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

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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

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

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 work

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 Second 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 second 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 preventive role of ERβ agonists in the blocking the growth of breast cancer using immune-competent animal models

    2. Identified mechanistic pathways that contributes to tumor suppressive actions of ERβ in murine models.

3. Elucidated how ERβ signaling network influences the role of ERβ agonists in blocking the proliferation hormone sensitive and breast cancer cells.

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 that ERβ agonists block the growth of breast cancer cells from immune-component tumor models (***Major Task 1-Subtask 2: 1-24 months).***

Objective 3: Determine the mechanisms that play a role in the tumor suppressive functions of ERβ using various endocrine therapy sensitive and resistant cells **(*Major Task 2-Subtask 2: 1-36 months; Major Task 3-Subtask 2:13-36)***.

Objective 4: Establish the role of phoshotyrosine switch involved in the endocrine-sensitizing activity of ERβ **(*Major Task 3 - Subtask 1: 1-12 months).***

3. Significant Results (outcomes):

Results for objective 1: *Establish that ERβ agonists prevent 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 first year studies 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. In the current year, we are continuing with a different group of mice using matured animals (6 months) and determining the change in the tumor incidence until they are 16 months of age, where majority of transgenic animals develop tumors and are morbid. We are in the final stages of determining tumor incidence. Results from this study will help to demonstrate the efficacy of ERβ agonists to prevent/decrease the tumor incidence.

Results for objective 2: *Establish that ERβ agonists block the growth of breast cancer cells from immune-component tumor models.* To test the therapeutic efficacy of ERβ agonists to block the tumor progression using immune-component breast cancer tumor models. During second year, we have used the D2A1 (BALB/c), MM51 (FVB) and E0771 (C57/B6) and established the dose-dependent effect of blocking cell growth. And as well as using (IC50) that blocks at least 50% or more we have also determined how both ERβ agonists (LY500307 and S-equol) were effective in blocking the growth alone or in the presence of combination of aromatase inhibitor (AI), letrozole that depletes intracellular production of estrogen.

As shown in figure 1, ERβ agonists (LY500307 and S-equol) were effective in blocking the cell growth in dose-dependent manner. Using effective dose (IC50) that is able to inhibit the cell growth by at least 50% in *in vitro* cultures, we tested the effect of both compounds on colony formation employing all three-tumor models. We have also

tested the effect of both these compounds alone or in combination with AI to block the growth in in vivo like conditions (colony formation).

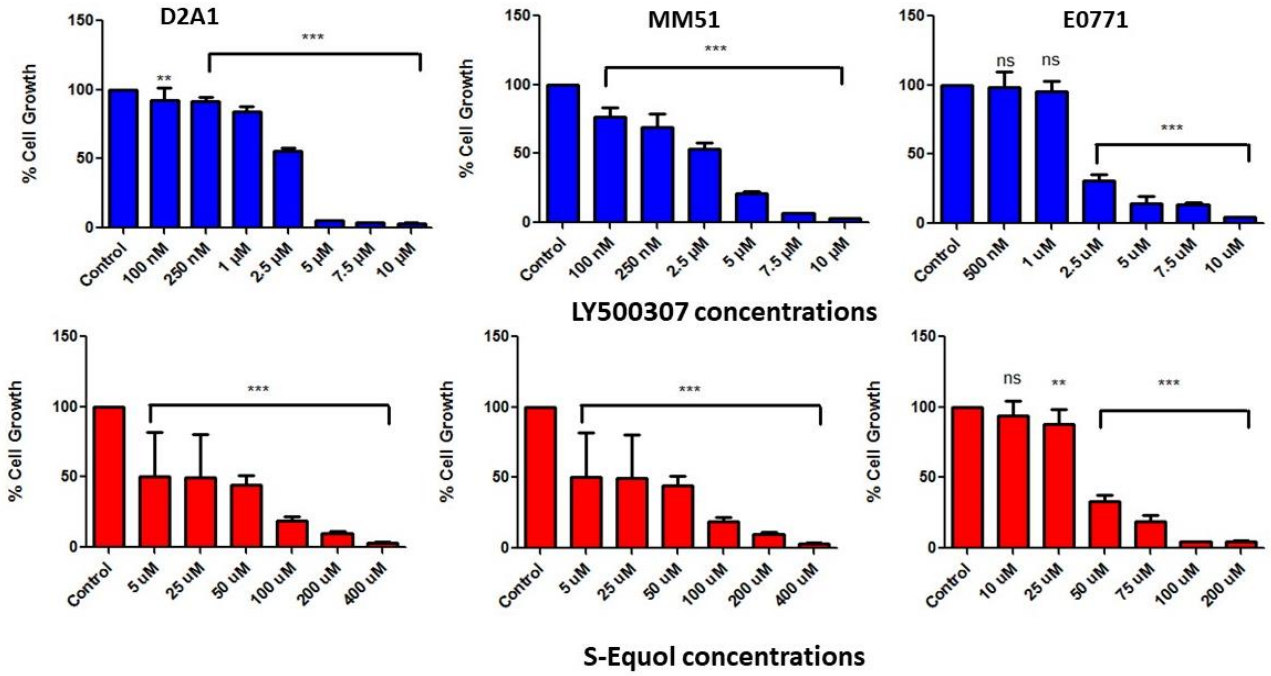


Figure 1. *Effect of ERβ agonists on in vitro growth of mammary cancer cells. LY500307 and S-Equol were effective in blocking the growth in dose dependent manner (p<0.01- p<0.001) in all three tumor models.*

