transcription. Therefore, there is some correlation between estrogen-mediated transcription in the reporter gene assay and cell cycle inhibition by these compounds.

The levels of the estrogen receptor itself may be decreasing in response to these compounds, which would account for the decline in transcriptional activity. Therefore, we measured the levels of ER by immunoblotting over the course of a 24-hr treatment regimen with the inhibitory molecules. ER is known to decline in response to estrogen treatment (17, 18), and we also observed this phenomenon since the levels of ER protein were reduced compared to the resting levels in starved cells after 24 hours. Treatment with PD098059 itself for 24 hours showed no decrease in ER levels, but when incubated in the presence of estrogen a decrease in ER levels, comparable to estrogen alone, was observed. LY294002 did result in some diminution of ER protein regardless of the presence or absence of estrogen. These studies on the impact of small molecule inhibitors on ER protein levels suggest that the effects observed with MAPK inhibitors are completely independent of ER levels. However, the lower ER levels in the presence of LY294002 suggests that the cell cycle arrest noted with this compound may result from degradation of estrogen's cognate receptor.

The decreased ER levels observed in the presence of LY294002 occurred between 9 and 24 hours after the addition of the drug, whereas the diminishment in the presence of ICI 182,780 was more pronounced and more rapid (occurring within the first 9 hours). This observation correlates with the moderate suppressive effect the PI3-K inhibitor had on estrogen-mediated transcription measured by reporter gene assay as compared to the complete loss of transcriptional activity noted with the ER antagonist. Furthermore, these assays were performed after 24 hours of treatment, at which time the cells are largely growth arrested. Decreased ER transcription may be a secondary effect of the arrest rather than a direct effect of the kinase inhibitors. Expression of immediate early genes occurs rapidly after estrogen treatment; therefore, we measured the expression of these genes directly. By Northern blotting, we observed maximal expression of the two putative estrogen-responsive immediate early genes, *c-myc* and *c-fos*, within one hour of estrogen treatment. Levels of *c-myc* remained highly elevated at 2 hours and declined by 6 hours after treatment while *c-fos* levels declined more rapidly. The ICI antiestrogen completely inhibited the induction of both *myc* and *fos* as expected. The PKA and PKC inhibitory controls, as well as PD098059, had no effect on the kinetics or magnitude of this induction. LY294002 did not prevent, but prolonged, the time needed to attain maximal expression of both genes. In the presence of the LY compound, *myc* and *fos* were significantly induced at 1 hour, however, a further increase in levels was observed at later time points. Levels of *myc* and *fos* protein were consistent with

the transcriptional induction of these genes. Where the pure antiestrogen completely inhibited immediate early gene expression, the kinase inhibitors allowed induction of both c-myc and c-fos, suggesting that the cell cycle block occurs after immediate early gene synthesis.

### **The Effects of PI3-K and MAPK Inhibitors on Estrogen-Induced Delayed-Early Cell Cycle Events**

Increased cyclin D1 Expression is considered to be a delayed-early cell cycle event occurring after immediate early genes are induced. The accumulation of cyclin D1 protein levels after estrogen stimulation is a critical event in cell cycle progression (19). In addition, overexpression of cyclin D1 can reverse cell cycle arrest in ER+ cells that was accomplished by antiestrogen treatment. As detailed last year, estrogen treatment of serum starved MCF-7 cells resulted in a 3-fold increase in cyclin D1 levels by 6 hours. LY294002, PD098059, and ICI 182,780 prevented cyclin D1 accumulation.

Downstream effects of cyclin D1 induction, in response to estrogen stimulation, include activation of cyclinE/cdk2 complexes and hyperphosphorylation of Rb (see Introduction). Experiments were performed to examine both of these phenomena. Activity of cdk2 was assayed by stimulating MCF-7 cells in the presence or absence of the small molecule inhibitors for 24 hours. Cells were then lysed and cdk2 was immunoprecipitated and incubated with histone H1 (a substrate for cdk2's kinase activity) in the presence of [ $\gamma$ -<sup>32</sup>P] dATP. Phosphorylation of histone H1 was determined by electrophoresis of the reaction on a 15% polyacrylamide gel and visualization by autoradiography. Estrogen alone stimulated a dramatic increase in cdk2 activity compared to serum starved samples, whereas, LY294002, PD098059, U0126, and ICI 182,780 all inhibited any noticeable increase in phosphorylation of histone H1. Not surprisingly, these drugs also prevented the hyperphosphorylation of Rb, which is required for cell cycle progression into the DNA synthesis stage. Therefore, it appears that the primary effect of PI3-K and MAPK inhibitors on estrogen induced cell cycle progression can be placed at or before the level of cyclin D1 induction (which is also prior to the decrease of ER levels noted with LY294002 treatment).

