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TITLE: Prevention of Breast Cancer and Therapy Resistance Using Novel Therapeutic Approaches

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CONTRACTING ORGANIZATION: University of Texas Health Science Center
San Antonio, TX 78229

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Fort Detrick, Maryland 21702-5012

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University of Texas Health Science Center at San Antonio
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14. ABSTRACT
A number of recent studies including form our group have shown that exogenous ERβ expression or its induction/activation by ERβ agonists result in blocking the growth of ERα-positive breast cancer cells. The central hypothesis of this study is that ERβ activation and/or its overexpression shifts the balance from oncogenic functions of ERα to tumor-suppressing actions of ERβ, thus preventing initiation and progression of breast cancer. In addition, ERβ activation and/or its overexpression prevent and or delay the development of resistance and restores hormonal sensitivity resistant tumors. This study is focused on testing the therapeutic efficacy of ERβ agonists in preventing and as well as treating and blocking the progression of breast cancers. Significant findings during first year funding period are: a) Using transgenic animal model we have shown both LY500307 and S-Equol reduced mammary growth and initiation of malignant changes; b) this is the first study to investigate the role of ERβ in the context of breast cancer prevention; c) ERβ agonists were able to restore sensitivity to endocrine therapy resistant cells to hormonal therapy; and d) we have identified novel signaling molecules associated with ERβ agonists mediated protection.

15. SUBJECT TERMS
Endocrine therapy resistant breast cancers; breast cancer prevention; estrogen receptor beta agonists, therapeutic efficacy of estrogen receptor beta agonists

16. SECURITY CLASSIFICATION OF: U			***17. LIMITATION OF ABSTRACT***	***18. NUMBER OF PAGES***	***19a. NAME OF RESPONSIBLE PERSON: USAMRMC***
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1. INTRODUCTION:

A number of recent studies including form our group have shown that exogenous ERβ expression or its induction/activation by ERβ agonists result in blocking the growth of ERα-positive breast cancer cells. Based on our findings, **we hypothesized** that ERβ activation and/or its overexpression shifts the balance from oncogenic functions of ERα to tumor-suppressing actions of ERβ, thus preventing initiation and progression of breast cancer. In addition, ERβ activation and/or its overexpression prevent and or delay the development of resistance and restores hormonal sensitivity of resistant tumors. This study is focused on testing the therapeutic efficacy of ERβ agonists in preventing and as well as treating and blocking the progression of breast cancers.

2. KEYWORDS:

Endocrine therapy resistant breast cancers; breast cancer prevention; estrogen receptor beta agonists, therapeutic efficacy of estrogen receptor beta agonists;

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

There are no changes in project direction or in the scope of Scientific work. *Before the completion of the third year an approval for a no-cost extension till the end of June 2020 (06/30/2020) was obtained from DOD. This progress report covers the work carried out during the year 3 of the funding. Final progress report will be submitted after completion of the no-cost extension period*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

During the Third-year funding period we have studied the following major goals (tasks):

Major goal (task) 1: Determine efficacy of ERβ agonists to prevent breast cancer and to prevent or delay the development hormonal resistance.

Subtask 1 (1-24 months): Establish that ERβ agonists prevent the incidence of breast cancer in genetically engineered animal models.

Subtask 2 (1-24 months): Establish that ERβ agonists block progression and recurrence of breast cancer immune-competent tumor models?

Subtask 3 (1-24 months): Does the antitumor activity of ERβ agonists depend on tumor-intrinsic and extrinsic ERβ.

Major goal (task) 2: Determine the efficacy of ERβ agonists to prevent or delay development of resistance in endocrine therapy naïve breast tumors.

Subtask 1 (1-12 months): Test whether ERβ agonists restore sensitivity to AE/AI endocrine therapy using various endocrine therapy sensitive and resistant cells.

Subtask 2 (1-36 months): Test whether ERβ agonists affect the growth of endocrine therapy naïve or AE/AI-resistant recurring breast tumors.

Subtask 1 (1-36 months): Test whether ERβ agonists overcome therapeutic resistance in PDX models.

Major goal (task) 3: Elucidate how ERβ signaling network influences the role of ERβ agonists in preventing and overcoming hormonal resistance.

Sub task 1 (1-24 months): Establish the role of the phosphotyrosine switch involved in the endocrine-sensitizing activity of ERβ

Sub task 2 (13-36 months): Determine the mechanisms by which ERβ agonists block progression of ERα-positive breast cancer.

subtask 3 (1-24 months): Establish the mechanisms of prevention/tumor suppressive functions of ERβ

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. Major Activities:

During the third-year funding period we have continued work of ongoing sub-tasks and initiated the work of new sub-tasks as stated in Statement of Work

- Initiated experimental work (Major tasks) as indicated in statement of work is shown below:

    1. Established the effect of ERβ agonists in the blocking/delaying the tumor incidence in genetically engineered mice models.
    2. Determined the role of phosphotyrosine switch on the tumor suppressor activity of ERβ
    3. Tested whether ERβ agonists affect the growth of ER positive breast PDX models

4. Identified mechanistic pathways that contributes to tumor suppressive actions of ERβ in human hormone therapy sensitive and resistant models.

2. Specific Objectives:

Objective 1: Establish that ERβ agonists prevent the incidence of breast cancer in genetically engineered animal models (***Major Task 1-Subtask 1:1-24 months***).