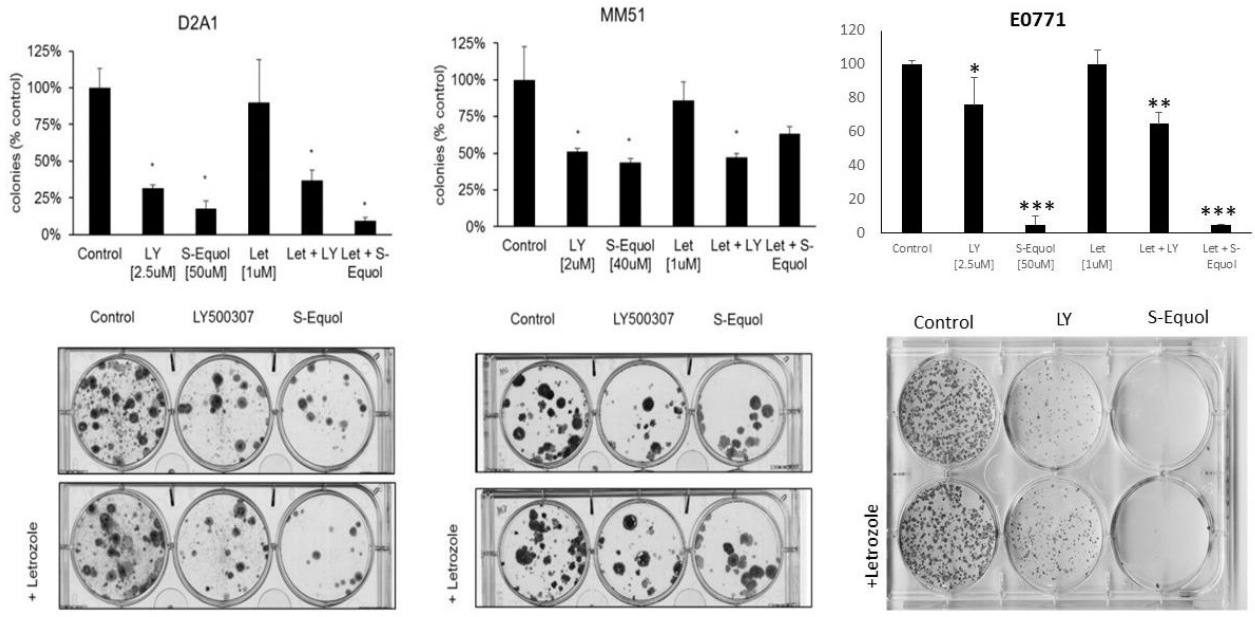
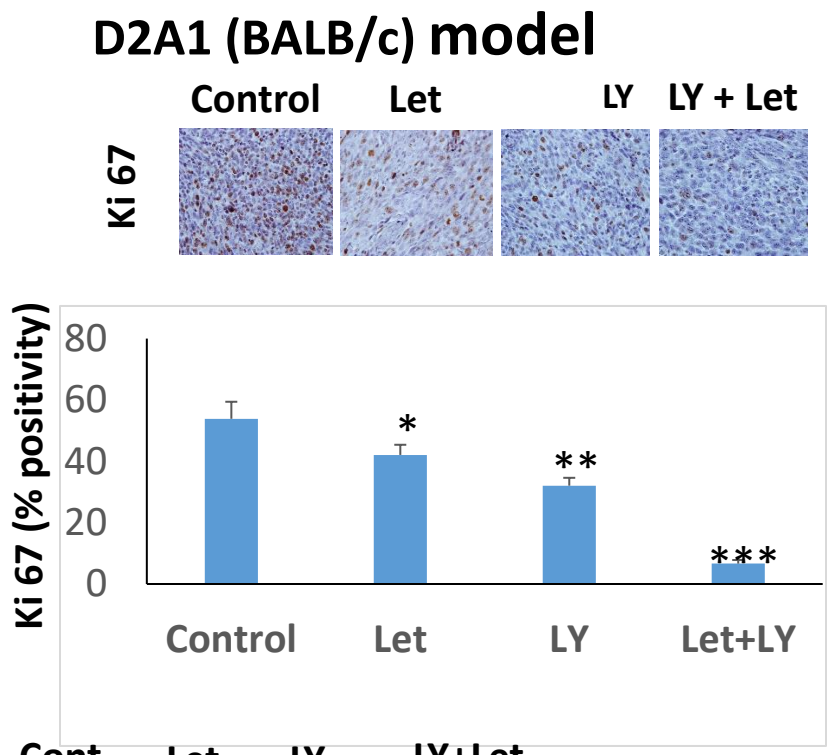


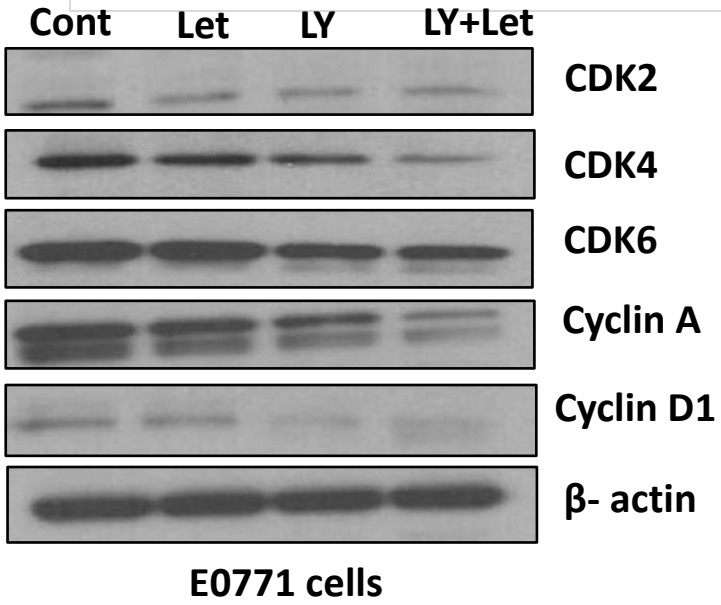
Figure 2. *Effect of ERβ agonists on colony formation of mammary tumor cells. LY500307 and S-Equol were effective in blocking the colony formation (p<0.01). Unlike these compounds alone, the effect of blocking colony formation was even more effective in combination with Letrozole (Let) p<0.001) in all three tumor models*

As shown in figure 2, ERβ agonists (LY500307 and S-equol) were effective in blocking the colony formation. Unlike ERβ agonists alone, these compounds in combination with AI were even more effective in blocking the formation of colony formation. In addition, the response appears to vary based on their genetic background. These observations suggest importance of combination therapy in blocking or preventing the tumor progression.



