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: Thomas Jefferson University**

2. **Reporting Period (start and end date of grant award period):** 01/01/10 – 2/31/13

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Joy Soleiman, MPA

4. **Grant Contact Person's Telephone Number:** 215-955-5684

5. **Grant SAP Number:** 4100050910

6. **Project Number and Title of Research Project:** 2 - Phosphorylated Proteins in Breast Cancer Prognosis

7. **Start and End Date of Research Project:** 01/01/10 – 12/31/2013

8. **Name of Principal Investigator for the Research Project:** Andrew Quong, PhD

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:

   $ 351,248.53

   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
Andrew Quong	Principal Investigator	10%, Yr.2,3,4	39,565
Colin Wynne	Instructor	100% ,Yr3	23,526
Rong Meng	Postdoc Fellow	15%, Yr1&2	8,998
Alimatou Minkeu	Postdoc Fellow	25%, Yr.1	9,659
Huang, Xiaofang	Graduate Student	100%, Yr.1&2	21,443
Gormley, Michael	Research Assistant	70% Yr.1&2	29,709
Chen, Yong	Postdoc Fellow	25% Yr4	9,591

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	Cost
NONE			

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
Mass Spectrometer	New capabilities for protein and metabolite identification	$ 49,965
Isoelectric Focusing	New capability for small molecule separations	8,602
Air Generator	Needed to operate the Mass Spectrometer	4,999

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_____ No _____x_____

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

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:
Discovery of Novel Proteins to Identify Aggressive Papillary Thyroid Cancer	■NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	5/2011	$426,250	$0
Proteomic Characterization of Adenoid Cystic Carcinoma	■NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	3/2012	$426,250	$0

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?

None

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 ____x_____ No_____

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

	Undergraduate	Masters	Pre-doc	Post-doc
Male			1	5
Female				
Unknown				
Total			**1**	**5**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				3
Unknown			1	2
Total			**1**	**5**

	Undergraduate	Masters	Pre-doc	Post-doc
White				2
Black			1	
Asian				3
Other				
Unknown				
Total			**1**	**5**

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.

This research project led to new capabilities for proteomics and metabolomics.

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

Estrogens are members of the family of steroid hormones that stimulate the development and maintenance of female characteristics and sexual reproduction. 17-β-estradiol (E2) is the most abundant estrogen in the human body [1, 2]. Estrogen circulates through the bloodstream and targets organs containing estrogen receptors (ER). Higher levels of estrogen exposure are associated with increased breast cancer risk [3] and the expression of estrogen receptor varies in breast cancer subtypes.

The well-established classical pathway of E2 signal transduction is genomic and ERα-dependent. Once E2 binds to ERα located mainly in the cytoplasm, the activated receptor complex moves to the nucleus where it dimerizes and binds to specific DNA sequences called estrogen response elements (ERE) via the DNA binding domain of ER [4]. The activated EREs are able to regulate the expression of specific genes located downstream of the sequence [5] and with the help of other activators, impact transcription of genes involved in cell proliferation and differentiation. The ligand-bound ERα also binds to DNA indirectly through protein-protein interactions with other transcription factors such as AP-1 and Sp1 [6, 7].

In addition to activating genomic pathways, E2 has also been shown to activate its receptor present on the cell surface and to participate in the activation of other membrane bound receptors leading to the mitogen-activated protein kinase (MAPK) signaling cascade [8], a process that occurs non-genomically within minutes of stimulation [9]. These non-genomic protein processes occur independently of gene transcription and in the case of E2, are assumed to take place through steroid-induced modulation of membrane-bound regulatory proteins. Estrogen has been linked to additional pathways that are ER-independent such as the regulation of angiotensin II type 1 to activate early cell survival mechanisms [8] and growth inhibition of some ER negative breast cancer cells [10]. In-depth knowledge of these pathways will enable us to better understand estrogen mediated breast cancer.

Several laboratories have studied the effects of E2 using gene expression microarrays, identifying specific genes affected by estrogen [11-21]. While genomic analysis provides valuable information about the transcriptional effects of estrogen, its impact on cell signaling is only inferred by the assumed correlation of gene to protein expression. There is growing evidence however, showing that this relationship is not always correlated [22]. Moreover, the fast non-genomic activity of E2 mentioned above occurs well before transcription, and therefore cannot be detected using gene microarrays. Protein profiling through mass spectrometry gives additional important information to gene expression profiling along with information on post-

translational modifications (e.g. phosphorylation and acetylation). Other laboratories have focused on global protein levels after E2 treatment [22-26]. Although these studies have increased our current knowledge on E2 responsiveness, much work is still required in understanding the differences associated with short versus prolonged E2 exposures on protein level and its impact on cell signaling.

Using a reciprocal flipped design and stable Isotope Labeling by Amino Acids in Cell culture (SILAC), proteins involved in regulating estrogen-induced cell growth and alteration in breast cancer cells were identified by measuring changes in protein levels resulting from E2 treatment of the ER-positive MCF-7 breast cancer cell line. In a parallel study, changes in the protein level induced by estrogen in the ER negative cell line MDA-MB-231 were identified. Cells were grown in amino acid deficient media supplemented with labeled amino acids, allowing for the total incorporation of the label into the cells proteome [27]. This design permits a more robust validation of the results by increasing confidence in the expression of proteins identified and decreasing errors associated with isotopic conversion of arginine to proline detected in SILAC-heavy media [28].

The aim of this parallel study using ER positive and ER negative cell lines is to identify unique non-genomic ER-dependent and ER-independent biological processes altered by estrogen in breast cancer cells.