Objective 2: Establish the role of phosphotyrosine switch involved in the endocrine-sensitizing activity of ERβ **(*Major Task 3 - Subtask 1: 1-12 months).***

Objective 3: Test the effects of ERβ agonists effect on the growth of ER positive breast PDX models (***Major Task 2 - Subtask 3: 13-36 months).***

Objective 4: Determine the mechanisms that play a role in the tumor suppressive functions of ERβ using various endocrine therapy sensitive and resistant cells **(*Major Task 3-Subtask 2:13-36)***.

3. Significant Results (outcomes):

Results for objective 1: *Establish that ERβ agonists prevent or delay the incidence of breast cancer in genetically engineered animal models.* To test the therapeutic efficacy of ERβ agonists to prevent the initiation of breast cancer, our studies during the previous years focused on MMTV-HER2/neu model and examined mammary growth (ductal elongation and ductal branching) for tumor initiating events in prepubertal age (8 weeks) mice with reach 4 months of continuous treatment. During the third-year, we extended this study with other group of mice using matured animals (6 months) and determining the change in the tumor incidence until they are morbid. As shown in figure 1, untreated or letrozole treated mice develop tumors by 24 weeks. Once tumor formation takes place, HER-2/neu mammary tumors grow very rapid. Treatment with either ERβ agonist LY50037 (LY) alone or in combination with letrozole the tumor incidence was delayed until 32 and 34 months respectively. Similar results were observed with another ERβ agonist, S-equol. These observations suggest that ERβ agonists are able to delay the incidence of tumor formation significantly in this model we tested. Studies with other transgenic models are in progress.

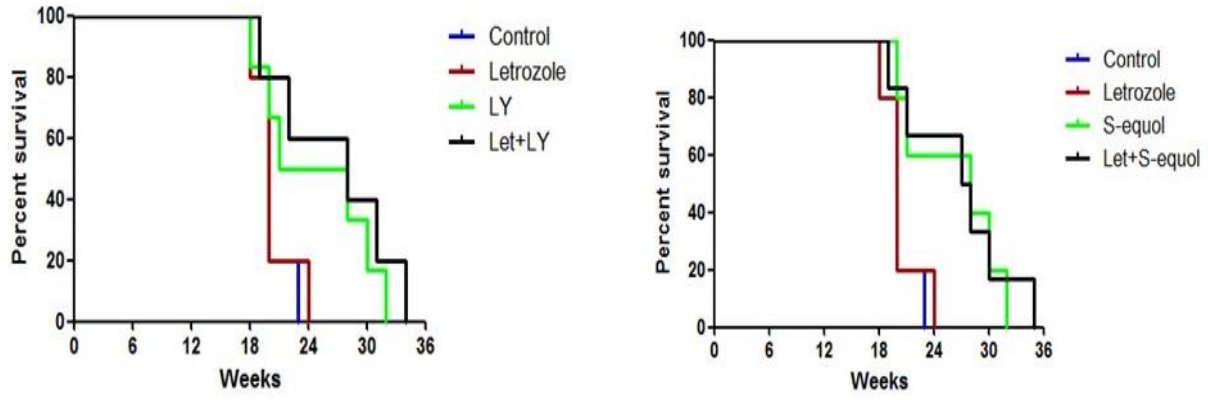


Figure 1. *Effect of ERβ agonists on tumor incidence in HER-2/neu transgenic mice. LY500307 (left panel) and S-equol (right panel) were effective in delaying the formation mammary tumors.*

Results for Objective 2: *Establish the role of phosphotyrosine switch involved in the endocrine-sensitizing activity of ERβ*

To establish the role of posttranslational modifications such as phosphorylation of ERβ specifically phosphotyrosine switch involved in the therapeutic response, we have knocked down the endogenous ERβ employ crisper-mediated methodology. We have established several clones tested the change in protein levels. We have confirmed the deletion of ERβ using nucleotide sequence determination (previously reported). As shown in figure 2 confirms the lack of wild-type ERβ expression and knock-in of muted ER expression proteins one that constitutively expresses phosphorylated form of ERβ (ERβ-36Y-E; tyrosine to glutamate) and other one without non-phosphorylation form (ERβ-36Y-F;tyorosine to phenylalanine) We have verified the effect of presence and absence of ERβ wild-type (ERβ-WT) and knock-down (ERβ-KD) form of ERβ on the growth of both hormone -therapy responsive (MCF7-Aro) and resistant cells (LTLT). As shown in figure 3, unlike breast cancer cells that express ERβ-WT, cells that lack this receptor expression (ERβ-KD) promotes the growth of these cells both in regular culture and colony formation assay. We next examined how these cells respond to ERβ agonist (S-equol). As shown in figure 4, the growth of MCF7-Aro cells with that express ERβ-WT is significantly affected its growth in dose dependent fashion compared to ERβ-KD cells.

