AWARD NUMBER: W81XWH-15-1-0387

TITLE: Gestational Exposure to Soy Isoflavones and Epigenetic Regulation of Breast Cancer Risk

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE

Gestational Exposure to Soy Isoflavones and Epigenetic Regulation of Breast Cancer Risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0387

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Tucson, AZ 85719-4824

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The **overarching challenge is to identify what makes the breast susceptible to cancer development**, and more specifically, the mechanisms that link epigenetic silencing of the BRCA-1 gene to the development of triple-negative breast cancers (TNBC). The main **purpose** of this project is to examine whether the DNA methyltransferase (DNMT) inhibitor and soy isoflavone, genistein prevents CpG methylation of the BRCA-1 gene and development of TNBCs. **Major findings**: Preliminary data acquired through the support of this grant indicate that: 1) in cell culture experiments with ERα-positive breast epithelial cells genistein exerted dose- and time dependent preventative effects on repression of BRCA-1 expression. In washout experiments, the post-treatment with genistein rescued BRCA-1 protein expression; also genistein reduced BRCA-1 CpG methylation and cell proliferation markers; 2) In ERα-negative breast cancer cells carrying hypermethylated BRCA-1, genistein reduced BRCA-1 and ERα (ESR1) promoter methylation while restoring BRCA-1 expression; 3) We have developed mice colonies of conditional Flox-CRE BRCA-1 heterozygous (BRCA-1 +/f). Pregnant mice from control (BRCA-1 +/+) and BRCA-1 heterozygous (BRCA-1 +/f) genotype are being treated during gestation with genistein. Mammary tissues from female pups are being harvested to examine the impact of gestational exposure to genistein on mammary morphology and parameters of TNBC development.

15. SUBJECT TERMS

BRCA-1, genistein, epigenetics, DNA methylation, TNBC, breast cancer prevention

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

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1. INTRODUCTION

Subject: The BRCA-1 gene encodes a tumor suppressor protein involved in DNA repair and transcription control (1-3). In women who carry a mutated BRCA-1 allele (BRCA-$1^{+/-}$), the silencing of the wild-type (WT) allele creates a BRCA-1-deficient phenotype, also referred to as loss of heterozygosity (LOH), which is associated with a high probability (~60-80%) of developing breast cancer (4,5). On the other hand, sporadic breast cancers, which represent the vast majority (~90%) of breast tumor cases, do not have mutations in the BRCA-1 gene (BRCA-$1^{+/+}$), but display a BRCAness phenotype characterized by absent or markedly reduced levels of BRCA-1 similar to those observed in hereditary BRCA-1 tumors, i.e. loss of estrogen receptor-a (ERα) and basal-like subtype (6-12).

Purpose: Epigenetics refers to modifications in chromatin structure (i.e. histone and DNA CpG methylation) and non-coding RNAs (13). Sporadic breast cancers that have CpG methylated BRCA-1 promoter share phenotypic characteristics (BRCAness) with hereditary BRCA-1 mutation tumors (14), i.e. they tend to be triple-negative with reduced or absent expression of the estrogen receptor–α (ERα), progesterone (PR), and epidermal growth factor receptor-2 (Her2) ($ER^-/PR^-/Her2^-$) (15). The CpG methylation of the BRCA-1 gene is associated with reduced BRCA-1 expression in 50-60% of higher histological grade sporadic tumors (16-19). A high degree of correlation (~75%) has been documented between hypermethylation of the BRCA-1 and ERα promoters, and reduced expression of BRCA-1 and ERα protein (20,21), which are invariably linked with resistance to endocrine therapies based on antagonists of the ERα (i.e., tamoxifen) [Murphy].

Scope: Genistein is a common dietary isoflavone with antagonistic properties toward the AhR (Denison) and DNMT enzymes that place methylation marks at CpGs in DNA (23,24) BRCA-1 is a molecular target for genistein (25). Rodents exposed in utero and through weaning to genistein had lower number of mammary tumors. Moreover, prepubertal genistein was found to reduce mammary tumorigenesis (26,27) associated with upregulation of BRCA-1 (28). Therefore, for this award we are investigating whether genistein may offer protection against epigenetic silencing of BRCA-1 in ERα-positive human breast cancer cells or ERα-negative sporadic breast cancer cells already harboring hypermethylated BRCA-1. Preventing the non-mutational mechanisms that contribute to silencing of BRCA-1 has important implications for therapy of both hereditary and sporadic breast cancers.

