Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Fox Chase Cancer Center

2. **Reporting Period (start and end date of grant award period):** 1/1/2010 – 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Maria Minko Gill

4. **Grant Contact Person's Telephone Number:** 215-728-2659

5. **Grant SAP Number:** 4100050895

6. **Project Number and Title of Research Project:** 2 – Estrogen Receptor-Mediated Gene Silencing in Tumorigenesis

7. **Start and End Date of Research Project:** 1/1/2010 – 12/31/2012

8. **Name of Principal Investigator for the Research Project:** Jeff Boyd, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $649,938

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Ariazi, Eric	Assistant Research Professor	50% Years 1-2	$114,387.46
Taylor, John	Scientific Associate	100% Year 1-2	$106,829.47

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Boyd, Jeff	Principal Investigator	15% Years 1-2

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes__X_____ No_____

If yes, please indicate the source and amount of other funds:

Institutional funds for salary support ($96,000)

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes__X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If

you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Estrogen receptor alpha-targeted silencing of gene expression via DNA methylation	☒ NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	October 2012	$490,875	not funded
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____ No___X_____

If yes, please describe your plans:

12. Future of Research Project. What are the future plans for this research project?

The project is being discontinued.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No____X____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

    Yes_____ No___X_____

    If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

    Yes___X_____ No_____

    If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

    We were able to start a new lab, and staffed the lab by hiring Eric Ariazi, PhD as an Assistant Research Professor and John Taylor, MS as a Scientific Associate.

16. **Collaboration, business and community involvement.**

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No___X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

INTRODUCTION

Estrogen receptor α (ERα, ESR1) has proven to be the single most important target in breast cancer. Indeed, approximately 70-80% of breast cancers are positive for ERα expression, for which the routine testing is used to predict response to antihormonal therapy (1). From genome-wide transcriptional profiling and ERα-binding site studies (2-8), it is evident that ERα is a global regulator of gene transcription that orchestrates a well-integrated hormonal response to impact numerous cell processes. Likely, because ERα directly and indirectly regulates a vast number of genes, it is the main driver in molecular classification of breast cancers as luminal and not basal with associated favorable and unfavorable prognoses, respectively (9, 10). It is well appreciated that 17β-estradiol (E2)-occupied ERα stimulates gene transcription, but it also represses transcription. Indeed, in MCF-7 cells, E2 represses expression of more genes than it stimulates (4, 5, 8). E2-regulated transcriptional activation and repression events act in concert to promote proliferation, survival and inhibit apoptosis. While mechanistic details of E2-stimulated gene transcription have been well described (11), relatively little is known about E2-repressed transcription. However, most of the limited number of studies examining E2-bound ERα-dependent transcriptional repression demonstrated that ERα binds promoter regions and recruits corepressors such as NRIP1/RIP140 or LCoR, and histone deacetylases (HDACs) (12-16). Further, E2-bound ERα directs histone modifications at target promoters including acetylation, phosphorylation, and methylation, which can alter chromatin structure and recruit specialized proteins for specific functions involved in stimulating or repressing transcription (17). However, our knowledge of how ERα coordinates such histone modifying activities to repress transcription is incomplete. We hypothesized that as a result of long-term ERα-dependent transcriptional repression, not only does ERα direct epigenetic silencing via histone modification, but also via DNA CpG methylation. This is plausible since multi-component corepressor complexes containing HDACs such as the NRIP1/RIP140 complex can recruit two key epigenetic repression systems, chromatin modifying histone methyltransferases (HMTs) such as EZH2 and DNA methyltransferases (DNMTs) (18). Further, ERα can directly interact with the epigenetic silencer EZH2, which, as a component of the polycomb protein repressor complex 2, recruits DNMTs. Thus, it is possible that ERα could silence targeted genes via DNA methylation by directly or indirectly recruiting corepressors, HDACs, HMTs and DNMTs.

In support of a role for ERα in regulating CpG methylation, genome-wide methylation profiling in human breast cancer specimens has revealed that specific CpG methylation biomarkers associate selectively with ERα-positive/negative status and risk of recurrence in human breast cancer specimens (19, 20). Differentially methylated genes have also been identified in antihormone-resistant vs. wild-type MCF-7 cells (21, 22), and in ERα RNAi-depleted vs non-depleted MCF-7 cells (23). Two studies have demonstrated that loss of ERα activity led to silencing of estrogen responsive genes such as progesterone receptor (PGR) (22, 23), but these studies indicate a passive role of ERα in preventing gene silencing. However, ERα may also play an active role in promoting silencing. One study (21) observed promoter hypomethylation more frequently than hypermethylation in antihormone (tamoxifen and fulvestrant)-resistant MCF7 cells compared to wild-type cells. Hence, blocking ERα activity associated with DNA hypomethylation. A second study (24) showed E2 repressed expression of HOXB13 and the demethylating agent decitabine increased its expression.

We identified high value candidate genes targeted for CpG methylation silencing by ERα using multiple antihormone-resistant breast cancer models which exhibit varying levels of ERα expression. These genes were inversely associated with ERα expression/activity and de-repressed by decitabine. Many basal-like breast cancer genes were identified, and among these, CpG methylation levels of LCN2 and TRIM29 were verified by pyrosequencing to associate with ERα expression/activity. We further demonstrate RNA and protein levels of the epithelial-mesenchymal transition (EMT) promoting factor LCN2 inversely associated with ERα, and that following LCN2 up-regulation, SNAI2/Slug was up-regulated. Therefore, ERα may enforce the luminal breast cancer subtype by DNA methylation silencing of basal-like gene such as LCN2 and TRIM29.