Using xenograft tumors (as reported during the first year) from immune competent models, we have examined the proliferative or antiproliferative effect of ERβ agonists using Ki 67 index. Ki67 immuno-staining directly correlates with the proliferative status of cells.

Figure 3. *Effect of ERβ agonists on proliferative index in mammary tumors immune-competent models. Unlike ERβ agonists alone, combination therapy with AI was very effective in blocking the proliferation (p<0.001). Inset represents immunostaining pattern of Ki67. Percent positivity was used for quantitative analysis (graph).*



Similar results were obtained with both MM51 (FVB) and E0771 tumors (data not shown).

As shown in figure 1, ERβ agonists (LY500307 and S-Equol) significantly affected the growth mammary cancer cells. Using protein from these treatment groups, we have determined how they affected cell cycle changes. Data presented in figure 4 shows that ERβ agonist LY alter the regulation of several genes involved in cell cycle.

Figure 4. *Effect of ERβ agonists on cell cycle proteins in EM0771 mammary tumor cells*

Results for objective 3: *Determine the mechanisms that play a role in the tumor suppressive functions of ERβ using various endocrine therapy sensitive and resistant cells*

To examine the molecular mechanisms that are involved in the blocking the growth of breast cancer cells in response to ERβ agonists, we used Total RNA and analyzed using microarray analysis. Using computational analysis, we have determined changes in the expression of various genes whose expression either significantly increased or decreased during ERβ agonists-mediated blocking of growth in breast cancer cells that sensitive to letrozole (MCF-7 Aro) or resistant letrozole (MCF-7 Aro, LTLT).

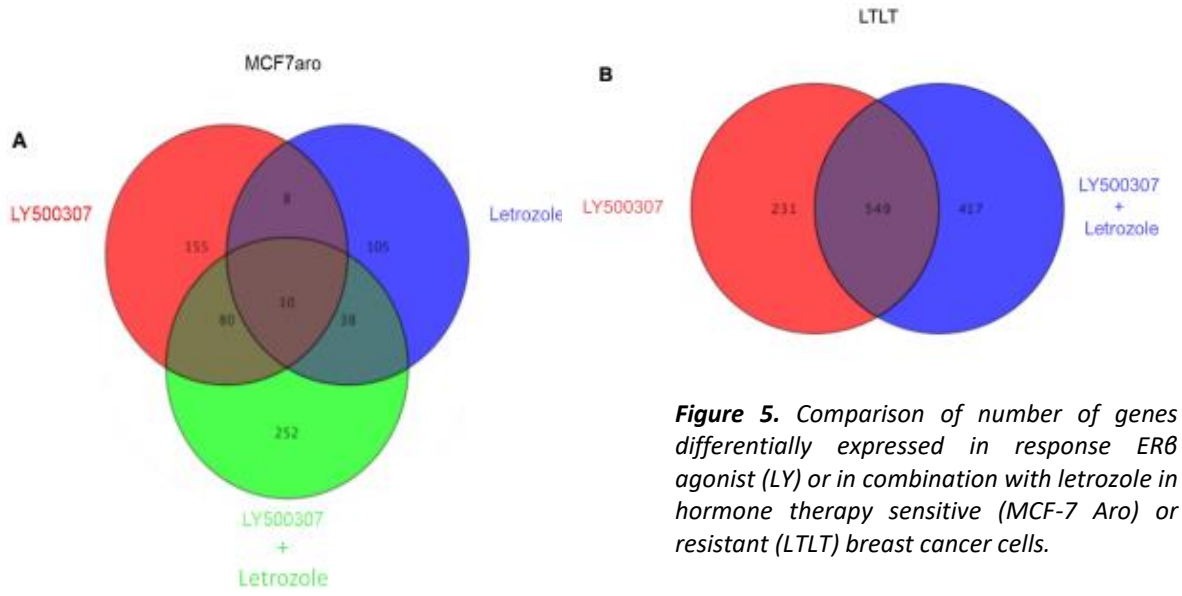


Figure 5. *Comparison of number of genes differentially expressed in response ERβ agonist (LY) or in combination with letrozole in hormone therapy sensitive (MCF-7 Aro) or resistant (LTLT) breast cancer cells.*

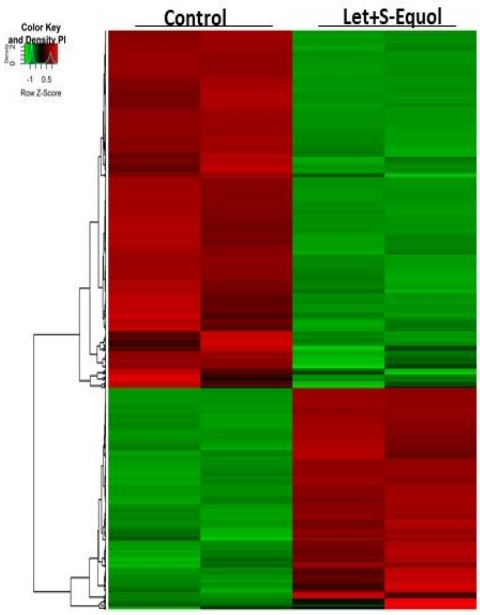
As shown in figure 5, treatment with ERβ agonist (LY) resulted significant changes in the expression of various genes. While ERβ agonist affected change in the expression of various genes and distinct set genes are modulated by this compound.