2. KEYWORDS

BRCA-1, genistein, epigenetics, DNA methylation, triple-negative breast cancers, prevention

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Goal 1. To examine how interactions between BRCA-1 genotype (i.e. BRCA-1 WT vs BRCA-$1^{+/f}$) and exposure to genistein impact CpG methylation of BRCA-1 and genes associated with the TNBC phenotype.

Goal 2. To examine how interactions between BRCA-1 genotype (i.e. BRCA-1 WT vs BRCA-$1^{+/f}$) and in utero exposure to genistein impact the development of TNBC in offspring.

- **What was accomplished under these goals?**

❖ *Accomplishment 1*

To determine the functional effects of genistein on regulation of BRCA-1 expression, we cultured ERα-positive MCF-7 cells in the presence of estradiol (E2), TCDD, and their combination. We adopted the MCF-7 cell line because it harbors wild-type, non-hypermethylated BRCA-1 and possesses a functional aromatic hydrocarbon receptor (AhR) pathway which can be activated with TCDD to repress BRCA-1 expression (29-31). The treatment with E2 induced within 24 h BRCA-1 protein expression, which however was reduced by cotreatment with TCDD (Fig. 1). We tested various concentrations of genistein, and found that doses ranging from 0.5 to 2.0 μM reversed the repressive effects of TCDD, whereas at higher levels (5.0 to 10.0 μM) genistein was not effective, or even repressed (20 μM), BRCA-1 expression.

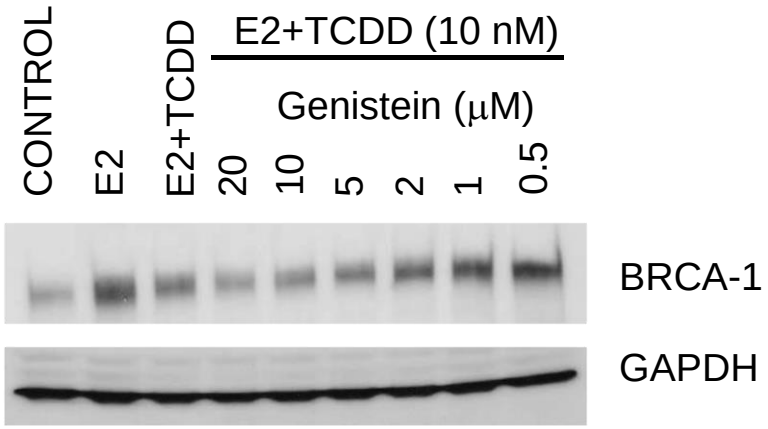

Fig. 1. Effects of genistein on BRCA-1 expression in MCF-7 breast cancer cells. Cells were cultured for 24 h in control phenol red-free media supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with TCDD (10 nM) and genistein. Bands are representative immunocomplexes detected by Western blotting for BRCA-1 and GAPDH from two independent experiments performed in duplicate.

Based on these results, we focused on the time-dependent effects of genistein, and found that its enhancing effects on BRCA-1 extended through a 72 h period (Fig. 2).

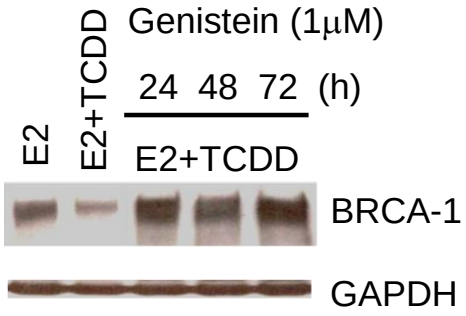

Fig. 2. Time-dependent effects of genistein on BRCA-1 expression in MCF-7 breast cancer cells. Cells were cultured for 24, 48, and 72 h in control phenol red-free media supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with TCDD (10 nM) and genistein. Bands are representative immunocomplexes detected by Western blotting for BRCA-1 and GAPDH from two independent experiments performed in duplicate.

As a control for proliferation, we confirmed that E2 induced expression of cyclin D1 and cyclin B1, which were, however, reduced upon cotreatment with E2 plus TCDD and genistein (Fig. 3).