RESULTS AND DISCUSSION

To begin to identify genes targeted by ERα for DNA methylation-mediated silencing, we took advantage of the effects of long-term estrogen deprivation and of the pure antiestrogen fulvestrant (FUL) on ERα expression in T47D and MCF-7 human breast cancer cell. Prior studies have shown that long-term estrogen deprivation promotes loss of ERα expression in T47D cells (25-28) but overexpression of ERα in MCF-7 cells (29, 30). Additionally, FUL binding to ERα induces an abnormal conformation that leads to accelerated ubiquitination and shuttling of ERα to the proteasome for degradation (31). Starting with wild-type T47D/A18 and MCF-7/WS8 cells, which themselves were clonally selected from T47D and MCF-7 cells for maximal estrogen sensitivity and responsiveness, we generated a panel of antihormone-resistant cell lines of varying ERα levels compared to wild-type counterparts. Long-term estrogen-deprived (ED) T47D/ED3 and T47D/ED2 cells were selected by culturing cells >1 year in estrogen-free media (phenol red-free RPMI-1640 media containing dextran-coated charcoal-stripped fetal bovine serum (FBS)). T47D/FUL and MCF7/FUL cells were derived by culturing cells for 8-36 weeks, depending on the particular cells, in estrogenized media (phenol red and whole FBS-containing RPMI-1640 media) supplemented with 100 nM FUL. As expected, long-term ED and FUL treatment led to a near complete loss of ERα mRNA and protein expression in

T47D-based cells; T47D/ED3, T47D/ED2, and T47D/FUL cells (at 36 weeks of FUL derivation) expressed only 0.1%, 5%, and 4%, respectively, of the ERα mRNA and protein levels in wild-type T47D/A18 cells (Fig. 1). Next, to generate a model capable of showing de novo effects of estrogen-activated ERα on gene expression and DNA methylation, we re-exposed ERα-low T47D/ED2 cells to 1 nM E2 in estrogen-free media for up to 38 weeks and generated T47D/ED2/E2 cells. Although T47D/ED2 expressed 5% the level of ERα as in wild-type T47D/A18 cells, T47D/ED2/E2 exhibited estrogenic responses such as a doubling in growth rate (data not shown), and appropriate regulation of responsive genes (discussed below). However, E2 did not promote a return of ERα expression back to similar levels as in wt T47D/A18 cells; rather, ERα remained repressed and actually decreased another 33% relative to parental T47D/ED2 cells (Fig. 1A). Thus ERα was irreversibly repressed, perhaps via an epigenetic mechanism. This irreversible loss of ERα in different long-term estrogen deprived T47D cells following re-exposure to E2 has previously been observed (27) Additionally, the further 33% decrease in ERα mRNA likely reflects an E2-mediated and ERα-dependent negative autoregulatory feedback loop (32). The changes in ERα expression in MCF7/FUL cells were more complex; 8 weeks of FUL exposure led to a substantial loss in ERα mRNA to 9% of wild-type MCF-7/WS8 cells, but continued FUL exposure reversed this trend such that at 21 weeks, ERα mRNA was actually overexpressed at 191% of the level in MCF-7/WS8 cells (Fig. 1A). These changes in ERα mRNA expression in all antihormone-resistant cells were confirmed at the protein level (Fig. 1B), except in MCF7/FUL cells derived for 21 weeks where ERα protein rebounded to just 75% of that in MCF7/WS8 cells rather than reflecting mRNA overexpression (Fig. 1B). Hence, MCF-7/FUL cells at 21 weeks of derivation adapted to FUL at least in part by overexpressing ERα mRNA to compensate for FUL-mediated degradation of ERα protein. Moreover, the ultimate retention of ERα during adaption to FUL, and overexpression of ERα during adaptation to long-term estrogen-deprivation in MCF-7 cells (29, 30), likely reflects an addiction of this cell type to the receptor.

To characterize the antihormone-resistant models and to identify genes which inversely associated with ERα expression, genome-wide transcriptional profiling was carried out using Agilent 4x44K v2 microarrays (Fig. 2A-D). Differentially expressed genes were identified using the method Significance Analysis of Microarrays (SAM) (33). Considering genes containing ERα-binding sites (7, 8, 34), E2-stimulated genes (TFF1/pS2, ERBB4 and PGR) were decreased and an E2-repressed gene (OASL) was increased in all ERα-negative/low cells (Fig. 2A). These genes were also induced or repressed as appropriate by E2 in T47D/ED2/E2 cells vs. parental T47D/ED2 cells. Observing that these ERα target genes inversely associated with ERα status/activity validated the models. Importantly, TFF1 and ERBB4 expression increased while OASL decreased as ERα expression rebounded in MCF7/FUL wk 21 cells vs. wk 8 cells, despite the presence of FUL. However, PGR did not increase along with TFF1 and ERBB4 in the same comparisons. This indicated that in the context of an antihormone-resistant cell type, changes in abundance of ERα can still regulate target genes, even if an antagonist is present, perhaps due to post-translational modifications in ERα. Further examination revealed that all antihormone-resistant ERα-negative/low T47D- and MCF-7-based cells compared to matched wt ERα-positive cells showed significantly decreased expression of luminal differentiation markers (ANXA9, TTC39A (35-37); Fig. 2B), while increasing expression of basal differentiation markers (SLPI, FGFBP1, KRT6A, KRT16 (35-37); Fig. 2B), epithelial-mesenchymal transition (EMT) markers (JAG1, FBLN5, SNAI2/Slug, FGFR2, RELB (38-43); Fig. 2C), and embryonic

stem cell markers (SOX2, KLF5, NTS, IFITM1, TRIM22, AASS, IFI16, LGR4 (44); Fig. 2D). Therefore, ERα was a major driver of luminal-like cell differentiation, and loss of ERα led to de-differentiation / trans-differentiation to more basal-like characterized by EMT and stem cell markers.

For our continuing studies, we chose to primarily employ the T47D-based models because of the clear time-dependent loss of ERα expression allowed a straightforward interpretation of acquired data. To identify genes likely regulated epigenetically by DNA methylation, wild-type T47D/A18 cells were treated for 96 h with 1 μM decitabine (DAC; 5-aza-2'-deoxycytidine), a cytidine analog that inhibits DNMTs, or with compound vehicle only (control). Cross-referencing 1) genes up-regulated in T47D/ED3, T47D/ED2 and T47D/FUL cells vs. wild-type T47D/A18 cells, 2) genes down-regulated in T47D/ED2/E2 vs. T47D/ED2 cells, and 3) genes up-regulated by DAC vs. control treatment in wild-type T47D/A18 cells identified 34 high-value candidates silenced by ER-mediated DNA methylation (Fig. 2E). Considering these candidate ER-methylated genes, 24 out of 34 are basal markers (35-37), and 6 of these are also EMT markers (LCN2, KRT17, KRT14, LAMB3, FBLN1, and PLAU (38-43)). Also, 19 of the 34 candidate ERα-methylated genes have previously been found as methylated in cancer cells, and 8 of these specifically in breast cancer (LCN2, GSTP1, SERPINB5/maspin, TRIM29, LAMP3, LAMB3, S100A2, and PLAU) (19, 20, 45-54).