### **The Effect of Estrogen on the Activation of the MAPK and PI3-K Cascades**

Inhibition of the PI3-K and MAPK cascades prevented efficient estrogen induced cell cycle entry in MCF-7 cells. Therefore, we investigated whether estrogen treatment activates these signaling pathways (9-13). Several recent reports have demonstrated that the MAP kinase pathway is activated by estrogen in an ER dependent fashion. MEK1 phosphorylates a threonine and a tyrosine residue in the regulatory sites of Erk1 and Erk2 resulting in the activation of these MAP kinases (reviewed in 20). We performed a series of experiments to determine whether Erk1 and/or Erk2 were activated under our conditions. The dually phosphorylated forms of Erk1 and Erk2 are specifically recognized on an immunoblot using a phospho-specific monoclonal antibody while total Erk1 and 2 are detected using a separate antibody after stripping and reprobing the same blot. Compared to untreated, serum-starved MCF-7 cells, EGF (epidermal growth factor) induced a dramatic increase in phosphorylated Erk1 and 2. Under the same conditions,  $2 \times 10^{-8}$  M estradiol, the most prevalent estrogen produced by the ovaries, had no effect on the levels of activated Erk 1 or 2. A number of repetitions of this experiment failed to show more than a 1.5-fold induction at any time point (from 1-20 minutes after treatment) or at any cell density. The strong activation of Erk1 and 2 by EGF was decreased but not entirely inhibited by the addition of PD098059, as previously shown (21). The ICI antiestrogen and the PI3-K inhibitor had no effect on EGF mediated activation. While estrogen failed to activate MAPK, PD098059 and U0126 completely inhibited the basal levels of phospho-Erk1 and 2. Neither ICI 182,780 nor LY294002 had any effect on active MAPK levels in estrogen or EGF stimulated cells.

Protein kinase B (PKB/Akt) is a primary downstream target of PI3-K activation and has been implicated in cyclin D1 protein stabilization (22). Stimulation with 30ng/ml insulin-like growth factor I (IGF-I), a known activator of PI3-kinase in MCF-7 cells (23), led to a pronounced increase in the phosphorylated (active) form of PKB as detected by a phosphospecific antibody. Treatment with estrogen had no effect on activated PKB levels, suggesting that PI3-kinase pathway does not respond to this hormone. The PI3-K drug was a potent inhibitor of PKB activation in all instances; it reduced both

the IGF-I induced and the basal levels of phospho-PKB to below the limit of detection. Therefore, estrogen fails to induce either the MAPK or PI3-kinase pathways to an appreciable degree; however, specific inhibitors reduce basal levels of the activated enzymatic substrates for these compounds to below the levels that are found in starved cells.

### Summary/Conclusions

In the work detailed above, we found that disruption of the PI3 and MAP kinase cascades by specific inhibitors prevented or reduced the ability of MCF-7 cells to enter S phase in response to estrogen. The small molecule inhibitors of these pathways arrested cells in G1 and did not induce any significant cytotoxicity. We did observe a modest effect on estrogen-mediated transcription assay on artificial estrogen response elements; however, the immediate early genes *c-myc* and *c-fos* were induced by estrogen even in the presence of these drugs. Both the PI3-K and the MAPK inhibitors prevent cyclin D1 expression, which typically increases shortly after the immediate early genes are induced in mitogen stimulated cells. The lack of cyclin D1 accumulation also correlated with an absence of cdk2 activity and retinoblastoma phosphorylation. We concluded that inhibition of estrogen-mediated mitogenesis by these drugs was likely due to this repression of cyclin D1. Finally, we also demonstrated that estrogen does not measurably activate either PKB or MAPK under conditions that result in a mitogenic response. The mechanism by which inhibitors of these pathways impacts estrogen activity is open to question; however, we hypothesized that estrogen initiates mitogenesis by inducing the transcription of immediate early genes, but MAPK and PI3-K play an important role in the control and regulation of subsequent cell cycle events.

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## Appendix

### Key Research Accomplishments

- ◆ Identification of signaling cascades important in estrogen-induced mitogenesis.
- ◆ Narrowing of the time interval (in terms of the cell cycle clock) when these kinases are necessary for DNA synthesis in response to estrogen stimulation in MCF-7 cells.

### Reportable Outcomes

- ◆ The data presented in this annual report has been submitted to the journal of Cell Growth and Differentiation and reviewed. This summary also includes the additional experiments requested by the reviewers and will be resubmitted by the end of September 1999.
- ◆ A poster of these experiments will be presented at the 1999 Duke University Medical Center Graduate Research Symposium in October.



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