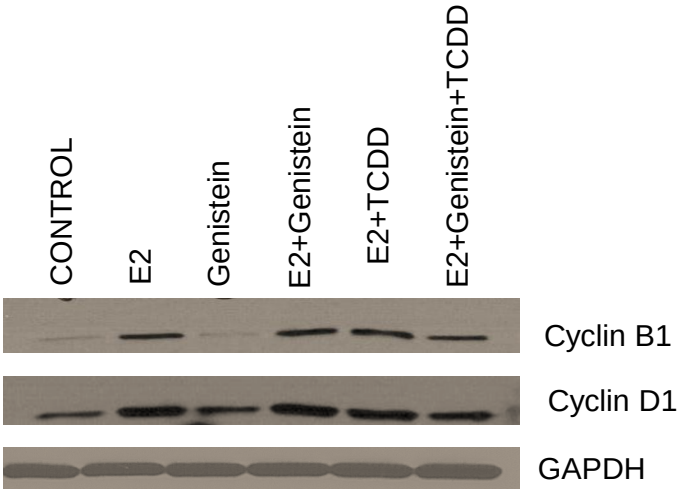

Fig. 3. Effects of genistein on expression of parameters involved in cell cycle progression in MCF-7 breast cancer cells. Cells were cultured for 72 h in control phenol red-free media supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with TCDD (10 nM) and genistein (1 μM). Bands are representative immunocomplexes detected by Western blotting for cyclin B1 and D1 and control GAPDH from two independent experiments performed in duplicate.

Next, we asked whether or not genistein could exert reversal effects on BRCA-1 expression after washout of the repressive AhR agonist TCDD. MCF-7 cells were first cultured in the presence or absence E2 or E2 plus TCDD for 24 h. Then, media were washed out and replaced with media containing E2 alone or E2 plus genistein. These experiments were extended to longer time periods (i.e. 7, 8, and 9 days). Western blot analysis of TCDD washout cell lysates showed that genistein rescued BRCA-1 expression (Fig. 4).

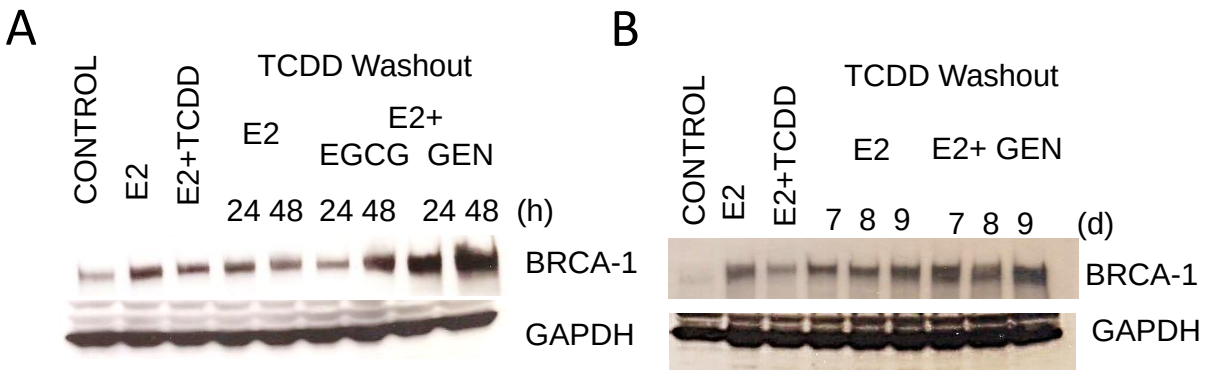

Fig. 4. Rescue effects of genistein on BRCA-1 expression in MCF-7 breast cancer cells. A) Cells were cultured for 24 h in control phenol red-free media supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with TCDD (10 nM) and genistein (GEN) (1 μM). Then, media was removed (washout) and replaced for 24 and 48 h with media containing E2, or E2 plus EGCG (positive control), or GEN. In B) MCF-7 cells were cultured with TCDD for 72 h and BRCA-1 expression was measured at 7, 8, and 9 days after TCDD washout and culturing with E2 or E2+GEN. Bands are representative immunocomplexes detected by Western blotting for BRCA-1 and GAPDH from two independent experiments performed in duplicate.

In previous studies, genistein was found to reactivate expression of tumor suppressor genes (Li 2013a) via promoter demethylation (Fang 2007; Fang 2005). Therefore, we analyzed the effects of genistein on TCDD-induced BRCA-1 CpG methylation and found that whereas TCDD elicited a 1.3-fold increase in BRCA-1 methylation, the addition of genistein (1 μM) lowered BRCA-1 methylation by ~50% compared to E2 control (Fig. 5) Similarly, EGCG, as a positive control, reduced BRCA-1 methylation of TCDD-treated MCF-7 cells even further (~80%) compared to E2 control. These methylation results were consistent with the protective effects of genistein on repression of BRCA-1 protein expression in MCF-7 cells.