LCN2 and TRIM29 were selected because of their biologic importance in breast cancer for validation of ERα-associated changes in CpG methylation. LCN2 (lipocalin-2; also neutraphil-gelatinase associated lipocalin, NGAL) is a 24 kDa intracellular and secreted glycoprotein that sequesters iron and transports it into cells (55, 56). Extracellularly, LCN2 forms a heterodimer with matrix metalloproteinase-9 (MMP9) to protect it from degradation (57, 58). LCN2 has roles in immunity, inflammation, apoptosis, tumorigenesis, metastasis and EMT (59, 60). LCN2 expression strongly associates with ER-negative and HER2-positive status, poor histologic grade, lymph node metastases, and high Ki-67 proliferation index. Also, LCN2 serves as an independent prognostic marker for disease-free survival in multivariate analysis (61). TRIM29 (tripartite motif containing 29, also ataxia-telangiectasia group D complementing gene, ATDC) contains multiple zinc fingers and a leucine zipper to presumably mediate nucleic acid and protein-protein interactions (62). TRIM29 exhibits pleomorphic functions depending on cell type; it acts as an oncogene in multiple cell types such as pancreatic cancer (63, 64) but as a tumor suppressor in nontumorigenic MCF10A breast cells and MCF-7 breast cancer cells (65). As an oncogene, TRIM29 binds p53 antagonizing its function (66) and promotes Wnt signaling by stabilizing β-catenin via Disheveled-2 (63). As a tumor suppressor in MCF-7 cells, it suppresses estrogen-stimulated of genes by inhibiting ERα binding to EREs (65).

LCN2 and TRIM29 were validated by pyrosequencing of bisulfite-modified DNA. Bisulfite chemically converts unmethylated cytosines to uracils while 5meC is protected from modification. Pyrosequencing essentially couples luciferase-mediated light output to a sequencing reaction to provide quantitative measurement of incorporated nucleotides. PCR amplification of bisulfite-treated DNA converts uracils to thymines, and pyrosequencing quantifies the proportion of protected 5meC to T bases at CpG sites. Initially, we mapped the hypervariable CpG methylation region in LCN2's upstream transcriptional regulatory region from -1000 to +100 bases relative to the transcriptional start site (TSS) by pyrosequencing (Fig.

3A). The LCN2 upstream transcriptional regulatory region contains a validated ERE with only a single base deviation from the consensus sequence located at -819 upstream of the TSS (2). However, CpGs near LCN2's ERE remained fully hypermethylated in T47D/ED2 cells following 24 and 36 weeks of estrogen deprivation despite having lost ERα expression, whereas the region from -279 to -100 was hypervariable (Fig. 3B). Interestingly, the LCN2 CpG site at position -177 overlaps a validated NFκB site required for induction of LCN2 expression (67) (Fig. 3A). This suggests that ERα was interacting with NFκB to target methylation near the LCN2 TSS to silence expression. LCN2 and TRIM29 CpG methylation was further characterized with respect to losing ERα and then stimulating ERα with E2 across all of the T47D-based models (Fig. 4A-D). LCN2 and TRIM29 CpG sites near their TSSs were hypermethylated in ERα-positive wild-type T47D/A18, hypomethylated in ERα-negative/low T47D/FUL, ED3 and ED2 cells (Fig. 4A, 4D), and again shifted towards hypermethylated upon E2 exposure in T47D/ED2/E2 cells (Fig. 4B, 4E). With regard to the MCF-7 models, LCN2 and TRIM29 CpG methylation increased in MCF7/FUL wk21 vs. wild-type MCF7/WS8 cells (Fig. 4C, 4F). As discussed above, it is likely that MCF7/FUL week 21 cells exhibited increased rates of ERα protein synthesis, as indicated by increased ERα mRNA levels, and/or decreased rates of ubiquitin-mediated degradation to compensate for FUL-mediated ERα protein degradation. Hence, increased ERα synthesis in MCF7/FUL week 21 cells likely promoted increased methylation of LCN2 and TRIM29.

We examined the inverse relationship between LCN2 and ERα expression. In addition, the key EMT transcription factor SNAI2/Slug (Fig. 5) was examined because LCN2 promotes EMT by up-regulating Slug (68), and we had found Slug overexpression in our antihormone-resistant models (Fig. 2C). Within 1 week of estrogen deprivation, ERα RNA and protein expression dramatically decreased in T47D/ED3 and T47D/ED2 cells. Importantly, after ERα had decreased to very low levels, LCN2 RNA and protein expression began increasing at 3 weeks of estrogen deprivation (Fig. 5A-B). After increases in LCN2 were observed, Slug protein expression began increasing starting at 10 weeks of estrogen deprivation. E2 re-exposure in T47D/ED2/E2 cells, which contain ~5% the ERα levels compared to wild-type T47D/A18 cells but are capable of inducing ERα target genes such as PgR (Fig. 2A), led to progressive decreases in LCN2 protein. By 16 weeks of E2 re-exposure, LCN2 levels were very low, and after LCN2 levels had dropped, Slug levels began decreasing at 24 weeks of E2 (Fig. 5C). A similar profile of ERα, LCN2, and Slug expression changes were observed in T47D/FUL and MCF7/FUL cells. In T47D/FUL cells as FUL exposure led to loss of ERα protein, LCN2 protein began increasing after 1 week of FUL and Slug was increased after 8 weeks (Fig. 5D). In MCF7/FUL cells, when ERα protein expression was very low at 8 to 12 weeks of FUL treatment, LCN2 protein expression was high. As ERα became re-expressed from 16 – 23 weeks, LCN2 expression was lost, but Slug protein became expressed (Fig. 5E). This indicates that LCN2 initiated Slug expression to facilitate EMT despite the presence of ERα.

Taken together, our data indicate that ERα can silence genes via DNA hypermethylation such as the EMT promoter LCN2 and the tumor suppressor TRIM29. These data have important implications in tumorigenesis and antihormone-resistant breast cancer. In tumorigenesis, overexpression of ERα which occurs in 70-80% of cases could lead to silencing tumor suppressors such as TRIM29. In antihormone-resistant breast cancer, long-term blockade of ERα activity could lead to de-repression of EMT genes such as LCN2 that promote disease

progression by transitioning luminal-like to more basal-like and stem-like breast cancer cells with worse clinical outcome.