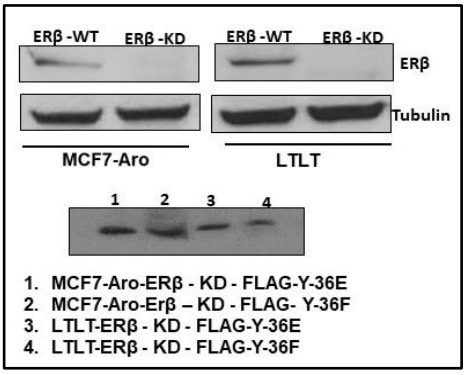


Figure 2: Demonstration of lack of ERβ expression: After knocking down ERβ expression using CRISPR approach, modified ERβ proteins were expressed to test the importance of phosphotyrosine switch

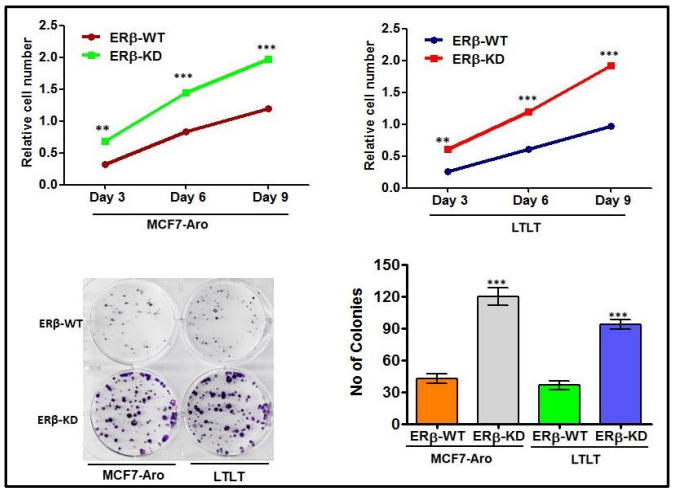


Figure 3: Lack of ERβ (knockdown) affects the growth of breast cancer cells in culture and colony formation

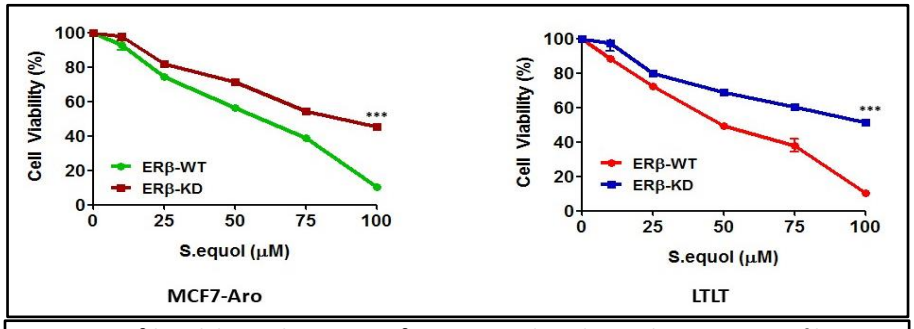


Figure 41: ERβ knockdown abrogates ERβ agonist-mediated growth suppression of breast cancer cells.

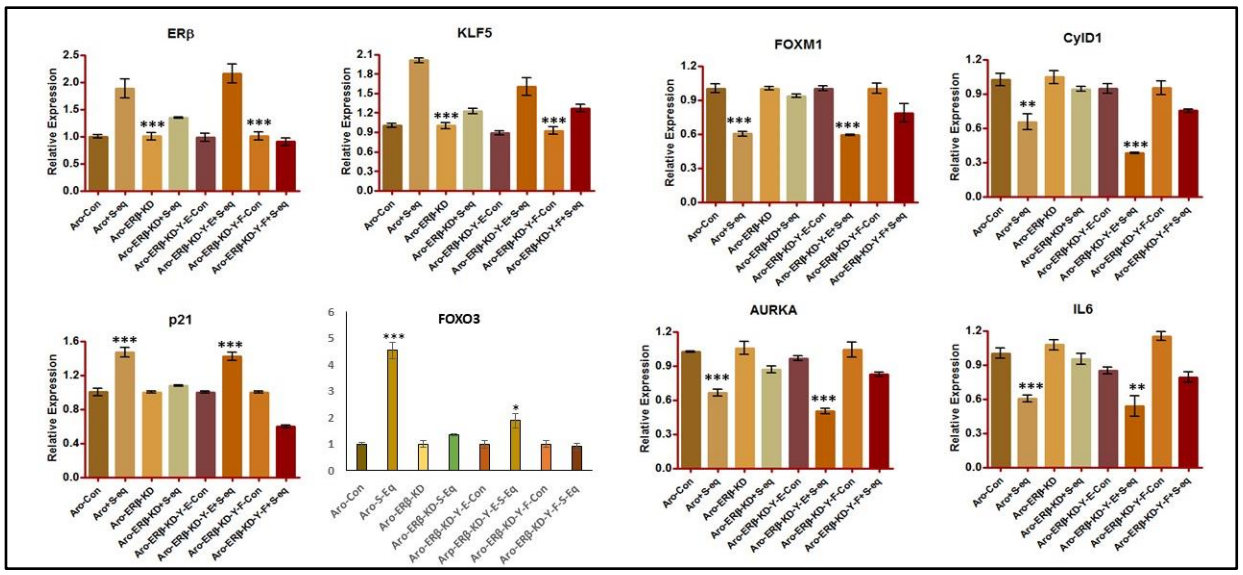


Figure 5: Constitutive activation of ERβ (phosphorylation) affects the regulation of a number of ERβ dependent genes in the presence of its agonist S-equol.