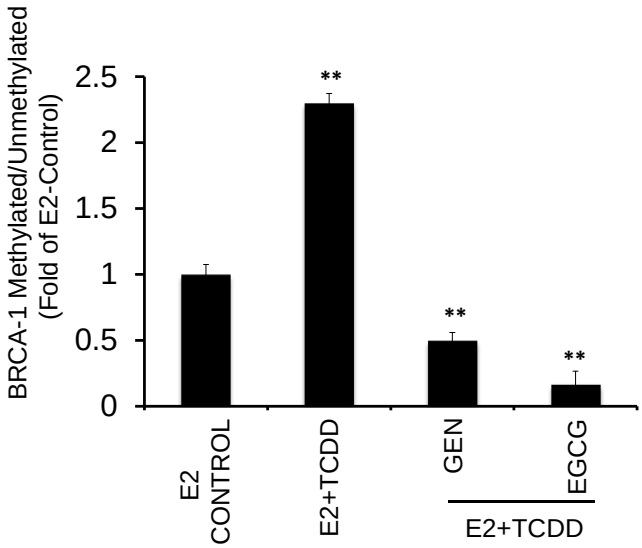

Fig. 5. MCF-7 cells were cultured for 72 h in control phenol red-free media supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with 1 μM genistein (GEN) or EGCG (positive control). Bars represent changes in BRCA-1 CpG methylation compared to E2-control.

❖ *Accomplishment 2*

Previously (32), we observed that ERα-negative sporadic breast cancer UACC-3199 cells with hypermethylated BRCA-1 had low levels of BRCA-1 compared to ERα-positive breast cancer cells. Therefore, we asked whether or not the reactivating effects of genistein observed in MCF-7 cells could be extended to UACC-3199 cells. In dose-dependent experiments, we found that compared to control, genistein at doses ranging from 1 nM to 100 nM did not affect BRCA-1 levels, which, however, were induced ~20 and 30% by 1 and 5 μM genistein, respectively (Fig. 6). The BRCA-1 induction was more pronounced (~1.0-fold) at doses of 10 and 20 μM genistein.

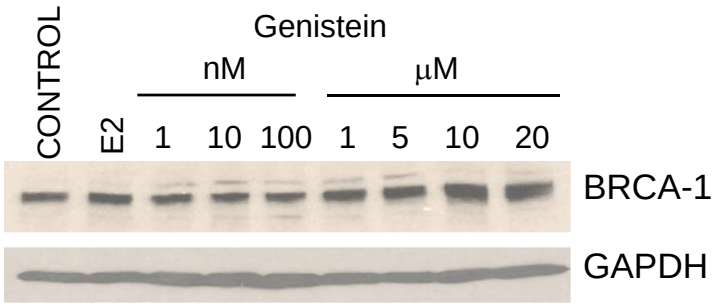

Fig. 6. Effects of genistein on BRCA-1 expression in UACC-3199 breast cancer cells. Cells were cultured for 72 h in control phenol red-free media supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with genistein. Bands are representative immunocomplexes detected by Western blotting for BRCA-1 and GAPDH from two independent experiments performed in duplicate.

The upregulation of BRCA-1 protein was paralleled by reduced BRCA-1 and ERα promoter CpG methylation, as determined by amplification of bisulfonated DNA obtained from UACC-3199 cells (Fig. 7), These data suggested that the reactivating effects of genistein on BRCA-1 and ERα expression were paralleled by differential regulation of DNMT activity on the BRCA-1 and ERα (ESR1) genes.