FIGURES

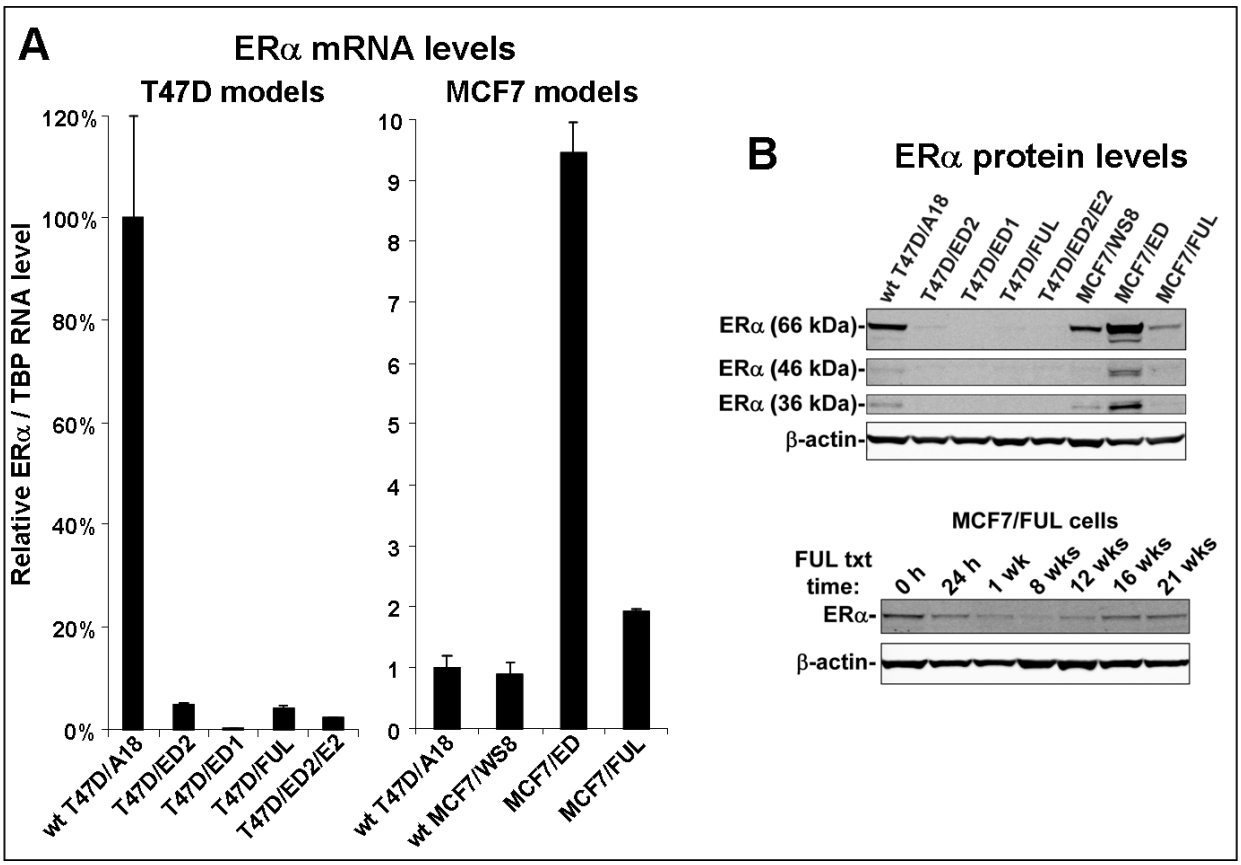

Figure 1. ERa expression in estrogen deprivation (ED) -resistant and fulvestrant (FUL)-resistant T47D and MCF-7 cells. (A) ERα mRNA expression measured by qRT-PCR. (B) ERα protein expression measured by Western blotting. Also shown are the 46 and 36 kDa ERα splice variant isoforms. Western blots were visualized using an Odyssey Infrared Imaging System (Licor).