RESULTS

Estrogen increases the rate of cell division in ER positive cells grown in SILAC media but not that of ER negative cells

To verify the proliferative effect of E2 on both cells types (MCF-7 and MDA-MB-231) cultured in SILAC media, we treated them with varying concentrations of E2 (0-20 nM), and cell proliferation was measured using the MTT assay. The range of E2 concentration used was derived from an estimation of our MCF-7 responsiveness to E2 and concentrations used in similar studies [8,9,11-13,20,29]. There was a sustained proliferation difference in MCF-7 over time depending on the E2 dosage. No difference was observed in MDA-MB-231 cells. At day 3 (Figure 1), MCF-7 E2 treated cells differed from the 0 nM treatment ($p = 0.04$, using two samples student t-test assuming equal variances). The difference between E2 treatment and no treatment was also significant at other time points (data not shown). We chose a saturating dose of 10 nM for the protein expression studies. The same dose was also used in several gene and protein expression studies [8, 9, 11-13, 20, 29].

214 proteins are significantly differentially expressed after estrogen treatment

MCF-7 cells were treated with ethanol vehicle control and 10 nM E2 for 0.25-, 4-, 8-, 24- 48- and 72- hours. MDA-MB-231 cells were treated with ethanol vehicle control and 10 nM E2 for 24 hours. The incorporated amino acids are isotopically distinct and can be easily distinguished by mass spectrometry. This allows for protein expression between treated and control cells to be quantified based on the relative peak intensities of the isotopic peptide pairs. A light-to-heavy ratio was obtained by contrasting the light and heavy isotopic versions of arginine (10 Da shift) and lysine (6 Da shift).

Close to 1500 proteins were significantly identified ($p<0.05$) with a false discovery rate less than 4%. We defined differentially expressed proteins as those (a) appearing in both replicates, (b) whose expression pattern occurred in the same direction (i.e. X identified protein was up-regulated in treated-light to control-heavy and up-regulated in treated-heavy to control-light and vice versa) and (c) at least one of the log 2 transformed expression values was > 0.5 or < -0.5.

We categorized the identified significant proteins based on enriched biological processes ($P<0.05$) as defined by Gene Ontology using DAVID [30, 31] (see method) to expose those genomic and ER-dependent processes that are affected by E2. Table 1 lists all enriched biological processes at each treatment period and the expressions of the proteins involved.

Time dependent changes on protein levels after estrogen treatment
15 minutes MCF-7

36 proteins are differentially expressed after 15 minutes of estrogen treatment. DAVID analysis of these proteins shows protein folding ($p=0.013$) and response to temperature stimulus ($p=0.033$) are enriched biological processes at this time point. Four proteins that assist the folding of other proteins, namely that of cytoskeletal proteins such as actin and tubulin upon ATP hydrolysis and prevent aggregation of misfolded protein, were up-regulated at this time point (T-complex protein 1 subunit zeta, beta, gamma, Protein disulfide-isomerase A6). However, two proteins with similar functions were down regulated (T-complex protein 1 subunit delta and RuvB-like 2). It was shown that the E2-ERα complex associates with microtubules to modulate rapid estrogen signaling in MCF-7 cells [32]. This association directly leads to phosphorylation of AKT and activation of MAPK and occurs within minutes of treatment in order for subsequent cell signaling to occur. Our data shows that estrogen (possibly in conjunction with its receptor) regulates the expression of proteins responsible for proper folding of microtubule proteins. This regulation probably occurs via the activation of protein translational or degradation machinery. Proteins involved in translation elongation were differentially expressed at this time point and so were members of proteolytic complex although this process was not enriched.

Three proteins were grouped into the response to temperature stimulus process. The expression of Latexin and Superoxide dismutase was up-regulated and ATP-dependent RNA helicase A was down-regulated.

4 hours MCF-7

35 proteins are differentially expressed after 4 hours of E2 treatment. As enriched biological processes at this time point, we found regulation of apoptosis ($p=7.61e-05$), positive regulation of transcription ($p=0.001$), cell to cell signaling ($p=0.001$), translational elongation ($p=0.002$) and proteolysis ($p=1.01e-04$). Five proteins are present in the translation elongation process and these are different proteins then were present found at 15 minutes. Within apoptosis, negative regulation of apoptosis was significantly enriched ($p=1.01e-04$). In this category are proteins such as endoplasmin (also known as heat shock protein 90) and nucleophosmin that negatively regulate apoptosis and both proteins were significantly up-regulated at this time point. Endoplasmin is a chaperone protein to the estrogen receptor. It is important for the proper post-translational folding and stability of proteins, and its inhibition leads to the degradation of key

oncogenes [33]. Nucleophosmin is an estrogen-regulated nucleolar phosphoprotein whose expression is induced by estrogen, however its expression is highest in MCF-7 cells that became estrogen independent and resistant to anti-estrogen treatment [34] implying a role in the acquired resistance known to take place in treated breast cancer cells. This protein inhibits p53 and prevents the DNA binding to interferon regulatory transcription factor 1 (IRF1) decreasing its tumor suppression activity. Other proteins with known functions in apoptosis including actinin alpha 4 calreticulin and valosin-containing-protein were also up-regulated. It was recently demonstrated tha Actinin alpha 4 enhance transcription activity of ERα in MCF-7[35]. Taken together this data shows the potential pathways used by estrogen to prevent cell death and increase cell survival.

Furthermore, cell to cell signaling included the down-regulation of ubuquitin, and Phosphatidylethanolamine-binding protein 1. The latter protein is a known inhibitor of raf-1 kinase activity and MAP kinase signaling [36, 37]. It directly binds to raf-1 and disrupts its interaction with MEK-1, a kinase that activates the extracellular signal regulated kinases (ERK) [37]. This protein also inhibits NF-κB and AP-1 signaling. The fact that estrogen is able to negatively regulate the expression of this protein further links at its role in early activation of MAPK signaling. Protein DJ-1 was up-regulated and categorized in this process. It functions as a transcription factor and translocates to the nuclei from cytoplasm in response to mitogens [38]. It has also been implicated in PI3K survival pathway as a negative regulator of PTEN-mediated tumor suppression [39]. The fact that this process is enriched at this time, points to the cytoplasmic signaling of E2 and the proteins directly involved. This signaling either probably precedes or occurs independently of the transcriptional (genomic) activities of estrogen.