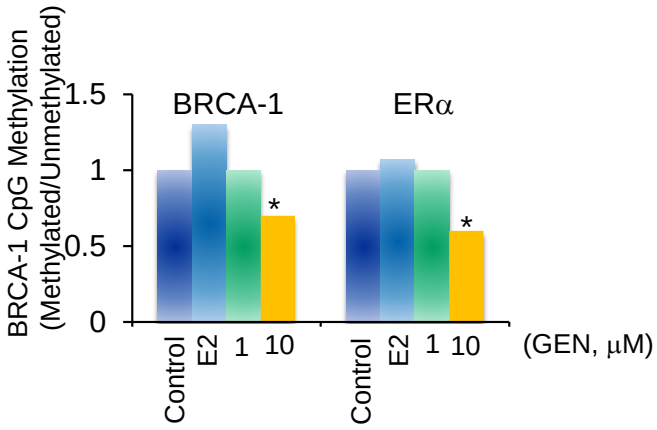

Fig. 7. UACC-3199 cells were cultured for 72 h in control phenol red-free media RPMI supplemented with 10 % charcoal-stripped FCS in the presence of 10 nM E2, and with 1 or 10 μM genistein (GEN). Bars represent changes in BRCA-1 CpG methylation compared to control.

❖ *Accomplishment 3*

Through approved breeding protocol conducted at the Experimental Mouse Shared Services (EMSS) of the University of Arizona Cancer Center, we have derived the mice colonies to test in vivo how interactions between BRCA-1 genotype and gestational treatment with genistein influence BRCA-1 CpG methylation and mammary tumor development in offspring. The colonies are BRCA-1 conditional Flox knock-out and heterozygous models expressing Cre recombinase in the mammary gland; the groups are as follows:

1. WT BRCA-$1^{+/+}$ (Control);
2. BRCA-1 conditional Flox-CRE) heterozygous (BRCA-$1^{+/f}$)

Pregnant mice for each genotype are being assigned at conception to a control diet or a diet supplemented with genistein through the end of gestation. So we have a total of four experimental gestational groups as planned [2 genotypes x 2 dietary treatments (control vs genistein)]. Female pups from each group are being assigned to foster mothers immediately after birth. Mammary tissues from offspring of the corresponding genotype are being collected at day 60 of age (postpuberty). Mammary tissue are being processed for analyses of morphology; markers of proliferation; and BRCA-1 expression and methylation.

Synopsis

We investigated the effects of genistein on epigenetic regulation of BRCA-1 via promoter hypermethylation in ERα-positive (MCF-7) and -negative sporadic (UACC-3199) breast cancer cells. In MCF-7 cells, we analyzed by western blotting the dose- and time dependent effects of genistein on BRCA-1 expression and washout experiments. We then measured the effects of genistein on BRCA-1 promoter methylation by quantitative PCR amplification of bisulfonated genomic DNA; and cyclin D1 and B1 expression by Western blotting. In ERα-negative UACC-3199 cells carrying hypermethylated BRCA-1 promoter, we tested the effects of genistein on BRCA-1 protein expression and BRCA-1 and ERα (ESR1) promoter CpG methylation; Genistein exerted dose- and time dependent preventative effects on BRCA-1 expression, and rescued BRCA-1 protein expression after washout. In UACC-3199 cells, genistein reduced BRCA-1 promoter methylation while restoring BRCA-1 expression. Results suggest that genistein prevents *BRCA-1* promoter methylation and silencing in ERα-positive breast cancer cells. Data also indicate that genistein may rescue BRCA-1 expression via CpG demethylation in ERα-negative sporadic breast cancer cells harboring hypermethylated *BRCA-1* gene. We have derived the mice colonies to test in vivo how interactions between BRCA-1 genotype and gestational treatment with genistein influence BRCA-1 CpG methylation and mammary tumor development in offspring.

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- **What opportunities for training and professional development has the project provided?**

  This project is providing a training opportunity for Jeff Tolson, undergraduate junior student in Biomedical Sciences and Micah Donovan who are assisting with various aspects of the project including qRT-PCR, Western blot, BRCA-1 methylation, and proliferation analyses.

  Additional opportunities for development included:

  - Attendance to Arizona Cancer Center Retreat by Dr. Romagnolo and Dr. Selmin on April 8, 2016.
  - Invitation to Dr. Romagnolo to participate in the Metastatic Breast Cancer Working Group, Arizona Cancer Center, The University of Arizona.

- **How were the results disseminated to communities of interest?**

  - Presentation by Dr. Romagnolo of instructional lectures on BRCA-1 and promoter regulation, Nutritional Biology NSc408, Department of Nutritional Sciences.
  - Seminar: Epigenetics of breast cancer – Cancer Biology Interdisciplinary Program – Dec 3, 2015. The University of Arizona.
  -

- **What do you plan to do during the next reporting period to accomplish the goals?**

Mammary tissues from female pups with various BRCA-1 backgrounds are being harvested and analyzed for BRCA-1/ERα methylation. Also we will perform analyzes of mammary morphology, tumor burden, various genes associated with TNBC and BRCA-1 and ERα expression by RT-PCR and Western blotting.