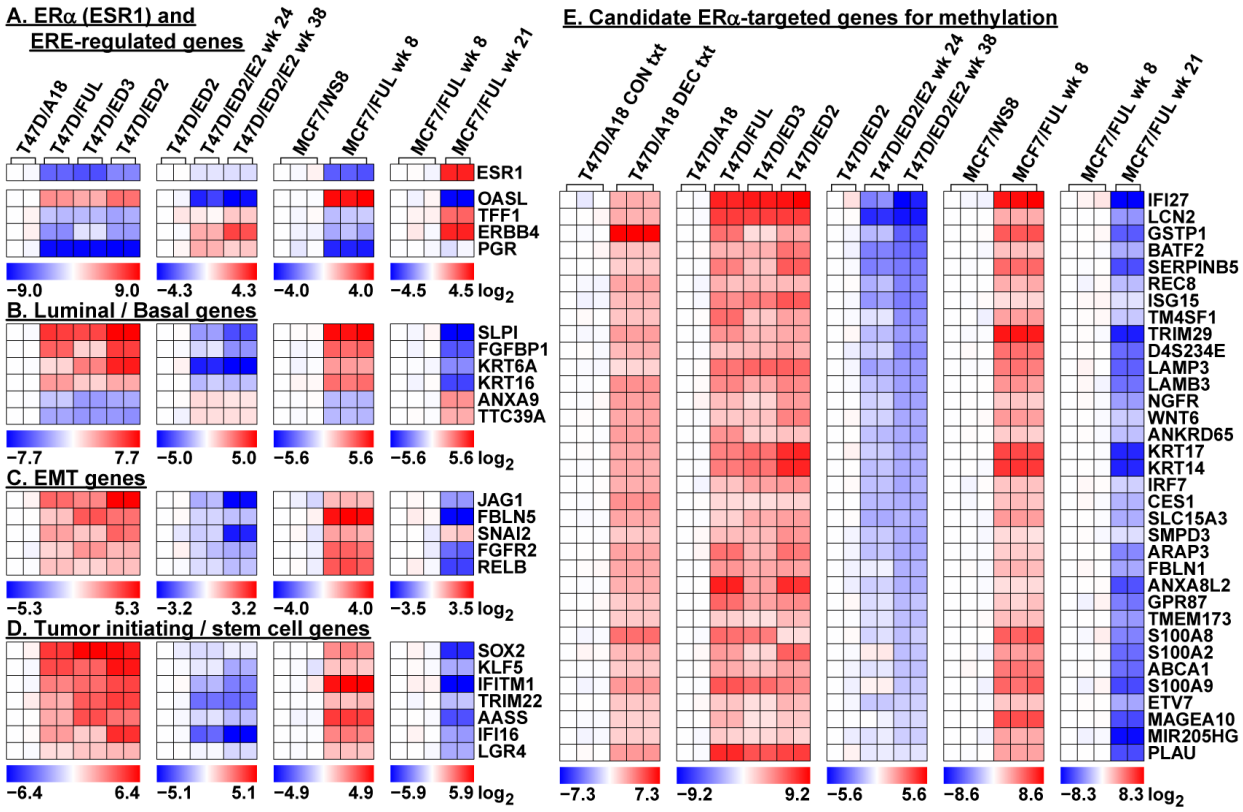

Figure 2. Gene expression microarray identification of genes inversely associated with ERα and induced by decitabine. (A) ERα (ESR1) and ERα inversely associated genes with identified ER-binding sites from (7, 8, 34). (B) Luminal and basal marker genes from (35-37). (C) EMT genes from (38-43). (D) Embryonic stem cell genes from (44). (E) Candidate ERα-targeted genes for methylation. These genes were induced by decitabine (DAC) in wild-type T47D/A18 cells and inversely associated with ERα expression across all antihormone-resistant models. Global transcriptional profiling was carried out using Agilent 4x44K v2 microarrays and one color-labeled (cyanine 3-CTP) c-RNA. Differentially expressed genes were determined by pairwise comparisons using SAM (significance analysis of microarrays (33)) at a false discovery rate (FDR) of 0%.

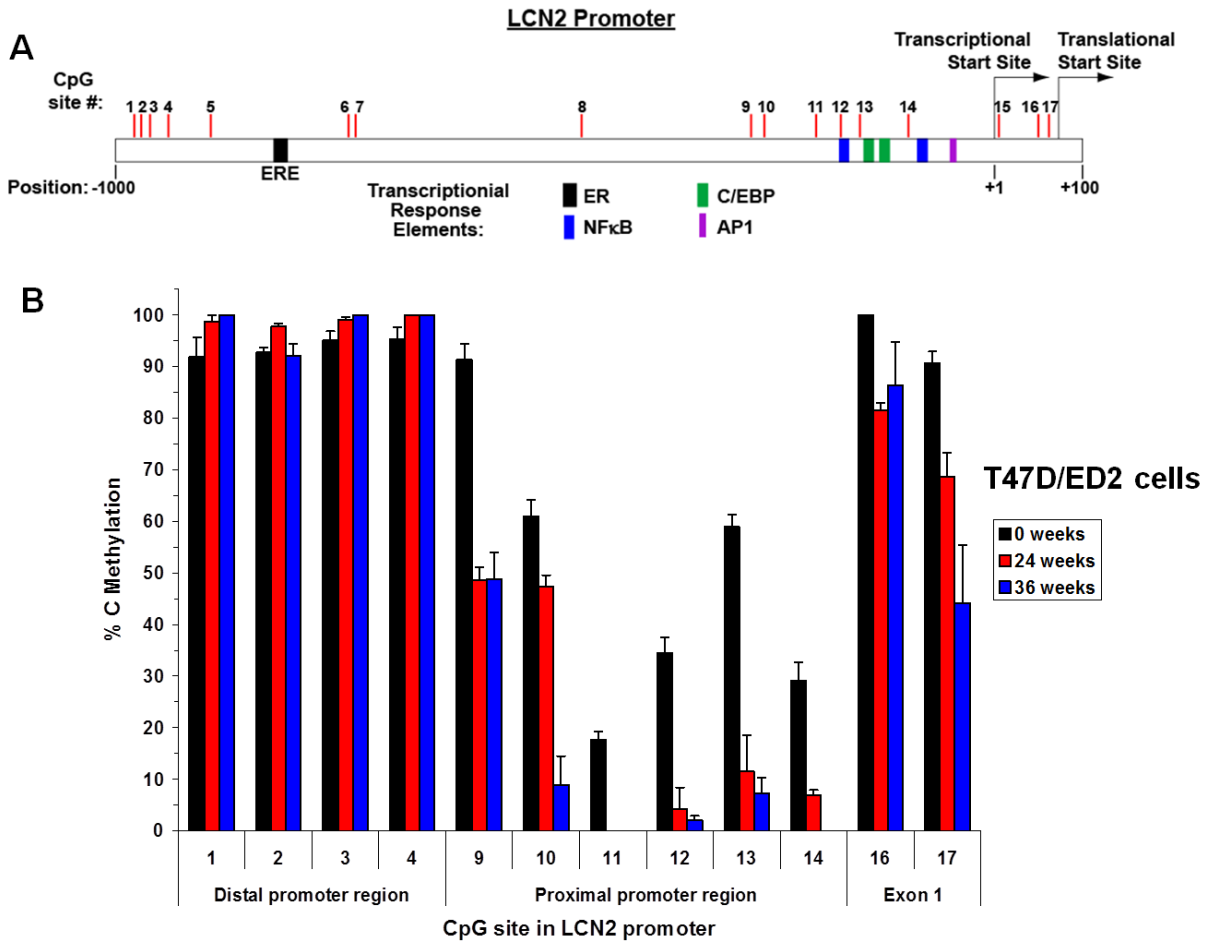

Figure 3. Mapping of hypervariable CpG methylation sites by pyrosequencing in the LCN2 transcriptional regulatory region. (A) Schematic of the LCN2 transcriptional regulatory region from -1,000 bases upstream of the transcriptional start site (TSS) to 100 bases downstream. CpG sites and transcriptional response elements are indicated. (B) Selected CpG sites in the LCN2 promoter were analyzed by pyrosequencing of bisulfite-treated DNA. DNA was isolated from T47D/A18 (0 weeks of estrogen deprivation) and T47D/ED2 cells after 24 and 36 weeks of estrogen deprivation. DNA was bisulfite-modified and PCR amplified. Single-stranded DNA (sense strand) was isolated, by means of an incorporated 5'-biotinylated reverse primer, and pyrosequenced using an internal primer and the PyroMark Q96 MD system. The hypervariable CpG methylation region extended from CpG site 9 through 14, which corresponds to bases -279 to -100 relative to the TSS of LCN2.