Using MCF7-Aro and LTLT-cells with mutant ERβ expression, we have examined the importance of ERβ phosphotyrosine switch on the growth as well change in the genes involved in tumor suppressive role of ERβ. As shown in figure 5, like in control MCF-7-Aro cells, MCF7-Aro-ERβ-36Y-E cells (that constitutively expressing phosphorated form of ERβ) respond well to ERβ agonist-mediated induction of ERβ expression and also affect the regulation of number of genes that are up regulated (agonist induced ERβ, KLF-5, p21 and FOXO3) or down regulated (FOXM1, cyclin D1, AURKA, IL-6) by the ERβ-WT or ERβ-36Y-E. These studies conclude that ERβ tumor suppressive functions are mediated by phosphorylated form of ERβ and in the absence of this phosphorylation switch tumor suppressive function of ERβ are significantly diminished.

Results for Objective 3: *Testing the effects of ERβ agonists effect on the growth of ER positive breast PDX models*

To evaluate the antitumor activity of ERβ agonists, in a biologically relevant *in vivo* models, we used three different PDX models. Mice were engrafted with an ER+ PDX, COHSC-31 (kind gift from Dr. Chen, City of Hope) and other two obtained from Jackson laboratory. Both the tumors were ER+ tumor models in which estrogen-mediated ER. activation became the major driving force of growth. The third PDX model (TM 00107) (Jackson laboratory) was ERα+ve when tested using primary tumor sample, but lost ERα+ve, but still expresses ERβ In brief, surgically resected tumors were implanted subcutaneously in 6-week-old female NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, NSG (The Jackson Laboratory, CA), under isoflurane anesthesia. Two days before tumor inoculation, a 17β-estradiol pellet was subcutaneously implanted into the dorsal flank to support establishment of ER-positive tumors. Tumor tissues were extracted and cultured on gelatin sponges in the absence or presence of ERβ agonists, letrozole alone or in combination. A representative data (Fig.6) is shown with PDX models COHSC-31 and TM00107 using LY50037, letrozole alone or in combination with both. Compared control, LY decreased the proliferation (as determined using Ki 67 staining pattern) of PDX tumors