As shown in figure 6 we have also identified pathways that are affected by ER ERβ agonist (S-equol) both in sensitive and resistant cells.

To further validate these observations, we have examined top few genes whose expression is significantly affected (either increased or decreased) using qRT-PCR analysis using data set from LY treatment group.

Data presented in figure 7 confirms the observations seen with microarray analysis. These observations will be further examined form other ERβ agonist group to learn the mechanism of action of ERβ agonist in blocking the tumor growth.

MCF-7 Aro (letrozole sensitive) breast cancer cells

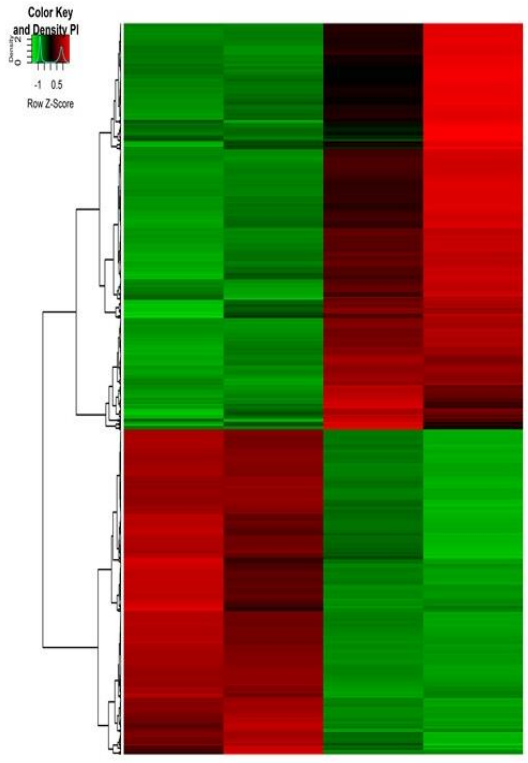


Molecular and cellular Functions

Name	P-value	#Molecules
Cell Cycle Progression	2.62E-20	138
Cell Death and Survival	1.11E-18	314
Cellular Movement	2.66E-14	195
Cellular growth and Proliferation	1.16E-10	159
Cellular Assembly and organization	8.59E-08	93

Top upstream regulators

Upstream regulator	p-value of overlap	Predicted activation state
ESR1	2.5E-29	Inhibited
Beta-Estradiol	1.23E-25	Inhibited
VEGF	1.28E-25	Inhibited
IL-6	5.68E-12	Inhibited
CDKN1A (p21)	7.02E-25	Activated
TP53	1.83E-24	Activated



Name	P-value	#Molecules
Cell Death and Survival	3.26E-18	776
Cellular Assembly and organization	3.82E-11	367
Cellular growth and Proliferation	2.11E-10	384
Cellular Movement	3.42E-10	200
Cell cycle	2.76E-08	167

Upstream regulator	p-value of overlap	Predicted activation state
CCDN1	3.98E-14	Inhibited
CTNNB1	3.51E-06	Inhibited
FOXM1	4.89E-06	Inhibited
CDKN1A (p21)	1.55E-09	Activated
NFE2L2	5.08E-06	Activated

LTLT (letrozole resistant MCF-7 Aro cells)

Figure 6. *Identification of pathways that are affected with letrozole or S-equol alone or in combination in hormone therapy sensitive (Top, MCF-7 Aro) or resistant (Bottom, LTLT) breast cancer cells.*