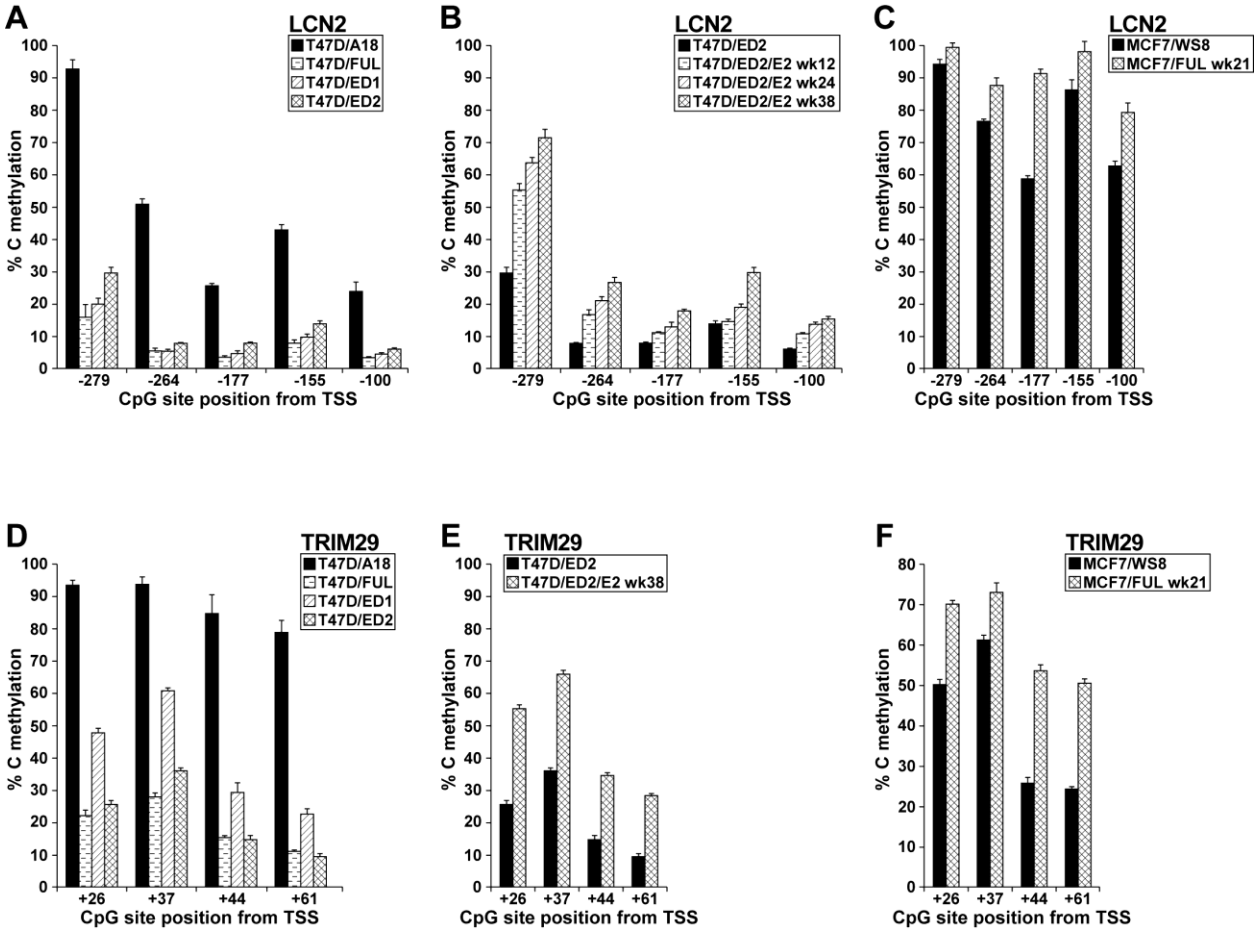

Figure 4. LCN2 and TRIM29 methylation levels as measured by pyrosequencing were associated with ERα expression/activity. (A) LCN2 and (B) TRIM29 CpG methylation decreased in ERα-negative/low T47D/FUL, ED3, and ED2 cells vs. ERα-positive T47D/A18 cells. (C) LCN2 and (D) TRIM29 CpG methylation increased upon 1 nM E2 re-exposure in T47D/ED2/E2 cells compared to parental T47D/ED2 cells. (E) LCN2 and (F) TRIM29 CpG methylation also increased in MCF-7/FUL cells vs. MCF-7/WS8 cells. Note that MCF-7/FUL cells show increased ERα synthesis to compensate for FUL-mediated ERα degradation as in Fig. 1. CpG site positions relative to the gene's transcriptional start site (TSS) are indicated. Pyrosequencing was conducted as in Fig. 3.