Positive regulation of transcription was also enriched (p=0.01) at this time point with the increased expression of proteins such as Probable ATP-dependent RNA helicase DDX5, Cullin-associated NEDD8-dissociated protein 1 that enhance transcription.

8 hours MCF-7

46 proteins are differentially expressed after 8 hours of E2 treatment with two biological processes enriched that are related to metabolism (ribonucleotide metabolic process, p=0.013; membrane lipid metabolic process, p=0.015). Proteins present in these categories serve as scaffolding molecules that coordinate the assembly of catalytic subunits, proton transport across mitochondrial membrane, Acetyl CoA synthesis, ATP production, and degradation. This suggests that there is an increase in protein synthesis and glycolysis, processes enriched during active cell development and growth and preparation for mitosis.

24 hours MCF-7

60 proteins are differentially expressed after 24 hours of estrogen treatment and the processes enriched are translation elongation (p=0.014), response to inorganic substance (p=0.014), angiogenesis (p=0.048) and regulation of cell proliferation (p=0.049).

Three proteins are present in the angiogenesis process: nucleolin (up), myosin 9 (up) and Beta F1 ATPase (down). The adhesion molecule nucleolin functions in conjunction with myosin 9 to regulate migration, invasion and tubule formation in endothelial cells [40]. It is found on the cell surface of MCF-7 cells but not of normal epithelial cells [41]. Beta F1 ATPase is a mitochondria

protein that drives ATP synthesis. It is down-regulated in multiple cancers and this reduction leads to tumor progression (Cuezva 2002). Its translation and localization is regulated by the mitochondria and it was found that its expression is repressed post-transcriptionally by breast carcinoma extracts [42]. The regulation of angiogenic proteins in our data agrees with previous studies and highlights the global effects of E2 even on mitochondrial proteins.

48 hours MCF-7

39 proteins are differentially expressed after 48 hours of estrogen treatment and the processes enriched are: regulation of cell proliferation (p=0.009), ribonucleotide metabolic process (0.002), response to protein stimulus (p=0.031) and inorganic substance (p=0.009).

Most of the proteins involved in the regulation of cellular uptake of ion and membrane mediated endocytosis were up-regulated. Chloride intracellular channel protein 1 transitions from cytoplasmic monomer to form membrane chloride channels via oxidation [43]. Serpin H1 is a molecular chaperone involved in the maturation and stabilization of collagen [44]. Actin-related protein 3 (up) is an ATP-binding component of a complex involved in actin polymerization that leads to increased cell motility [45]. Transferrin receptor protein 1 is responsible for cellular uptake of iron through the plasma membrane. Signal transducer and activator of transcription 1-alpha/beta activates transcription after serine and tyrosine phosphorylation by kinases and/or growth factors [46]. Other proteins responsible for cellular uptake of iron through the plasma membrane, destruction of toxic radicals produced within the cell, chromatin organization and ATP hydrolysis were also up-regulated. Among the down-regulated proteins were Clathrin heavy chain 1, a major cell and vesicle membrane protein and Catechol O-methyltransferase, disturb microtubule dynamics and inhibits estrogen signaling [47-49]. The data from this time point give details of specific proteins targeted by E2 to increase cellular transport which lead to proliferation.

72 hours MCF-7

46 proteins are differentially expressed proteins after 72 hours of estrogen treatment and the processes enriched were translation elongation (p=0.002) and actin filament capping (p=0.028), and there were Spectrin alpha chain, F-actin-capping protein subunit alpha-1, and F-actin-capping protein subunit beta were all up-regulated and are involved in actin filament capping, a crucial step for actin cytoskeleton remodeling. Dysregulation in actin based processes is a factor in cell motility and transformation [50]. The changes to the cytoskeleton after estrogen treatment could be linked to increased migration and invasion.

24 hours MDA-MB-231

50 proteins are differentially expressed after 24 hours of estrogen treatment in the estrogen receptor negative cells (MDA-MB-231). The processes enriched are translational elongation (p=1.48e-04), proteolysis (p=0.035), cell cycle checkpoint (p=0.007).

Translation elongation usually occurs in the cytoplasm and is regulated by ribosomes, which consist of two subunits that work as one to create a polypeptide chain from mRNA during protein biosynthesis, and each subunit is comprised of a set of proteins (ribosomal proteins) whose functions expand beyond protein synthesis [51]. We identified as up and down regulated proteins that primarily aid at stabilizing both subunits during protein translation. Furthermore,

subunits of the 26S proteasome, involved in controlling the concentration of proteins in diverse regulatory pathways by post-translational ubiquitination and degradation, led to the enrichment of the proteolysis process in these cells. Cell cycle check point was enriched at this time point and it included three proteins: DNA damage-binding protein 1 (down), cell division protein kinase 1 (up), and 60S ribosomal protein L24 (down). These proteins hint the E2 induced changes in cell cycle in ER negative cells. It is known that E2 treatment causes cell death and/or decreases the proliferation rate of ER negative cells [10]. We did not however observe a difference in growth between E2 treated MDA-MB-231 cells and control (figure 1), and this could be due to the dose of estrogen used and treatment period.

Comparison with Known Estrogen Responsive Genes

Forty published E2 responsive genes are present in our data as differentially expressed E2 proteins. These include known oncogenes like AGR2, IF5A1, and PA2G4 all exhibiting similar expression patterns. The mRNA of FK506-binding protein 4, involved in protein binding, is up-regulated after 4, 12 and 24 hours of E2 treatment [18]; in our data, the protein FK506-binding protein 4 is up-regulated after 4, 24 and 72 hours. Interestingly, changes in gene expression over time do not always correlate with changes in protein levels. Endoplasmin plays a role in estrogen signaling in cells through the estrogen receptor and we found that its protein level is up-regulated at an earlier time point (4 hours) than its gene expression (24 hours) (see table 2). Although the expression of more than 85% of these proteins/genes agreed in at least one time point, we noticed that protein expression followed an up-down pattern over-time. This observation was somewhat expected given that proteins are continuously being produced and degraded depending on their role in the specific regulatory pathway taking place. For this reason, amount of mRNA transcripts are not always direct indicators of the level of their corresponding protein. Our data demonstrates the increased transcriptional-induced expression of E2 proteins already known through gene expression studies and provides the expression patterns of proteins regulated by rapid E2 signaling processes and those that are post-transcriptionally regulated.