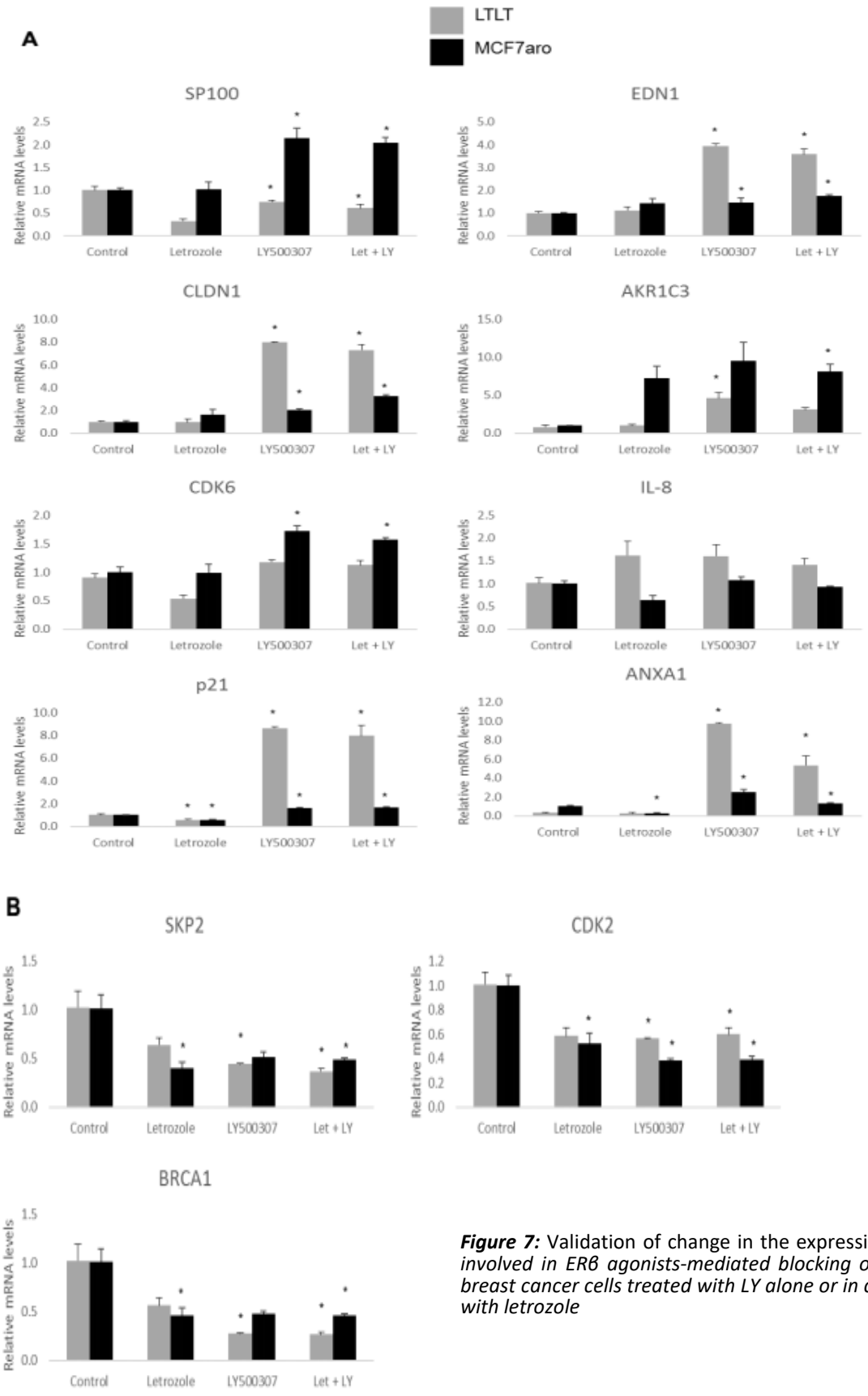


Figure 7: Validation of change in the expression of genes *involved in ERβ agonists-mediated blocking of growth of breast cancer cells treated with LY alone or in combination with letrozole*

Results for Object 4: *Establish the role of phoshotyrosine 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. As shown below, we have confirmed the deletions using nucleotide sequence determinations. Stable clones with deleted ERβ expression is being used for expression of ERβ with phosphotyrosine switch. We have over some difficulties in generating required clones and work is progress to accomplish the proposed goals.

T73: LTLT-KO using pX330-hER-B-CRISPR-#7
KI Inserted 1 C after 53 Base
ATGGATATAAAAAACTCACCATCTAGCCTTAATTCTCCTTCCTCCTACAACTGCCAGTCAATCCATCTTAC
CCCTGGAGCACGGCTCCATATACATACCTTCCTCCTATGTAGACAGCCACCATGAATATCCAGCCATGAC
ATTCTATAGCCCTGCTGTGATGAATTACAGCATTCCCAGCAATGTCACTAACTTGGAAGGTGGGCCTGGT
CGGCAGACCACAAGCCCAAATGTGTTGTGGCCAACACCTGGGCACCTTTCTCCTTTAGTGGTCCATCGCC
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GCTGCTGCTGGACGCCCTGAGCCCCGAGCAGCTAGTGCTCACCCTCCTGGAGGCTGAGCCGCCCCATGTG
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CGGCAGAGGACAGTAAAAGCAAAGAGGGCTCCCAGAACCCACAGTCTCAGTGA

WT protein
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NVTNLEGGPGRQTTSPNVLWPTPGHLSPLVVHRQLSHLYAEPQKSPWCEARSLEHTLPVN
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CAGKAKRSGGHAPRVRELLLDALSPEQLVLTLLEAEPPHVLISRPSAPFTEASMMMSLTK
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VLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAMILLNSSMYPLVTATQDA
DSSRKLAHLLNAVTDALVWVIAKSGISSQQQSMRLANLLMLLSHVRHASNKGMEHLLNMK
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MW 55KD
KO Stopped after 41aa of ER-B
MDIKNSPSSLNSPSSYNCQSIHLTPGARLHIHTFLLCRQPP-ISSHDIL-PCCDELQHSQ
QCH-LGRWAWSADHKPKCVVANTWAPFSFSGPSPVITSVCGTSKESLV-SKIARTHLTCK
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--LYLSSYKSVYNR-KPAQELPGLPTSEVLRSGNGEVWLPEREMWVPPCAETEKCRRAAA
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KO ORF Started at 46aa of ER-B

WI-KTHHLALILLPPTTASQSILPLEHGSIYIPSSYVDSHHEYPAMTFYSPAVMNYSIPS
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MW50KD

M74: MCF-7/Aro-KO using pX330-hER-B-CRISPR-#7
KO deleted 1 A after 49 Base
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KO Stopped after 36aa of ER-B
MDIKNSPSSLNSPSSYTAVNPSYPWSTAPYTYLPPM-TATMNIQP-HSIALL--ITAFPA
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KO ORF Started at 46aa of ER-B

GYKKLTI-P-FSFLLHCSQSILPLEHGSIYIPSSYVDSHHEYPAMTFYSPAVMNYSIPSN
VTNLEGGPGRQTTSPNVLWPTPGHLSPLVVHRQLSHLYAEPQKSPWCEARSLEHTLPVNR
ETLKRKVSGNRCASPVTGPGSKRDAHFCAVCSDYASGYHYGVWSCEGCKAFFKRSIQGHN
DYICPATNQCTIDKNRRKSCQACRLRKCYEVGMVKCGSRRERCGYRLVRR ERβ QRSADEQLHC
AGKAKRSGGHAPRVRELLLDALSPEQLVLTLLEAEPPHVLISRPSAPFTEASMMMSLTKL
ADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMMGLMWRSIDHPGKLIFAPDLV
LDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAMILLNSSMYPLVTATQDAD
SSRKLAHLLNAVTDALVWVIAKSGISSQQQSMRLANLLMLLSHVRHASNKGMEHLLNMKC
KNVVPVYDLLLEMLNAHVLRGCKSSITGSECSPAEDSKSKEGSQNPQSQ-

Major Findings/Conclusions:

- Using immune-competent mammary cancer models, we have shown that both LY500307 and S-Equol reduced tumor growth by altering the mechanisms involved in cell cycle and other pathways.