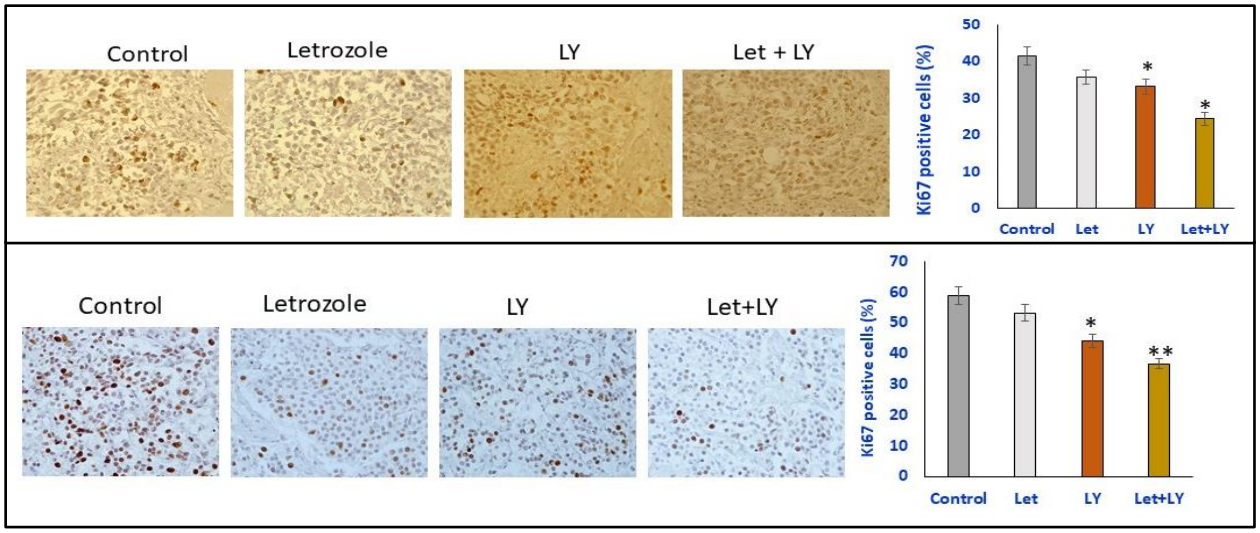


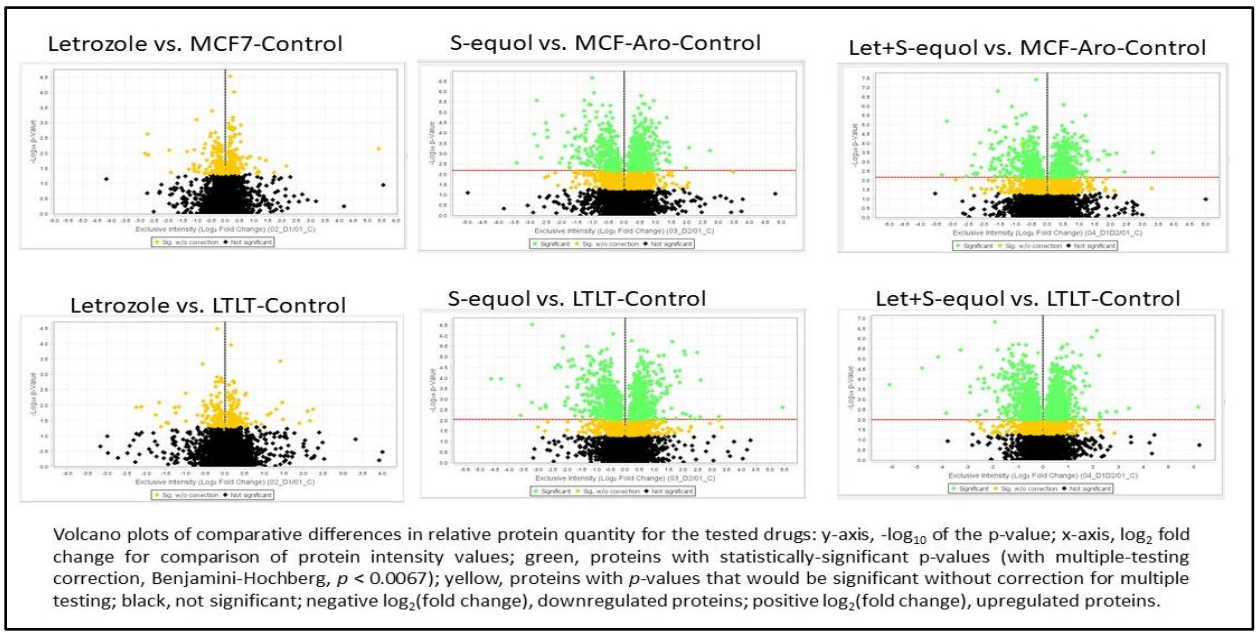
Figure 6: Effect of ERβ agonists on the growth PDX tumors. PDX model tumor COHSC-31 (top panel). PDX model TM00107 (lower panel). ERβ agonist, LY500307 (LY) was very effective in blocking the proliferation of PDX tumor alone or in combination with aromatase inhibitor letrozole (Let).

and effect was highly significant when combined with letrozole. We have seen similar effect with two other ERβ agonists S-equol and liquiritigenin (data not shown).
Data presented here shows like in established epithelial breast cancer cell models, ERβ agonists were very effective in blocking the growth of PDX tumor which retains full components of all cell types (histological representation) and behaves like primary tumor. These observations also provide very valuable new findings that ERβ agonists when used alone or in combination with aromatase inhibitors should be very effective in blocking breast tumor growth.

Results for Objective 4: *Determine the mechanisms that play a role in the tumor suppressive functions of ERβ using various endocrine therapy sensitive and resistant cells*

To study what mechanisms regulates the tumor suppressive functions of ERβ, we have used both genomic and proteomic approaches. During the previous year, we have reported the findings using genomic approach. During this year we have focused on what are the different pathways are affected in response to ERβ and its agonist-mediated actions. Both hormone therapy sensitive (MCF7-Aro) and resistant (LTLT) cells were treated with vehicle (DMSO), Letrozole (1 µM) and S-equol (75 µM) and in combination for 48 hrs. After the treatment, cells were pelleted, snap-frozen, lysed in 5% SDS in 50 mM TEAB (in the presence of protease/phosphatase inhibitors and a nuclease preparation to degrade DNA) and applied to S-Traps (mini; Protifi) for reduction/alkylation, tryptic digestion and cleanup, starting with 100 µg of protein. Protein concentrations were determined by EZQ Protein Quantitation kit (Thermo Fisher) and peptides by Pierce Quantitative Fluorometric Peptide Assay (Thermo Fisher). A pool was made of all of the samples, and 2-µg peptide aliquots were analyzed by HPLC-electrospray ionization data-independent mass spectrometry (HPLC-ESI-DIA-MS) on a Thermo Fisher Orbitrap Fusion Lumos using gas-phase fractionation and 4-m/z windows (120k resolution for

precursor scans, 30k for product ion scans, all in the orbitrap) to create a DIA chromatogram library by searching against a panhuman spectral library (doi: 10.1038/sdata.2014.31). Experimental samples were blocked by replicate and randomized within each replicate. Injections of 2 µg of peptides and a two-hour HPLC



Volcano plots of comparative differences in relative protein quantity for the tested drugs: y-axis, $-\log_{10}$ of the p-value; x-axis, $\log_2$ fold change for comparison of protein intensity values; green, proteins with statistically-significant p-values (with multiple-testing correction, Benjamini-Hochberg, $p < 0.0067$); yellow, proteins with p-values that would be significant without correction for multiple testing; black, not significant; negative $\log_2$(fold change), downregulated proteins; positive $\log_2$(fold change), upregulated proteins.

gradient were employed. MS data were acquired in the orbitrap using 12-m/z windows (staggered; 120k resolution for precursor scans, 30k for product ion scans) and searched against the chromatogram library. Scaffold DIA (v1.3.1; Proteome Software) was used for all DIA data processing. As shown in figure 7 levels of a number of proteins (2-fold increase or decrease) in response to ERβ agonist treatment alone or in combination with letrozole both in hormone sensitive (MCF7-Aro) or letrozole resistant cells (LTLT). Pattern of changes suggests ERβ agonist affects a different set of proteins that changes the proliferation of hormone sensitive breast cancers or resensitizes letrozole resistant cells so they respond to ERβ agonist therapy and results in decreased proliferation.

Based on pathway specific analyses ERβ agonist S-equol in combination with letrozole modulates a number of pathways both in MCF7-Aro (hormone sensitive) and letrozole resistant (LTLT) cells as showed by Reactome pathway analysis.