DISCUSSION

It is well known that E2 is able to increase the rate of cellular transcription in mammary cells primarily through the ERα receptor, the most abundant ER in mammary cells. MCF-7 cells are ER positive; MDA-MB-231 cells are ER negative [52, 53]. We first verified the mitogenic effects of estrogen by treating both cells lines with E2 and observed changes on cell proliferation. We found increased proliferation only in the cell line expressing ER. We next examined the effects of E2 on global protein expression over multiple time points in ER cells, as well as compared protein changes in an ER-positive system to that of an ER-negative system.

Our proteomic approach combines the in-culture labeling techniques (SILAC) with two-dimensional peptide separation (HILIC and reverse-phase) and allows for direct comparison between treated and control samples with minimized variations caused by sample preparation. SILAC media has been shown to have no effect on cell morphology and rate of proliferation [27, 54]. We used dialyzed fetal bovine serum (FBS) supplement rather than charcoal stripped FBS that is usually used in estrogen studies as a supplement to the cell culture media. In dialyzed FBS, most molecules below a molecular weight of 10,000 are removed; this includes a low but

uncontrollable amount of steroid hormones like E2 and free unlabeled amino acids that can alter the labeling efficiency. A cell proliferation assay (MTT) allowed us to verify that the cells remained estrogen responsive with the dialyzed FBS.

The importance of the flipped label design is illustrated by the discovery that the number of significant differentially expressed proteins identified in the same sample differed from one experiment run to another. This is potentially caused by recent mentions of problematic conversion of isotopic arginine to proline in cells grown in SILAC-heavy media [28]. The resulting conversion causes inaccuracies when comparing heavy peptide ion signals to their light counterparts. Our flipped replicate design helped in decreasing these errors affecting protein quantification.

Genomic Biological Processes

The well described classical genomic pathway of estrogen involves the transcriptional regulation of specific estrogen responsive genes through direct binding of the activated estrogen receptor on EREs. This can only take place in ER-positive cell lines. In addition, the resulting changes in protein expression caused by the genomic pathway can only be detected experimentally at later time points given the time required for gene transcription and mRNA translation into proteins takes hours. We used 8-, 24-, 48-, and 72-hours as time points influenced by the genomic pathway. This pathway appears in our data to directly affect the synthesis of proteins involved in cell proliferation, angiogenesis, and actin filament capping. Increased intra-cellular transport, namely nuclear import and export to and from the cytoplasm were enriched at early time points (i.e. 4 hours). An early process of estrogen genomic pathway involves the localization of estrogen bound estrogen receptor from the cytoplasm to the nucleus. This cellular transport is controlled by specific chaperone and transport proteins such as heat shock protein 90 and importin alpha 2, both of which were differentially expressed after 4 hours of estrogen treatment. Once inside the nucleus, the ligand bound receptor activates transcription via specific transcription factors (ERE). Positive regulation of transcription is another process that was enriched after only 4 hours of estrogen treatment and this agrees with the described signaling genomic events.

We found that estrogen affects cancer specific processes required for metastasis such as increased cell migration and invasion through regulation of different stages of cell cycle and changes in cytoskeletal dynamics. The present analysis suggests that this only occurs in the presence of the estrogen receptor and is a result of the classical genomic pathway. MDA-MB-231 cells do not express ER but this receptor is expressed in MCF-7 cells. ER is responsible for most of the proliferative effects of estrogen on cells and is the primary target of selective estrogen receptor modulator therapies used in women with estrogen receptor positive breast cancers. The cancer specific processes mentioned above were only observed in the MCF-7 cells phenotype (increase cell proliferation fig. 1), genomic (published E2 responsive genes[18]), and proteomic (enriched biological processes table 1) data support our hypothesis.

Our data agrees with previous genomic studies. Gadal et al. [11] stimulated MCF-7 cells with 10nM E2 for 15 hours and identified E2-inducible genes whose functions include cytoskeletal and ECM remodeling, cell-cycle progression, and catechols/quinone synthesis regulation. Inoue

et al. also used 10nM E2 to stimulate MCF-7 cells for 6, 12, 24, and 72 hours using a custom-made microarray to measure the gene expression levels of 138 genes [12]. A comparison of the differentially expressed proteins identified in our studies and the genes reported in these previous studies show correlation between gene and protein expression. In addition, our data allowed us to identify E2 proteins whose expression is only altered post-transcriptionally and E2 mitochondrial proteins.

Our data also agrees with previous proteomic studies. One such study compared the tryptic peptide ion intensities of E2 treated and control whole cell lysates after 1-D SDS PAGE band fractionation [25]. Another study used 2-D PAGE gels [24] and yet another relied on isobaric tags [23]. These studies report proteins such as FK506-binding protein 4 that also appear in our data displaying similar levels. Although slightly different E2 doses were used in the other studies, all studies utilized a saturating dose of E2 so the results should be comparable. The overall agreement is fairly good, but unfortunately, there was only a limited overlap in the proteins that were identified in the various studies. This is not unexpected with the different methods employed in the studies. Our data also reveals proteins involved in some non-genomic and ER-independent processes not previously associated with estrogen.