- Alteration of pathways that contribution of tumor suppressive functions of ERβ appears to be different based on the type ligand that induced this receptor.

- We have identified novel signaling molecules pathways associated with ERβ agonists mediated tumor suppression in hormone therapy sensitive and resistant breast cancers using in vitro models

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 **second year**, this project provided training opportunities for 6 students (1 medical and 5 high school) and two post-doctoral fellows (one MD and one Ph.D. fellow). All the students/fellows was 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 spend 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 was 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 2017 AACR-SABCS and 2018 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 2017 San Antonio Breast Cancer Symposium (international meeting attended by ~7500 from over 80 countries) and 2018 American Association of Cancer Research (AACR)

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

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 journal

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. 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 ovarian and endometrial cancers. Gynecological Oncology fellow 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.

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.*

Nothing to Report

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).*

A manuscript is submitted to report findings to scientific community based on studies with one of estrogen receptor beta ligand. Another manuscript was published using shared resources (cell lines, reagents etc) from this study.

- Samayoa, C., Ramasamy, K., Kota, A., Ganapathy, Krishnegowda, N.K., Vadlamudi, R.K., and **Tekmal, R.R**.: Liquiritigenin, a plant-derived Estrogen Receptor β agonist, inhibits the growth of therapy-sensitive and therapy-resistant breast cancer cells. Breast Cancer Research and Treatment (submitted).

- Raj, G.V., Sareddy, G.R., Ma, S-H., Lee, T-K., Viswanadhapalli, S., Li, R., Murkami, S., Chen, C-C., Le, W-R., Mann, M., Krishnan, S.M., Manandhar, B., Gonugunta, V.K., Strand, **Tekmal, R.R**., Ahn, J-M., and Vadlamudi, R.K.: Estrogen receptor coregulatory binding modulators (ERXs) effectively target estrogen receptor positive human breast cancers. Elife. 2017 Aug 8;6. pii: e26857. doi: 10.7554/eLife.26857. PMID: 28786813

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).*

- Ramasamy, K., Samayoa, C., Naveen Krishnegowda, and **Tekmal, R.R**.: Therapeutic use of estrogen receptor beta agonists in prevention and treatment of endocrine therapy resistant breast cancers: Observations from pre-clinical models. In Progress in Molecular Biology and Translational Science 151: Approaches to Understanding Breast Cancer. Lakshmanaswamy, R. (Ed), Elsevier, UK, pp 177-194 2017.
  http://dx.doi.org/10.1016/bs.pmbts.2017.08.002.

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, N.K., Vadlamudi, R.K. and Tekmal, R.R.: Efficacy of estrogen receptor β agonists in the prevention of breast cancer progression to therapy resistance. 40th Annual San Antonio Breast Cancer Symposium, P1-09-09, 2017.

- K. Ramasamy, K., C. Samayoa, C., Krishnegowda, N.K., Chen, S., Vadlamudi, R. K., and Tekmal, R.R.: Estrogen receptor β agonists suppress the growth and progression of mammary tumors in immune-competent mouse models. . Proc. Am. Assn. Cancer Res., 109th Annual Meeting, Abstr. 3734, 2018

- Abstract is accepted for presentation 41st Annual San Antonio Breast Cancer Symposium to be held during December 2018

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.

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 replaced Dr. Krishnegowda and he was responsible for maintenance and generation of all required animals from animal models and carried 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: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

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.

Cancer Research

Poster Session Abstracts

Abstract P1-09-09: Efficacy of estrogen receptor β agonists in the prevention of breast cancer progression to therapy resistance

K Ramasamy, C Samayoa, NK Krishnegowda, RK Vadlamudi, and RR Tekmal

DOI: 10.1158/1538-7445.SABCS17-P1-09-09 Published February 2018 Check for updates

Abstracts: 2017 San Antonio Breast Cancer Symposium; December 5-9, 2017; San Antonio, Texas

Abstract

Estrogen plays an important role in the initiation and progression of breast cancer (BCa). Approximately, 70% of breast tumors are estrogen receptor (ER) positive at the time of presentation. Endocrine therapy using aromatase inhibitors (AI), or anti-estrogen (AE) molecules are widely used for treating ER+ve BCa. However, their efficacy is limited by intrinsic and acquired therapy resistance and most patients develop resistance to these drugs. The transcriptional effects of estrogen are mediated by two ERs (ERα and ERβ) and both are expressed in normal breast tissue. Unlike ERα, ERβ functions as tumor suppressor. However, role of ERβ specific agonists in the prevention of BCa progression remains elusive. In this study, we investigated the effectiveness of two ERβ agonists (S-Equol and LY500307) in the prevention of BCa progression using endocrine therapy sensitive (MCF7-aro) and letrozole resistant (MCF7-aro-LTLT) cells. Our results demonstrated that treatment with ERβ agonists inhibit short- and long-term growth of both endocrine therapy sensitive and resistant BCa cells. In addition, ERβ agonists treatment inhibited invasion and migration of both MCF7-aro and MCF7-aro-LTLT cells. Importantly, cell cycle analysis revealed that ERβ agonists induced cell cycle arrest. Our gene microarray analysis demonstrated that both ERβ agonists significantly modulated genes involved in the cell cycle progression, DNA replication and cell death pathways. Further, gene enrichment analysis of differentially expressed genes revealed that genes involved in the cell cycle checkpoints emerged as significant pathway modulated by ERβ agonists treatment in MCF7-aro cells. Interestingly, in letrozole-resistant MCF7-aro cells, DNA replication was significantly affected by ERβ agonists

treatment. Pathway analysis also identified enrichment for chemokine signaling pathways. We confirmed pathway analysis by qRT-PCR and western blot analysis. Accordingly, treatment of in vivo syngeneic xenografts with ERβ agonists significantly inhibited BCa progression. Collectively, these results from this study suggest that ERβ agonists have potential to prevent the progression of BCa progression.