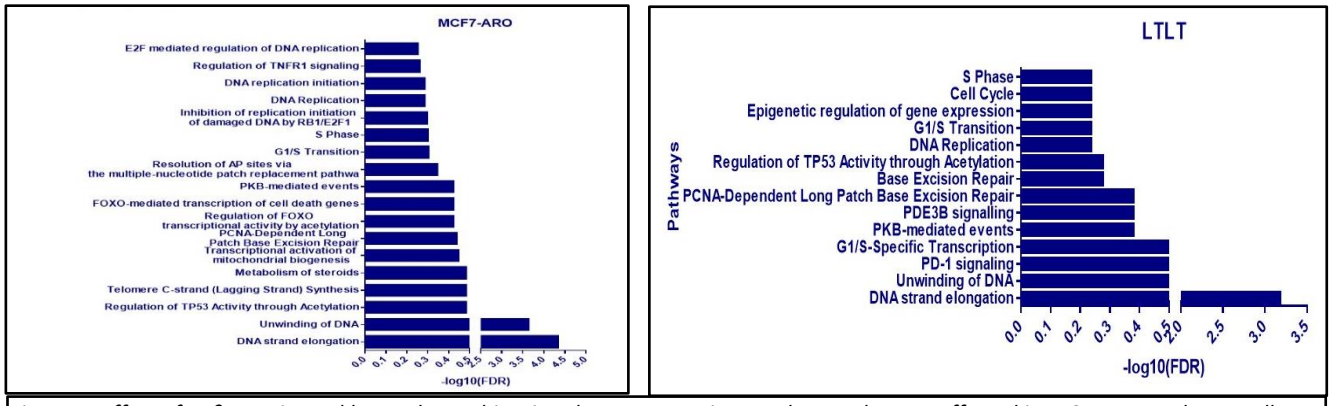


Figure 7: Effect of ERβ agonist and letrozole combination therapy on various pathways that are affected in MCF7-Aro and LTLT cells.

These findings suggest ERβ agonists affects the regulation of ERβ-dependent actions by regulating different pathways both in hormone therapy sensitive and resistant breast cancer cells. Once we validate proteins that are affected in various pathways using both biochemical and molecular approaches, the identified biomarkers will help to design specific therapeutic approaches and evaluate therapeutic efficacy using identified biomarkers.

Major Findings/Conclusions:

- Tumor suppressive function of ERβ/its agonists mediated actions. ERβ agonists are able to block/delay the tumor incidence in genetically engineered mice models.

- Activated ERβ (phosphorylated form of ERβ) is critical for its action that are critical for its tumor suppressive effects in both hormone therapy sensitive and resistant tumors.
- ERβ agonists are able to exert tumor suppressive functions in both ERα+ve and -ve breast tumors as determined using breast PDX models

- We have identified novel signaling molecular pathways associated with ERβ agonists mediated tumor suppression in hormone therapy sensitive and resistant breast cancers using Reactome pathway analyses (proteomic approaches).

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

During the **third year**, this project provided training opportunities for 4 students (1 medical and 3 high school) and two post-doctoral fellows (one MD and one Ph.D. fellow). All the students/fellows were trained in conducting oncology research using BC model cells, preclinical animal models, designing/analyzing research experiments and interpreting the data. In addition, Post-doctoral fellow was given an opportunity to train students, this provided an opportunity to sharpen his mentoring skills. Postdoctoral fellow was provided an opportunity to serve as mentor of rotating and high school students. Every week, PI spent an hour of assigned time mentoring about their research project, interpreting their results and discussing their career development plans. All students participated in weekly project meetings and presented their research progress. In addition, students/fellows were provided several professional development opportunities including participation in journal clubs, participation in UTH cancer center workshops, and attendance of grand rounds, and attendance of ethics seminar series. Further, postdoctoral fellow attended 2018 AACR-SABCS and 2019 AACR annual meeting to present our research findings.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Findings was presented to scientific community at 2018 San Antonio Breast Cancer Symposium (international meeting attended by ~7500 from over 80 countries) and 2019 American Association of Cancer Research (AACR)

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We are planning to present our findings at both national and international meeting at least once a year. We also are in the process of preparing manuscript to be published in a peer reviewed scientific journals and complete remaining experiments and data analyses.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

These studies provided evidence for therapeutic efficacy of estrogen receptor-β agonists in the prevention of breast cancer and to restore the sensitivity to endocrine therapy resistant breast tumors to hormone therapy in preclinical models as well as in PDX models. Once completed these studies will form a basis for clinical trial since the compounds tested are being explored for clinical use for other indications.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The results of this study provided a rationale to examine the efficacy of ERβ agonists blocking the growth of cervical, endometrial and ovarian cancers. Gynecological Oncology faculty and Ob-gyn residents are engaged in these studies at our institute. We are pursuing independent funding to expand these observations in both ovarian and endometrial cancers. Involvement of Gyn-Oncology faculty helped to demonstrate the scope of research areas available to train Gyn-Oncology fellows through newly ASGME approved fellowship program in PI's department.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Some of these findings helped to new funding to develop and test novel more potent ERβ agonist supported by Texas Cancer Prevention and Research Institute funds.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes.
Remember that significant changes in objectives and scope require prior approval of the agency.

No changes and there is nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

It took more time to develop ERβ knock in cell clones to test the phosphotyrosine switch involved in the endocrine-sensitizing activity of ERβ. We have now made needed cell clones and the work is in progress (Major goal 3; subtask 1).