Non-Genomic Biological Processes

At the earliest time point after treatment with E2, mRNA transcription is not expected to change substantially, but protein production can be post-transcriptionally regulated through phosphorylation of initiation/elongation factors and response to iron levels [55]. The increased expression of proteins found at early time point (15 minutes) is due to this mechanism and cannot be a result of transcription activation (genomic effects of E2). Similarly, the rapid degradation of proteins can also occur. Hence, estrogen appears to regulate the rapid signaling pathways through increased translation of key proteins.

Apoptosis, protein folding and cell-cell signaling all appear to be regulated via non-genomic processes. They were all enriched after 0.25 hours and/or 4 hours of estrogen treatment. There are known non-genomic pathways described as occurring within minutes of estrogen treatment via activation of MAPK signaling through membrane bound ER that lead to anti-apoptosis. This process may involve proteins such as ubiquitin and nucleophosmin 1 which all negatively regulate apoptosis and were differentially expressed after 4 hours in our data. Furthermore, microtubule associated proteins are linked to these early non-genomic actions of estrogen as modulators [32]. We found at our earliest time point (15 minutes), protein folding was enriched. The proteins present in this category specifically aid in the folding of microtubule proteins (actin and tubulin).

ER-Independent Biological Processes

Biological processes enriched specifically after 24 hours of estrogen treatment in both cell lines are those we represent here as ER-independent processes since their regulation occurred regardless of the presence of the estrogen receptor. 50 proteins were differentially expressed in the estrogen receptor negative systems. Estrogen is able to alter the expression of these proteins while relying on increased translation of specific already-present mRNA and protein degradation. These regulations take place independent of transcription activation. Translation elongation was enriched after 24 hours of E2 treatment in both cell lines but also after 4 and 72 hours in MCF-7 cells. It is interesting to note that translational elongation enriched in the MDA-MB-231 cells did not lead to increased cell proliferation. Proteolysis was enriched after 24 hours in MDA-MB-231 cells and 4 hours in MCF-7 cells. Taken together, we see that estrogen affects the post-transcriptional regulation of protein expression using translation elongation and protein degradation in an ER-independent manner.

Other ER-independent pathways of estrogen have been described as occurring through membrane receptors namely G-protein coupled receptors, epidermal and insulin growth factor receptors [56, 57]. The expression level of some proteins were similar in both cell lines however key proteins needed for increased cell division were either differentially expressed in different directions or only different in the MCF-7 cells.

CONCLUSIONS

In conclusion, we present biological processes perturbed after estrogen treatment and hypothesize the pathways involved. Estrogen negatively regulates apoptosis through non-genomic pathways, and we present the proteins involved. Increased translation elongation and proteolytic activity occurs independently of the estrogen receptor. Estrogen causes an increase in cell proliferation, and molecular analysis suggest that it also increases cell motility, which could lead to invasion and metastasis only in the presence of the estrogen receptor. This data supports and complements gene expression studies and implies the need for a combination of gene and protein expression analysis to determine the response elicited by cellular stimulants such as hormones. Together they highlight the intricate mechanisms of actions of estrogen and its role in breast cancer onset and development. Taken together, our data increases our knowledge of estrogen signaling in cells and reveals targets that could be used to block the estrogen induced progression of breast cancer.

Materials and Methods

Reagents

Chemicals used in this study included 17-β-estradiol (E2) (MP Biomedicals); SILAC™ media, all supplements and dialyzed fetal bovine serum (Invitrogen); Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin (Fisher Scientific); fetal bovine serum (Tissue Culture Biological); MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)reagent (Research Products International corp.); DMSO (Sigma).

Cell Culture

All cells were grown at 37°C in a humidified incubator with 5% CO_2. The MCF-7 (ER positive) and MDA-MB-231 (ER negative) human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin. SILAC^{TM} medium was prepared according to the manufacturer's instructions. In brief, glucose, L-glutamine, sodium pyruvate, penicillin, and streptomycin were added to phenol red free flex medium lacking the essential amino acids L-Lysine and L-Arginine. [U-$^{13}C_6$]-L-Lysine and [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine were added to form the "Heavy" medium; normal L-Lysine and L-Arginine were added to form the "Light" media. Once normal cell growth was established in DMEM, both cell types were moved to Heavy or Light SILAC media supplemented with 10% dialyzed fetal bovine serum and grown for at least six passages before the start of experiments to allow for complete incorporation of the labeled amino acids into the entire proteome.

Estrogen treatment and MTT Assay

We performed an MTT colorimetric proliferation assay to determine cell proliferation. The cells were grown in phenol-free DMEM containing 5% dialyzed FBS for 48 hours prior to E2 treatment. Cells were plated in 96-well plates (2500-5,000 cells/well). Twenty-four hours after cell plating (day 0), E2 was added to the medium (final concentrations of 5 nM, 10 nM and 20 nM), and an equal volume of ethanol was used as vehicle-control (0 nM of E2). Following the appropriate stimulation period, MTT reagent was added to each well and incubated for 4 hours. DMSO was used to dissolve the crystals and optical density readings at 570 nm were obtained using a spectrophotometer.

For the proteomics experiment, the MCF-7 cells were treated with 10nM E2 or ethanol control for 15 minutes, 4 hours, 8 hours, 24 hours, 48 hours, and 72 hours, 4 plates per time point. In addition, we performed the experiment in duplicate with light-label serving as control in the first case, and heavy-label in the second. The flipped label control allowed us to run the same biological sample twice: heavy treated combined with light control and light treated combined with heavy control. The MDA-MB-231 cells were treated similarly with 10nM E2 or ethanol (vehicle control) for 24 hours only. At the end of each treatment period, the cells were washed twice with Tris-buffered saline, scraped with 500 µl of 8 M urea in 50 mM tris-HCl buffer pH. 8.3 supplemented with protease inhibitors (Roche Complete Mini), then incubated on ice for 15 minutes. Using cell disruption by pressure cycling technology [58], samples were processed through a pressure barocycler^{TM} (Pressure Biosciences Inc.) for 15 cycles (30 seconds at 35 kpsi and 20 seconds at atmospheric pressure (0 kpsi)) then centrifuged for 15 minutes at 12,000 x g and the supernatant was collected. Protein concentration was determined using BCA assay kit (Pierce). This harvest was performed at approximately 70% cell confluence.