Citation Format: Ramasamy K, Samayoa C, Krishnegowda NK, Vadlamudi RK, Tekmal RR. Efficacy of estrogen receptor β agonists in the prevention of breast cancer progression to therapy resistance [abstract]. In: Proceedings of the 2017 San Antonio Breast Cancer Symposium; 2017 Dec 5-9; San Antonio, TX. Philadelphia (PA): AACR; Cancer Res 2018;78(4 Suppl):Abstract nr P1-09-09.

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icantly differentially expressed genes following E2 treatment. Fulvestrant inhibited the majority of E2-induced differentially expressed genes, confirming that these genes are dependent upon ERα. Gene Set Enrichment Analysis (GSEA) indicated that the Hallmark early and late estrogen responses are enriched in the E2 dataset, confirming that classical ER activity is intact in these cells. Furthermore, we identified G_2/M checkpoint as positively enriched in our dataset, indicating that proliferation genes are upregulated by ERα in these cells. Additionally, we found that apoptosis was negatively enriched in our dataset, indicating that apoptosis pathway genes are suppressed by E2 treatment. Future investigation in this project will center on exploring the mechanisms of ERα transcriptional activity through examining the ERα-dependent cistrome, regulation of target genes, and its interactions with other cofactors. These data will give us insight into how ER is regulated and what pathways and processes ER is driving. Additionally, we will use CRISPR/Cas9 screening to uncover the key genes downstream of ER that are executing the estrogen-dependent effects on proliferation and survival. We believe that these studies will provide additional drug targets that may suggest a combinatorial therapeutic approach in conjunction with endocrine therapies for the treatment of ovarian cancer.

#3732 Insights into the non-coding genome of parathyroid tumors. Annamaria Morotti,[1] Irene Forno,[1] Valentina Andrè,[2] Vito Guarnieri,[3] Andrea Terrasi,[1] Rosa Maria Silipigni,[4] Silvana Guerneri,[4] Chiara Verdelli,[2] Alfredo Scillitani,[3] Leonardo Vicentini,[4] Filomena Cetani,[5] Edoardo Beretta,[6] Sabrina Corbetta,[2] Valentina Vaira[1]. *[1]University of study of Milan, Milano, Italy; [2]IRCCS Istituto Ortopedico Galeazzi, Milano, Italy; [3]IRCCS Casa Sollievo della Sofferenza, Foggia, Italy; [4]IRCCS Cà Granda, Ospedale Maggiore Policlinico, Milano, Italy; [5]University Hospital of Pisa, Italy; [6]Ospedale San Raffaele, Milano, Italy.*

Background: Parathyroid tumors are characterized by genetic and epigenetic alterations resulting in aberrant expression of protein coding genes and non-coding RNAs. Although long non-coding RNAs (lncRNAs) play a regulatory role in endocrine cancer pathogenesis, a lncRNAs signature in human parathyroid tumors is still missing. Here we investigated the lncRNAs alterations, both at genomic and transcriptional level, in human non-familial parathyroid tumors. Methods: The expression of 90 lncRNAs was investigated in 4 parathyroid carcinomas (PCas), 12 adenomas (PAds) and 2 normal glands (PaNs) using a commercial array. Both unsupervised (hierarchical clustering-HCL and Principal Component Analysis-PCA) and supervised (Significance Analysis of Microarray, SAM) analyses were performed to identify differences in lncRNAs expression between the 3 tissue types. Significant lncRNAs were validated in a second set of parathyroid tissues including 7 PCas, 26 PAds, 6 atypical PAds (aPAds) and 4 PaNs. Genomic characterization of 21 PAds was performed by array Comparative Genomic Hybridization (aCGH). CDC73 and Multiple Endocrine Neoplasia 1 (MEN1) genes mutations were detected by Sanger sequencing. Results: HCL analysis of lncRNAs expression identified 2 major groups in which PaNs and PCas were distinguished. Nine lncRNAs were differentially expressed in parathyroid tissues. Specifically, KCNQ1OT1 and SNHG6 were enriched in PaNs, HAR1B, MEG3, HOXA3as and NEAT1 expression characterized PAds, whereas BC200, HOXA6as and WT1-AS were significantly upregulated in PCas. Besides confirming previous data, validation analysis highlighted a different lncRNAs expression pattern in PCas and aPAds according to CDC73 mutation status, with mutated tumors overexpressing the majority of the lncRNAs. Interestingly, BACE1-AS, KCNQ1OT1, NEAT1 and SNHG6 levels in PAds were significantly correlated with MEN1 levels while HAR1B upregulation was associated with chromosome 11 loss of heterozygosity (LOH). Conclusions: Overall these findings shed light on lncRNAs deregulation in parathyroid pathobiology. Parathyroid tumors histotypes are characterized by different lncRNAs signatures that are related to chromosome 11 derangements and to MEN1 inactivation. Finally, MEN1 may play an epigenetic role in lncRNAs regulation, supporting the important role of chromosome 11 in parathyroid tumorigenesis.