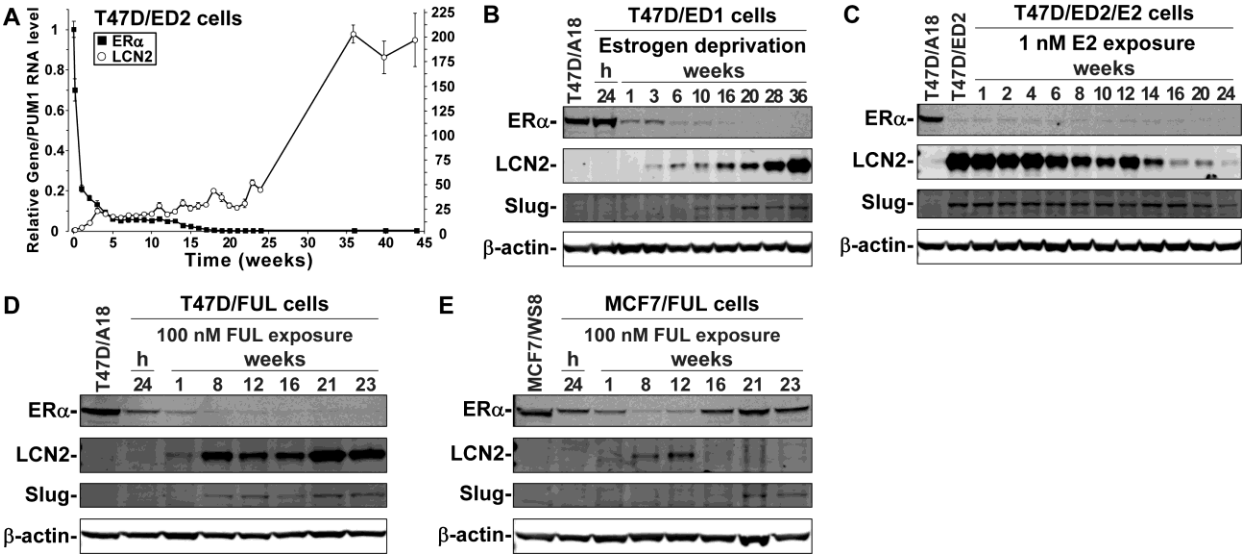

Figure 5. Time-dependent inverse relationship between ERa expression/activity and LCN2 and Slug expression. (A-B) Estrogen deprivation led first to decreases in ERα mRNA *(A)* and protein levels *(B)*, followed by increases LCN2 mRNA *(A)* and protein *(B)* in T47D/ED2 *(A)*and T47D/ED3 cells *(B)*. (C) Re-exposure to 1 nM E2 decreased LCN2 in T47D/ED2/E2 cells. (D) In T47D/FUL cells, long-term FUL (100 nM) supplementation to estrogenized media led to 95% loss of ERa followed by de-repression of LCN2. (E) In MCF-7/FUL cells, long-term FUL supplementation to estrogenized media led to an initial decrease in ERα and an increase in LCN2, but after 8 weeks of FUL, ERα rebounded and LCN2 was again silenced. Proteins levels were assessed by Western blots probed with specific antibodies. Blots were scanned and quantitated using an Odyssey Infrared Imaging System (Licor).

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18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics

_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X___No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the

publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No____X_____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No___X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

See below.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Jeff Boyd, Ph.D.	Professor Senior Vice President, Molecular Medicine

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Duke University, Durham, NC	B.S.	1976-80	Psychology/Chemistry
North Carolina State University, Raleigh, NC	M.S.	1980-82	Toxicology/Biochemistry
North Carolina State University, Raleigh, NC	Ph.D.	1982-86	Toxicology/Biochemistry
University of North Carolina, Chapel Hill,	Postdoctoral	1986-88	Pathology
NIH/NIEHS, Research Triangle Park, NC	Postdoctoral	1988-90	Molecular Carcinogenesis

POSITIONS

Senior Staff Fellow and Head, Gynecologic Pathobiology Group, Laboratory of Molecular Carcinogenesis, NIH/NIEHS, Research Triangle Park, NC 1990-1994

Associate Professor, Departments of Obstetrics and Gynecology and Genetics 1994-1997
Director, Gynecologic Oncology Research Laboratory, University of Pennsylvania Medical Center, Philadelphia, PA

Associate Attending Biologist, Departments of Surgery and Medicine 1997-2003
Director, Gynecology and Breast Research Laboratory
Associate Member, Memorial Sloan-Kettering Cancer Center, New York, NY

Attending Biologist, Departments of Surgery and Medicine 2003-2006
Director, Gynecology and Breast Research Laboratory, Department of Surgery
Director, Diagnostic Molecular Genetics Laboratory, Department of Medicine
Member, Memorial Sloan-Kettering Cancer Center, New York, NY

Member and Director, Curtis and Elizabeth Anderson Cancer Institute 2006-2008
Vice President, Oncology and Research, Memorial Health University Medical Center, Savannah, GA; Professor of Obstetrics and Gynecology, Surgery, Internal Medicine, and Division of Basic Medical Sciences, Mercer University School of Medicine, Savannah
Assistant Dean for Research, Mercer University School of Medicine, Savannah
Distinguished Cancer Scholar, State of Georgia

Senior Vice President, Chief Scientific Officer; The Robert C. Young, MD, Chair in Cancer Research; Professor, Women's Cancer Program, Fox Chase Cancer Center, Philadelphia, PA 2008-2010

Senior Vice President, Molecular Medicine; The Robert C. Young, MD, Chair in Cancer Research; Executive Director, Cancer Genome Institute; Chief, Division of Molecular Pathology; Professor, Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA 2010-

Professor (adjunct), Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA 2013-

REPRESENTATIVE PEER-REVIEWED PUBLICATIONS
(from a total of 194 papers, reviews, and chapters)

Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, **Boyd J**. Genetic instability of microsatellites in endometrial carcinoma. Cancer Res 53: 5100-5103, 1993.

Risinger JI, Berchuck A, Kohler MF, **Boyd J**. Mutations of the E-cadherin gene in gynecologic cancers. Nature Genet 7: 98-102, 1994.

Rubin SC, Benjamin I, Behbakht K, Takahashi H, Morgan MA, LiVolsi VA, Berchuck A, Muto MG, Garber JE, Weber BL, Lynch HT, **Boyd J**. Clinical and pathological features of ovarian cancer in women with germ-line mutations of *BRCA1*. N Engl J Med 335: 1413-1416, 1996.

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Olshen AB, Gold B, Lohmueller KE, Struewing JP, Satagopan J, Stefanov SA, Eskin E, Kirchhoff T, Lautenberger JA, Friedman E, Norton L, Ellis NA, Viale A, Lee CS, Borgen PI, Clark AG, Offit K, **Boyd J.** Analysis of genetic variation in Ashkenazi Jews by high density SNP genotyping. BMC Genet 9: 14, 2008. PMCID: PMC2393811

Pothuri B, Leitao MM, Levine DA, Viale A, Olshen AB, Arroyo C, Bogomolniy F, Olvera N, Lin O, Soslow RA, Robson ME, Offit K, Barakat RR, **Boyd J.** Genetic analysis of the early natural history of epithelial ovarian carcinoma. PLoS ONE 5(4)e:10358, 2010. PMCID: PMC2859950

The Cancer Genome Atlas Network. Comprehensive genomic characterization of squamous cell lung cancers. Nature 489: 519-525, 2012. PMCID: PMC3466113

The Cancer Genome Atlas Network. Integrative analysis of genomic and molecular alterations in clear cell renal cell carcinoma. Nature 499: 43-49, 2013. PMCID: PMC3771322