Protein Preparation and Digestion

For each stimulation period, a 1:1 (200 µg total) mixture of protein lysate from heavy and light sample was combined and digested with trypsin as follows: 10 mM DTT in 50 mM Tris-HCl buffer, pH 8.3 incubation for 1 hour at 37°C followed by 20 mM iodoacetamide in 50 mM Tris–HCl buffer, pH 8.3, incubation for 1 hour at room temperature in the dark. The protein solution was diluted by a factor of 4 with 50 mM Tris-HCl pH 8.3. Trypsin was then added at ratio of 1:50 (trypsin: protein) by weight and incubated overnight at 37°C (16 hours).

Peptide Analysis

The digested peptide mixtures were desalted using Supelco ENVI-18 cartridges. The dried peptides were re-suspended in 250 µl 90 % ACN/ 0.1 % TFA and fractionated by hydrophilic-interaction liquid chromatography (HILIC) through a 4.6 x 250 mm TSKamide-80 HILIC column (Tosoh Bioscience LLC). All reagents for the mobile phases were of HPLC grade (Sigma). Buffer phase A contained 2% ACN and 0.1% TFA; buffer phase B contained 98% ACN and 0.1% TFA. Peptides were eluted at 0.5 ml/min total flow through a linear gradient of mobile phase B from 90% to 85% in 5 minutes, then from 85% to 70% in 15 minutes. Fractions were collected every 2 minutes.

From the 60 collected fractions, 12 were determined to contain peptide fractions based on the absorbance. The fractions were transferred to 1.5 ml micro-centrifuge tubes and speed-vacuumed to dryness, then resuspended in 18 µl of 0.1% TFA in water. The mass spectrometry analysis was performed using LC/MS/MS on a Thermo-Electron ProteomeX LC/MS workstation, consisting of a LCQ DecaXP Plus Ion-Trap mass spectrometer equipped with a nanospray ion source and an LC system with two Surveyor HPLC pumps and Surveyor auto-sampler. One third of each fraction was loaded onto a trap column (Agilent Zorbax SB-C18, 5µm, 5x0.3mm) by 3% acetonitrile/0.1% formic acid at a flow rate of 15 µl/min. Peptides were eluted from the trap onto an in-house packed reverse phase capillary column (3 µm C18, 300 Å pore size particles slurry-packed in a 12 cm long 75 µm ID PicoTip from New Objectives) in the nanospray source for separation. The elution gradient was 3-40 % acetonitrile/ 0.1 % formic acid in 46 min, at a flow rate of 180 µl/min. The nanospray source spray voltage was 1.8 keV. The LCQ DecaXP Plus Ion-Trap mass spectrometer was set to acquire one full MS scan from 380 – 1600 m/z, followed by three data-dependent MS/MS scans of the top 3 most intense ions, using a normalized collision energy of 35% and an isolation width of 2.3 m/z. To ensure adequate coverage of co-eluting peptides, dynamic exclusion was used with a repeat count of 2, repeat duration of 0.5 min, and exclusion duration of 2 min.

Protein Identification and Quantification

The peptide mass spectra acquired in ".RAW" format were converted to ".mzXML" and then to Mascot Generic files (.mgf) using the Trans-Proteomic Pipeline (TPP) web interface (Institute for Systems Biology, version 3.4). The individual ".mgf" files from each fraction were searched against the SwissProt database using Mascot Daemon (Matrix Science version 2.1.6). The searches were performed with a peptide tolerance of 1.2 dalton and MS/MS tolerance of 0.8 dalton; peptide charge of "2^+ and 3^+"; fixed modification at carbamidomethyl (C); variable modifications to account for the 6 and 10 dalton shift of Lysine (K) and Arginine (R) respectively, methionine oxidation (M), and a maximum of 2 missed cleavages.

Mascot files containing the identified proteins were converted to "pepXML" files using TPP. Protein identification was determined using TPP tools PeptideProphet and ProteinProphet. Only protein identification established with a $p < 0.05$ were used for downstream analysis. The relative expression levels of each protein was determined by Automated Statistical Analysis on Protein Ratio (ASAPRatio) that specifically quantifies ratios of the differentially labeled proteins and return a value light/heavy with their corresponding confidence intervals [59]. The biological processes and KEGG pathways were obtained from a web-based **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID) [30, 31].

FIGURES

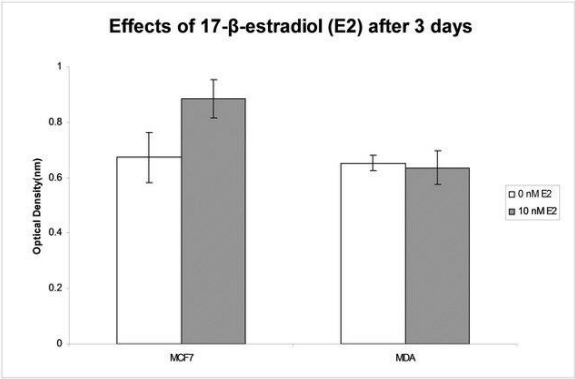

Figure 1. Effect of 17-β-estradiol on the proliferation of MCF-7 and MDA-MB-231 cells
Cells grown in 5% dialyzed FBS and treated with 0 and 10 nM E2 for 3 days. MTT assay was performed to quantify the number of viable cells in 96 well plates. Y axis represents Optical Density readings obtained from an observance reading 570nm on a spectrophotomer. The error bars represent the standard deviation between quadruplet wells. Estrogen treatment has no effect on the observed growth of the estrogen receptor negative cell line (MDA-MB-231) but does affect the growth of the estrogen receptor positive cell line (MCF-7).