#3733 Combined targeting of estrogen receptor alpha and nuclear transport pathways remodel metabolic pathways to induce autophagy and overcome endocrine resistance. Zeynep Madak Erdogan,[1] Eylem Cotul-Kulkoyluoglu,[1] Kinga Wrobel,[1] Sunati Sahoo,[2] Barbara Haley,[2] Yosef Landesman[3]. *[1]Univ. of Illinois at Urbana-Champaign, Urbana, IL; [2]University of Texas Southwestern, Dallas, TX; [3]Karyopham Therapeutics, MA.*

Majority of breast cancer specific deaths in women with ERα (+) tumor occur due to metastases that are resistant to endocrine therapy. There is a critical need for novel therapeutic approaches to resensitize recurrent ERα (+) tumors to endocrine therapies. The objective of this study was to elucidate mechanisms of improved effectiveness of combined targeting of ERα and XPO1, a nuclear transport protein in overcoming endocrine resistance. Selinexor (SXR), an XPO1 antagonist, has been evaluated in multiple later stage clinical trials in patients with relapsed and /or refractory hematological and solid tumor malignancies. Using Cignalfinder to profile kinase signaling pathways, we found that 4-OH-Tam, SXR or their combination induced differential Akt phosphorylation profiles, changing the localization and activity of the kinase. Since we observed dramatic changes in Akt activity we hypothesized that metabolic profile of breast cancer cells would change in the presence of 4-OH-Tam and SXR. Using Seahorse metabolic profiler and cell viability experiments in limited media conditions we showed that tamoxifen resistant cells were more dependent on mitochondria for energy production. Their glucose and fatty acid dependency decreased in the presence of SXR and cells were more dependent on glutamine as the mitochondrial fuel source. In order to examine metabolic pathways that might result in the observed phenotype we performed transcriptomics and GC/MS whole metabolite profiling and identified aminoacid metabolism pathways to be upregulated when cells were treated with SXR+4-OH-Tam. We demonstrated that combined targeting of XPO1 and ERα rewires metabolic pathways and shuts down both glycolytic and mitochondrial pathways that would eventually lead to autophagy. Remodelling metabolic pathways to regenerate new vulnerabilities in endocrine resistant breast tumors is novel, and given the need for better strategies for improving therapy response of relapsed ERα(+) tumors, our findings show great promise for uncovering the role ERα-XPO1 crosstalk plays in reducing cancer recurrences.

#3734 Estrogen receptor β agonists suppress the growth and progression of mammary tumors in immune-competent mouse models. Kumaraguruparan Ramasamy, Cathy Samayoa, Naveen K. Krishnegowda, Shaorong Chen, Ratna K. Vadlamudi, Rajeshwar R. Tekmal. *UT Health Science Ctr. at San Antonio, San Antonio, TX.*

Background: Despite medical advances in early detection and treatment, breast cancer still has a relatively high mortality rate in women due to recurrence and metastasis. Many human cancers are able to suppress the activity of the immune system. With the emerging importance of the immune system in tumor surveillance, the need to employ immunocompetent in vivo models to study breast cancer progression is evident. Syngeneic tumor mouse models are a useful tool to study drug development and therapeutic utility of novel drugs. Emerging evidence suggest that Estrogen receptor (ER) β functions as a tumor suppressor in many cancers including breast cancer. Therefore, targeting ERβ with selective agonists may provide therapeutic benefit in the treatment of breast cancer. Herein, we examined the therapeutic efficacy of ERβ agonists on the growth of syngeneic mouse mammary tumors. Experimental design: To test the effects of ERβ agonists on growth, we carried out cell proliferation, invasion and migration, and clonogenic assays in all three-mouse mammary tumor models with different genetic background. Cell cycle was analyzed using FACS analysis. D2A1 (BALB/c) and MM51 (FVB) syngeneic models and ex-vivo culture of E0771 (C57/B6) cells were used to evaluate the antitumor effects of ERβ agonists. Results: First, we assessed the effects of ERβ agonists on cell proliferation of these three mouse mammary tumor cells. Cells were treated with different concentrations of LY500307 (100nM-10μM) and S-Equol (1μM-100μM) for 72 and 96 hrs. D2A1 cells and E0771 cells showed IC_{50} of 2.5μM for LY500307 and 50 μM for S-Equol and MM51 cells had an IC_{50} of 2 μM for LY500307 and 40 μM for S-Equol. Our results also showed that ERβ agonists reduce the colony formation ability of D2A1 and MM51 cells. In D2A1 and MM51 cells, LY500307 treatment decreased colonies by 32% and 50%, and S-Equol reduced colonies to 18% and 40% as compared to control respectively. Cell cycle analysis showed that LY500307 and S-equol treatment in D2A1 and E0771 cells resulted in a significant accumulation of cells in S phase. Further, we analyzed the therapeutic efficacy of LY500307 in two syngeneic mouse tumor models from D2A1 and MM51 cells. Our results demonstrated that LY500307 inhibited the tumor growth and the effect was more pronounce in combination with aromatase inhibitor letrozole. Further, using ex-vivo model of tumor explants from E0771 cells, we showed that ERβ agonists inhibited the mammary tumor growth. Conclusions: Our results suggested that ERβ agonists have potential to prevent the progression mammary tumors in immunocompetent hosts.

#3735 Somatostatin receptor-based imaging and treatment of murine pancreatic neuroendocrine tumors induced by MEN1-loss. Janet W. Li,[1] Hanwen Zhang,[1] Sean D. Carlin,[2] Nitya Raj,[1] David S. Klimstra,[1] Steven K. Libutti,[3] Wolfgang A. Weber,[1] Diane Reidy-Lagunes,[1] Brian R. Untch[1]. *[1]Memorial Sloan Kettering Cancer Center, New York, NY; [2]University of Pennsylvania, Philadelphia, PA; [3]Rutgers Cancer Institute of New Jersey, New Brunswick, NJ.*

Background: Somatostatin receptor ligands are used for the detection and treatment of neuroendocrine tumors. Mutations of MEN1 are frequently observed in human pancreatic neuroendocrine tumors (PanNETs). We utilized a