Boyd J, Luo B, Peri S, Wirchansky B, Hughes L, Forsythe C, Wu H. Whole exome sequence analysis of serous borderline tumors of the ovary. Gynecol Oncol 130: 560-564, 2013. (Support provided by Sandy Rollman Ovarian Cancer Foundation)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Wisconsin, Madison, WI	B.S.	05/91	Biochemistry, Genetics; Double major
University of Wisconsin, Madison, WI	Ph.D.	08/98	Molecular & Cellular Biology, Human Cancer Biology; Double major
University of Wisconsin, Madison, WI	Postdoctoral	09/02	Oncology

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Positions and Employment

Research Assistant Professor, Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL 2002-2004
Staff Scientist, Fox Chase Cancer Center, Philadelphia, PA 2004-2009
Assistant Professor (adjunct appointment), Dept of Pharmacology, Temple University School of Medicine, Philadelphia, PA 2008-present
Assistant Research Professor, Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA 2010-2013

Honors and Awards

B.S. with Highest Honors (equivalent to *summa cum laude)* in Biochemistry, Genetics 1991
DoD BCRP Predoctoral Fellowship DAMD17-94-J-4041 1994-1997
DoD BCRP Postdoctoral Fellowship DAMD17-99-1-9452 1999-2002
Eli Lilly Research Fellowship for Breast Cancer 2002-2004
Avon Career Development Award for Breast Cancer 2004-2006
FCCC-PENN SPORE in Ovarian Cancer Career Development Award 2012-2013
Teal Tea Foundation Award for Translational Ovarian Cancer Research 2012-2013

SELECTED PEER-REVIEWED PUBLICATIONS (from a total of 28)

1. **Ariazi, E.A.**, Clark, G.M., Mertz, J.E. Estrogen-related Receptor α and Estrogen-related Receptor γ Associate with Unfavorable and Favorable Biomarkers, Respectively, in Human Breast Cancer. *Cancer Res*, **62**: 6510–6518, 2002.
2. Glidewell-Kenney, C., Weiss, J., Lee, E.J., Pillai, S., Ishikawa, T., **Ariazi, E.A.**, Jameson JL. ERE-independent ERalpha target genes differentially expressed in human breast tumors. *Mol Cell Endocrinol*, **245**: 53-59, 2005.
3. O'Regan, R.M., Osipo, C., **Ariazi, E.**, Lee, E.S., Meeke, K., Morris, C., Bertucci, A., Sarker, M.A., Grigg, R., Jordan, V.C. Development and therapeutic options for the treatment of raloxifene-stimulated breast cancer in athymic mice. *Clin Cancer Res*, **12**: 2255-2263, 2006.
4. Liu, S., Abdelrahim, M., Khan, S., **Ariazi, E.**, Jordan, V.C., Safe, S. Aryl hydrocarbon receptor agonists directly activate estrogen receptor alpha in MCF-7 breast cancer cells. *Biol Chem*, **387**: 1209-1213, 2006.
5. **Ariazi, E.A.**, Lewis-Wambi, J.S., Gill, S.D., Pyle, J.R., Ariazi, J.L., Kim, H.R., Sharma, C.G.N., Cordera, F., Shupp, H.A., Li, T., Jordan, V.C. Emerging principles for the development of resistance to antihormormonal therapy: Implications for the clinical utility of fulvestrant. *J Steroid Biochem Mol Biol*, **102**: 128-138, 2006. PMCID: PMC1810511.
6. **Ariazi, E.A.**, Kraus, R.J., Farrell, M.L., Jordan, V.C., Mertz, J.E. Estrogen-related receptor alpha 1 transcriptional activities are regulated in part via the ErbB2 (HER2) signaling pathway. *Mol Cancer Res*, **5**: 71-85, 2007.
7. **Ariazi, E.A.**, Leitão, A., Oprea, T.I., Chen, B., Louis, T., Bertucci, A-M., Sharma, C.G.N., Gill, S.D., Kim, H.R., Shupp, H.A., Pyle, J.R., Madrack, A., Donato, A.L., Cheng, D., Paige, J.R., Jordan, V.C. Exemestane's 17-Hydroxylated Metabolite Exerts Biological Effects as an Androgen. *Mol Cancer Ther*, **6**: 2817-2827, 2007. **Featured on the cover of the journal.*
8. Yu, J.J., Robb, V.A., Morrison, T.A., **Ariazi, E.A.**, Karbowniczek, M., Astrinidis, A., Wang, C., Hernandez-Cuebas, L., Seeholzer, L.F., Nicolas, E., Hensley, H., Jordan, V.C., Walker, C.L., Henske, E.P. Estrogen Promotes the Survival and Pulmonary Mestastasis of Tuberin-null Cells. *Proc Natl Acad Sci USA*, **106**: 2635-2640, 2009. PMCID: PMC2637277.
9. **Ariazi, E.A.**, Brailoiu, E., Yerrum, S., Shupp, H.A., Slifker, M.J., Cunliffe, H.E., Black, M.A., Donato, A.L., Arterburn, J.B., Oprea, T.I., Prossnitz, E.R., Dun, N.J., Jordan, V.C. The G Protein-Coupled Receptor GPR30 Inhibits Proliferation of Estrogen Receptor-Positive Breast Cancer Cells. *Cancer Res*, **70**: 1184-1194, 2010. PMCID: PMC2879282. **Evaluated by Faculty of 1000 Medicine member Chellakkan Selvanesan Blesson, Karolinska Institute, Sweden, 07 June 2010.*
10. Hu, Z-Z., Kagan, B.L., Zhang, L., **Ariazi, E.A.**, Rosenthal, D.S., Huang, H., Wu, C., Jordan, V.C., Riegel, A.T., Wellstein, A. Proteomic Analysis of Pathways Involved in Estrogen-induced Growth and Apoptosis in Breast Cancer Cells. *PLoS One*, 6: e20410, 2011. PMCID: PMC3124472
11. **Ariazi, E.A.**, Cunliffe, H.E., Lewis-Wambi, J.S., Slifker, M.J. Willis, A.L., Ramos, P., Tapia, C., Kim, H.R., Yerrum, S., Sharma, C.G.N., Nicolas, E. Balagurunathan, Y., Ross, E.A., Jordan, V.C. Estrogen-induces Apoptosis in Estrogen Deprivation-resistant Breast Cancer via Stress Responses as Identified by Global Gene Expression Across Time. *Proc Natl Acad Sci USA*, 108(47): 18879-18886, 2011. PMCID: PMC3223472. **Inaugural Article.*