Table 1. Enriched Biological Process

Genomic processes

Enriched biological process	Sample (P value)	Up-regulated proteins	Down-regulated proteins
Positive regulation of transcription	4 hours (0.001)	DD5X, CAND1	UBIQ
Ribonucleotide metabolic process	8 hours (0.015) 48 hours (0.002)	ATP5J, ACLY, PUR2 ATPB, PUR9	NDKA, PUR6 ATPA, ATPD
Membrane lipid metabolic process	8 hours (0.015)	2AAA	PPT1, SAP
Response to inorganic substance	24 hours (0.014)	TFR1, TFF1, PEBP1	PRDX1, PRDX6, EF1A2
Response to organic substance	48 hours (0.009)	CLIC1, SERPH, ARP3, TFR1, STAT1, SODC	SYAC, COMT
Angiogenesis	48 hours (0.020)	TFR1, SODC, LMNA, ATPB	CLH1
Actin filament capping	72 hours (0.028)	SPTA2, CAPZB, CAZA1	-
Cell proliferation	24 hours (0.049) 48 hours (0.009)	PP1B, IF5A1 STAT1, 1433S	ATPA, TPD54, E2AK2, PHB ATPA, TPD54, COMT, RS9

Non-genomic Processes

Enriched biological process	Sample (P value)	Up-regulated proteins	Down-regulated proteins
Regulation Apoptosis Anti-Apoptosis	4 hours (7.61e-05) 4 hours (1.01e-04)	ENPL, NPM, TERA, CALR, ACTN4 ENPL, NPM	UBIQ, SYAC, CH60 UBIQ
Protein folding	0.25 hours(0.013)	TCPZ, TCPB, TCPG, PDIA6	TCPD, RUVB2
Response to temperature stimulus	0.25 hours (0.033)	LXN, SODC	DHX9
Cell-cell signaling	4 hours (0.001)	PARK7	UBIQ, PEBP1

ER-independent Processes

Enriched biological process	Sample (P value)	Up-regulated proteins	Down-regulated proteins
Proteolysis	4 hours (1.01e-04) MDA (0.035)	ENPL, CAND1, TERA PSA, CDC2, CAN2, PRS6B	UBIQ, DPP3 PSMD2, UCHL3, PSD11, DDB1
Translational elongation	4 hours (0.002) 24 hours (0.014) 72 hours (0.002) MDA (1.48e-04)	RL19 RL6, RL29, RSSA RL3, RS15, RS4X, RS3A RSSA, RL13, RL18	UBIQ, EF1B, RS23, EFTU RL5, RS25, RL3, EF1A2, EFTU, RLA2 RL4, RL15, RSSA, RL12, RL7A RL7, EF1D, RS15, RS4X, RS18, RL23, RS10, RL24

Table 2: Comparisons of Genomic Data and Proteomic Data

Estrogen responsive genes at 4 hours

Protein/gene	Up in gene exp.	Down in gene exp.	Up in protein exp.	Down in protein exp.
1433S	4hrs, 24hrs	-	48hrs	-
C1TC	4hrs, 12hrs, 24hrs	-	72hrs	MDA
CAND1	4hrs, 12hrs, 24hrs	-	4hrs	48hrs
FKBP4	4hrs, 12hrs, 24hrs	-	4hrs, 24hrs, 72hrs	-
G6PD	4hrs, 12hrs, 24hrs	-	48hrs	24hrs
IF5A1	4hrs, 12hrs, 24hrs	-	24hrs	-
K6PP	4hrs, 24hrs	-	0.25hrs	-
PA2G4	4hrs, 12hrs, 24hrs	-	48hrs, 72hrs	-
PCNA	4hrs, 12hrs, 24hrs	-	-	24hrs
PCTL	4 hrs	-	0.25hrs, 4hrs	-
PRS6A	4hrs, 12hrs, 24hrs	-	8hrs, 72hrs	24hrs
PRS6B	4 hrs	-	MDA	-
PSA	4hrs, 24hrs	-	0.25hrs, MDA	24hrs, 72hrs
RAN	4hrs, 12hrs, 24hrs	-	-	24hrs, 72hrs
SRC8	4 hrs	-	0.25hrs	
TCPD	4hrs, 24hrs	-	8hrs	0.25hrs, MDA
TFF1	4hrs, 12hrs, 24hrs	-	24hrs	72hrs
TFR1	4hrs, 12hrs, 24hrs	-	24hrs, 48hrs	-
UGDH	4 hrs	-	48hrs	-

Estrogen responsive genes at 12 hours

Protein/gene	Up in gene exp.	Down in gene exp.	Up in protein exp.	Down in protein exp.
AGR2	12hrs, 24hrs	-	48hrs	-
HAT1	12hrs, 24hrs	-	24hrs	8hrs
HNRPQ	12 hrs	-	-	4hrs, 72hrs
HPRT	12hrs, 24hrs	-	MDA	-
HS105	12hrs, 24hrs	-	24hrs	-
PUR6	12hrs, 24hrs	-	0.25hrs	8hrs, 72hrs
RUXE	12hrs, 24hrs	-	-	8hrs
SYLC	12hrs, 24hrs	-	-	MDA
TRAP1	12 hrs	-	24hrs	-

Estrogen responsive genes at 24 hours

Protein/gene	Up in gene exp.	Down in gene exp.	Up in protein exp.	Down in protein exp.
ADT3	-	24 hrs	-	8hrs
ANXA3	-	24 hrs	MDA	
CPSF5	24hrs	-	24hrs	-
ENPL	24hrs	-	4hrs	-
H2AV	24hrs	-	72hrs	-
ILF2	24hrs	-	48hrs	24hrs
IMA2	24hrs	-	4hrs	24hrs
KPYM	24hrs	-	-	4hrs
PGK1	24hrs	-	0.25hr, 8hrs 24hrs	-