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No changes to report

Significant changes in use or care of vertebrate animals

No changes to report

Significant changes in use of biohazards and/or select agents

No changes to report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Two manuscripts are being submitted to report findings to scientific community based on studies with estrogen receptor beta ligand.

- Samayoa, C., Ramasamy, K., Kota, A., Ganapathy, Krishnegowda, N.K., Vadlamudi, R.K., and **Tekmal, R.R**.: Liquiritigenin with dual properties (as estrogen Receptor β agonist and as an aromatase inhibitor) inhibits the growth of therapy-sensitive and therapy-resistant breast cancer cells. Breast Cancer Research and Treatment (in submission).

- Ramasamy, K., Samayoa, C., Chen, S., Krishnegowda, N.K., Vadlamudi, R.K., and **Tekmal, R.R**.: Estrogen receptor β agonists inhibits growth and progression of mammary tumors in immunocompetent mice by altering the mechanisms involved in cell cycle and other pathways. Breast Cancer Research (in submission)

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or*

dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

- Ramasamy K, Samayoa C, Krishnegowda NK, Thurlapati, A, Vadlamudi RK, **Tekmal RR.**: Estrogen receptor β agonists inhibits syngeneic mammary tumor growth through cell-cycle arrest by modulating cell-cycle regulators 41st Annual San Antonio Breast Cancer Symposium, P1-04-01, 2018.

- Ramasamy, K., Samayoa, C., Chen, S., Li, R., Vadlamudi, R.K., Tekmal, R.R.: S-Equol inhibits breast cancer growth by regulating phosphorylation status of estrogen receptor β. Proc. Am. Assn. Cancer Res., 110th Annual Meeting, Abstr. 1008, 2019

Abstract is accepted for presentation 42nd Annual San Antonio Breast Cancer Symposium to be held during December 2019

Website(s) or other Internet site(s)

*List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section***.**

None

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

None

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a

meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Modified cell lines and in vitro as well as in vivo models will be available once the ongoing studies are completed to scientific community

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	**Rajeshwar Rao Tekmal, Ph.D**.
Project Role:	**Principal Investigator**
Researcher Identifier (e.g. ORCID ID):	UTHSCSA Faculty, Department of Ob-Gyn
Nearest person month worked:	3.6 months (30% paid effort on this project)
Contribution to Project:	Dr. Tekmal was involved in planning and execution of all research aspects and as well scientific and financial management of this grant
Funding Support:	Partially funded by USAMRC grant (BCRP151884; this grant), other grants and UTHSCSA funds.

Name:	**Ratna K. Vadlamudi, Ph.D.**
Project Role:	**Co-Investigator**
Researcher Identifier (e.g. ORCID ID):	UTHSCSA Faculty, Department of Ob-Gyn
Nearest person month worked:	0.48 months (4% paid effort on this project)
Contribution to Project:	Dr. Vadlamudi was involved in planning and analysis of biomarker studies that examined molecular pathways
Funding Support:	Partially funded by USAMRC grant (BCRP151884; this grant), other grants and UTHSCSA funds.

Name:	**Rong Li, Ph.D*.**
Project Role:	**Co-Investigator**

Researcher Identifier (e.g. ORCID ID): UTHSCSA Faculty, Dept. of Molecular Medicine
Nearest person month worked: 0.36 months (3% paid effort on this project)

Contribution to Project: Dr. Li was involved in planning and analysis of ERβ phsoprorylation using ERβ knock in and knock out models.
Rong Li, Ph.D. Separated few months back from UTHSCSA.* ***He is no longer is spending paid effort on this project during no-cost extension period**. He will serve as collaborator. Moreover, experiments involving his expertise (phosphotyrosine switch) have already been completed. His separation will not have any impact on the project.*

Funding Support: Partially funded by USAMRC grant (BCRP151884; this grant), other grants and UTHSCSA funds.

Name: **Kumaraguruparan Ramasamy, Ph.D.**
Project Role: **Postdoctoral Fellow**
Researcher Identifier (e.g. ORCID ID): UTHSCSA Staff, Department of Ob-Gyn
Nearest person month worked: 12 months (100% paid effort))

Contribution to Project: Dr. Ramasamy has performed all molecular and cell culture work and assisted with animal studies
Funding Support: USAMRC funded grant (BCRP151884; this grant)

Name: **Shaorong Chen, Ph.D.**
Project Role: **Research Associate**
Researcher Identifier (e.g. ORCID ID): UTHSCSA Staff, Department of Ob-Gyn
Nearest person month worked: 11 months

Contribution to Project: Dr. Chen is responsible for maintenance and generation of all required animals from animal models and carrying out animal work.
Funding Support: USAMRC funded grant (BCRP151884; this grant)

As there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

No change

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

See the attached copy of the abstracts presented at national and international meetings.