4. **IMPACT**

- **What was the impact on the development of the principal discipline(s) of the project?**

Genistein is a common food isoflavone with antagonistic properties toward enzymes that place methylation marks at CpGs. Therefore, this work and experimental data integrate etiological and dietary factors with broad impact for the prevention of TNBCs, for which prospects for treatment remain poor.

What was the impact on other disciplines?

- The findings reported so far are likely to make an impact on epigenetic approaches to understanding the etiology of breast cancer. Importantly, epigenetic modifications do not involve changes in the DNA sequence, and are potentially reversible. Therefore, understanding the underlying mechanisms of epigenetic regulation of BRCA-1 and TNBCs is critical for the development of therapeutic strategies.

- **What was the impact on technology transfer?** Nothing to report.

- **What was the impact on society beyond science and technology?** Impact beyond the bounds of science, engineering, and the academic world have been:

  - Reaching out to general public and breast cancer interest groups and patients who have expressed interest in the research as well as desire to learn more about the potential applications of our research findings.

  - Although TNBCs account for only 20% of breast cancer cases, their poor prognosis and lack of treatment options are responsible for a disproportionate number of breast cancer deaths. Therefore, our ultimate vision for the work is to identify the mechanisms that link epigenetic silencing of the BRCA-1 gene in BRCA-1 mutation carriers and women with no BRCA-1 mutation history to the development of TNBCs. Therefore, this work has broad impact affecting not only women dealing with breast cancer but also groups involved in food production, biomedical communities and society as a whole.

5. **CHANGES/PROBLEMS:** *Nothing to Report.*

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  - **Journal publications.**

Selmin OI, Papoutsis AJ, Donovan MG, Romagnolo, DF. 2016. Genistein reverses *BRCA-1* CpG methylation in human breast cancer cells. Breast Cancer Res. (In submission).

Selmin OI, Papoutsis AJ, Romagnolo DF. 2016. Reversal effects of genistein and (-)-epigallocatechin-3-gallate on repression of BRCA-1 expression in human breast cancer cells. FASEB/ASN Meetings, San Diego, CA.

Selmin OI, Papoutsis AJ, and Romagnolo DF. 2016. Reversal of BRCA-1 CpG hypermethylation by genistein and (-)-epigallocatechin-3-gallate in human breast cancer cells with activated AhR. Annual Meetings of the American Association for Cancer Research, New Orleans, LA.

- **Books or other non-periodical, one-time publications.** Nothing to report

- **Other publications, conference papers, and presentations.**

Conference participation: Nutrients beyond nutrition-5: nutrients before, during and after cancer. 2016. Conference organized by The University La Sapienza in Rome: From primary prevention to therapy and secondary prevention. October 7, 2016. Santa Margherita Ligure, Italy.

- **Website(s) or other Internet site(s)**
  https://uanews.arizona.edu/story/researchers-to-examine-soy-intake-breast-cancer-risk

  This is a web link to the University of Arizona Cancer Center. It highlights the research supported by this project and makes specific mention of the support received by this funding mechanism.

- **Technologies or techniques.** Nothing to report.

- **Inventions, patent applications, and/or licenses.** Nothing to report.

Other Products. Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Donato F Romagnolo, PhD University of Arizona	PI.
Ornella I. Selmin, PhD University of Arizona	Co-PI
Sueme Darah Lynne University of Arizona	Technician, assisting with breeding protocols and gestational treatments.
Micah Donovan	Graduate Student. Assisted with Western blotting analysis, RNA extractions, BRCA-1 promoter methylation from tumor samples.
Andreas Papoutsis, PhD	Postdoc. Assisted with preparation and submission of BRCA-1 manuscript
Tom Doetschman, PhD The University of Arizona	Collaborator on design of experiments with mouse models.
Jeff Tolson	Undergraduate student assisting with laboratory experiments.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to report.
- **What other organizations were involved as partners?** Nothing to report, all personnel was from The university of Arizona.

8. SPECIAL REPORTING REQUIREMENTS

a. **COLLABORATIVE AWARDS:** Nothing to report.

b. **QUAD CHARTS:** Nothing to report.

9. APPENDICES: Nothing to report.