SODC	24hrs	-	0.25hrs, 48hrs	-
TALDO	24hrs	-	4hrs	-
XPO2	24hrs	-	0.25hrs	-

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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.An Integrated Framework to Model Cellular Phenotype as a Component of Biochemical Networks	Gormley, M., Akella, V U, Quong J N, Quong AA	Advances in Bioinformatics	June 2011	☐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.*

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME **Andrew A. Quong, Ph.D.**	POSITION TITLE **Associate Professor**
eRA COMMONS USER NAME **axq002**	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of California, Berkeley	BA	1980-85	Physics & Applied Mathematics
University of California, Irvine	MS	1985-87	Physics
University of California, Irvine	PhD	1985-91	Physics

B. POSITIONS AND HONORS:

1985-1991	Member of Technical Staff, Senior, Aerojet Electronics Systems Division, Azusa, CA
1991-1993	National Research Council Research Associate, Naval Research Laboratory, Washington DC
1993-1998	Senior Member of Technical Staff, Sandia National Laboratories, Livermore, CA
1998-2004	Physicist, Lawrence Livermore National Laboratory, Livermore, CA
1999-2001	Program Manager, Office of Basic Energy Sciences, USDOE, Germantown, MD
2000-2001	Scientific Editor, Science and Technology Review, LLNL, Livermore, CA
2000-2001	Scientific Capabilities Leader, Computational Materials Science, LLNL, Livermore,CA
2001-2004	Scientific Capabilities Leader, Computational Systems Biology, LLNL, Livermore,CA
2003-2004	Deputy Director, Biosecurity and Nanoscience Laboratory, LLNL, Livermore, CA
2004-2005	Associate Professor, Department of Oncology, Georgetown University, Washington, DC
2004-2005	Division Director, Department of Oncology, Nanotechnology and Integrative Cancer Biology
2006-2008	Genomics Consultant, Food and Drug Administration, Center for Devices and Radiological Health, Silver Spring, MD
2004-	Adjunct Associate Professor, Department of Physics, Georgetown University, Washington, DC
2006- 2013	Associate Professor, Department of Cancer Biology and Department of Otolaryngology Head and Neck Surgery , Thomas Jefferson University, Philadelphia, PA
2006- 2013	Director, Proteomics and Molecular Characterization Shared Resource, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

2007-	Guest Associate Professor, School of Biomedical Engineering, Drexel University, Philadelphia, PA
2007- 2013	Assistant Director Shared Resources, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA
2006-2007	Associate Editor, Cancer Research
2008-	Associate Editor, American Journal of Pathology
2009-	Editorial Board, Cancer Research

Reviewer: NIH, DOD/CDMRP, Susan G. Komen for the Cure, Breast Cancer Alliance

C. SELECTED PUBLICATIONS

Most Relevant to Current Application

1. Caveolin-1-/- null mammary stromal fibroblasts share characteristics with human breast cancer-associated fibroblasts. Sotgia F, Del Galdo F, Casimiro MC, Bonuccelli G, Mercier I, Whitaker-Menezes D, Daumer KM, Zhou J, Wang C, Katiyar S, Xu H, Bosco E, Quong AA, Aronow B, Witkiewicz AK, Minetti C, Frank PG, Jimenez SA, Knudsen ES, Pestell RG, Lisanti MP. Am J Pathol. 2009 Mar;174(3):746-61.
2. Loss of caveolin-3 induces a lactogenic microenvironment that is protective against mammary tumor formation. Sotgia F, Casimiro MC, Bonuccelli G, Liu M, Whitaker-Menezes D, Er O, Daumer KM, Mercier I, Witkiewicz AK, Minetti C, Capozza F, Gormley M, Quong AA, Rui H, Frank PG, Milliman JN, Knudsen ES, Zhou J, Wang C, Pestell RG, Lisanti MP. Am J Pathol. 2009 Feb;174(2):613-29.
3. The anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. Juliana C, Fernandes-Alnemri T, Wu J, Datta P, Solorzano L, Yu JW, Meng R, Quong AA, Latz E, Scott CP, Alnemri ES. J Biol Chem. 2010 Jan 21. [Epub ahead of print]
4. Cyclin D1/cyclin dependent kinase 4-mediated phosphorylation of filamin A affects the migration and invasion potential of breast cancer cells. Yeow WS, Zou C, Zhong Z, Wassell R, Wang C, Pestell RG, Quong JN, Quong AA, Cancer Research, 2010 Mar 1;70(5):2105-14.
5. An integrated framework to model cellular phenotype as a component of biochemical networks. Gormley M, Akella VU, Quong JN, **Quong AA**. Adv Bioinformatics. 2011;2011:608295. Epub 2011 Nov 29.
6. Low abundance protein enrichment for discovery of candidate plasma protein biomarkers for early detection of breast cancer. Meng R, Gormley M, Bhat VB, Rosenberg A, **Quong AA**. J Proteomics. 2011 Dec 21;75(2):366-74. Epub 2011 Aug 7
7. Proteomic profiling of infiltrating ductal carcinoma reveals increased cellular interactions with tissue microenvironment. Gormley M, Minkeu A, Meng R, Zhong Z, **Quong AA**. J Proteome Res. 2012 Feb 22.
8. Growth of V-SRC-transformed cells in serum-free medium through the induction of growth factors. Deangelis T, **Quong A**, Morrione A, Baserga R., J Cell Physiol. 2012 Dec 18. doi: 10.1002/jcp.24303. [Epub ahead of print]
9. Tchafa, A. , Zhong, Z. , Meng, R. , Quong, J. and Quong, A. (2013) Increased Cellular Invasion and Proliferation via Estrogen Receptor after 17-*β*-Estradiol Treatment in Breast Cancer Cells Using Stable Isotopic Labeling with Amino Acids in Cell Culture (SILAC). *Advances in Breast Cancer Research*, **2**, 32-43. doi: 10.4236/abcr.2013.22007.