Estrogen receptor β agonists inhibits syngeneic mammary tumor growth through cell-cycle arrest by modulating cell-cycle regulators

Ramasamy K, Samayoa C, Krishnegowda NK, Thurlapati A, Vadlamudi RK, Tekmal RR UT Health San Antonio, San Antonio, TX

Breast Cancer is the main cause of cancer-associated mortality in women worldwide. The estrogen receptors (ER's) play an important role in normal mammary gland development, as well as in breast cancer. Estrogen Receptor α is expressed in 70% of breast cancers, where it contributes to increased cell proliferation and decreased cell death. Endocrine therapies such as anti-estrogens and aromatase inhibitors target ERα signaling and improve outcomes of these patients. Syngeneic, immunocompetent mouse models are essential for elucidating the mechanisms and for evaluating novel strategies for the treatment of breast cancer. In contrast to the tumor-inducing role of ERα, ERβ has been shown to have tumor suppressive activities in various cancer, including the breast cancer. Compounds that selectively activate ERβ hold promise because they could potentially avoid the unwanted effects of ERα activation, while exploiting the tumor-suppressive function of ERβ. In the present study, we assessed the antitumor effects of ERβ agonists using three different syngeneic mouse models; D2A1 (BALB/c) and MM51 (FVB) syngeneic models and *ex-vivo* culture of highly metastatic cell line E0771 (C57/B6). Effect on *in vitro* cancer cell growth was evaluated by cell proliferation and clonogenic assays. Cell cycle distribution was analysed by flow cytometry. Our results demonstrate that ERβ agonists LY500307 and S-Equol not only inhibited the growth of all three mouse mammary tumor cell lines, but also reduced the colony formation ability. ERβ agonists also induced the cell-cycle arrest in time and dose-dependent manner. In mechanistic studies, ERβ agonists LY500307 and S-Equol, modulated the protein levels of cyclin-dependent kinases (CDKs) (4, 6, and 2), cyclins (D1 and E), in a differential manner in these three cell lines. Our *in vivo* studies of D2A1 and MM51 cells demonstrates that ERβ agonist LY500307 inhibited the tumor growth and the effect was more pronounce in combination with aromatase inhibitor letrozole. Ex-vivo model of E0771 cells showed that LY500307 has potential to dramatically reduce the proliferation of mouse mammary tumor growth.Together, these results identify potential molecular targets and anticancer effects of ERβ agonists in mouse mammary tumors.

Session: Poster Session 1: Tumor cell and molecular biology: Cell cycle regulation (5:00 PM-7:00 PM)
Date/Time: Wednesday, December 5, 2018 - 5:00 pm
Room: Hall 1

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AACR American Association for Cancer Research ANNUAL MEETING 2019 ATLANTA
MARCH 29-APRIL 3 GEORGIA WORLD CONFERENCE CENTER

Session PO.EN01.01 - Endocrine-related Cancers

Add To My Itinerary

1008 / 9 - S-Equol inhibits breast cancer growth by regulating phosphorylation status of estrogen receptor β

April 1, 2019, 8:00 AM - 12:00 PM Section 2

Presenter/Authors

Kumaraguruparan Ramasamy, Cathy Samayoa, Shaorong Chen, Rong Li, Ratna K. Vadlamudi, Rajeshwar R. Tekmal. UT Health San Antonio, San Antonio, TX

Disclosures

K. Ramasamy: None. **C. Samayoa:** None. **S. Chen:** None. **R. Li:** None. **R.K. Vadlamudi:** None. **R.R. Tekmal:** None.

Abstract

Background: Breast cancer is the most common malignancy in females and second most common cause of cancer related mortality in women. Since 70% of all breast cancers are estrogen receptor-positive (ER+ve), endocrine therapy such as anti-estrogens or aromatase inhibitors, targeting the estrogen receptor (ER) pathway is the most common treatment used for ER+ve breast cancers. However, patients will develop de novo or acquired resistance to therapy, leads to tumor progression, and metastasis. It is well documented that ERβ functions as tumor suppressor in different cancers including breast cancer. We recently showed that phosphorylation status of ERβ is important for its antitumor activity. However, little is known about the role of ERβ phosphorylation status in hormone therapy and resistance; therefore, we investigated whether phosphorylation status of ERβ has role in overcoming hormone therapy resistance in ER+ve breast cancers.

Experimental design: To elucidate the importance of phosphorylation status of ERβ, we used CRISPR-Cas9 system to knockout ERβ in MCF7-Aro (therapy-sensitive) and Letrozole resistant (MCF7aro-LTLT) cells. Several mutant clones were identified for both MCF7-Aro cells and LTLT cells and the depletion of ERβ protein in both cell clones was confirmed by immunoblotting. The parental and knockout cells with or without treatment of S-equol were analyzed for cell proliferation, protein (Western) and RNA (RT-qPCR) analysis.

Results: First we analyzed the cell proliferation in parental (MCF7 Aro and LTLT) and ERβ knock out cells. The proliferation rate is increased in the ERβ knockout cells compared to the parental cells. Treatment with ERβ agonist S-Equol to the parental cells inhibited the cell proliferation whereas in the knock out cells, the effect of S-equol is compromised. RNA-seq analysis of S-equol treated parental cells showed the downregulation of ERβ target genes involved in tumor progression and resistance to hormone therapies. In contrast, compared to parental cells, ERβ knock out cells showed diverse effects to S-equol treatment. RT-qPCR analysis revealed that S-Equol could not modulate the ERβ-target genes in ERβ knock out cells compared to parental cells.

Conclusions: Our findings provide evidence that phosphorylation status of ERβ is important for elucidating its antitumor activity in therapy-resistant cells. The differential effects of S-equol on parental and ERβ knockout cells suggest that the antiproliferative action of S-equol is partly mediated by ERβ. We believe that our ongoing studies may further validate the role of phosphorylation status of ERβ by using both ERβ agonists and phosphorylation-regulating compounds in both therapy sensitive and resistant cells.