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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:

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.

1. KEYWORDS:

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

2. 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 06/30/2020 was obtained from DOD. ***This final progress report covers the work carried out during the no-cost extension period as well as during all previous years (1-3).***

What were the major goals of the project?

Specific aims/major goals of the project as approved in SOW:

During the no-cost extension period and as well as during the previous years (as reported through individual progress report for years 1-3), 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?

First Year Grant Period from: 07/01/2016-06/30/2017

Major activities carried out during the first year:

During the first year funding period we have initiated the work as stated in Statement of Work

- We have completed the approval of the IACUC protocol
- Obtained approval for collection of tissues from breast cancer patients from UTHSCSA tumor bank and institutional IRB under exemption category.
- Increased transgenic animal breeding for generating required transgenic mice.
- Initiated experimental work (Major tasks) as indicated in statement of work is shown below:
    1. Established the preventive role of ERβ agonists in the prevention of breast cancer using transgenic animal model

2. Determined efficacy of ERβ agonists to restore sensitivity to endocrine therapy in resistant breast cancer cells

3. Elucidated how ERβ signaling network influences the role of ERβ agonists in preventing breast cancer using transgenic models.

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 progression and recurrence of breast cancer immune-component tumor models (***Major Task 1-Subtask 2: 1-24 months).***

Objective 3: Test whether ERβ agonists restore sensitivity to AE/AI endocrine therapy using various endocrine therapy sensitive and resistant cells **(*Major Task 2-Subtask 1: 1-12 months)***.

Objective 4*:* Establish the mechanisms of prevention/tumor suppressive functions of ERβ ***(Major Task 3 - Subtask 3:1-24 months).***

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 initial studies focused on MMTV-HER2/neu model. We have used the pubertal animals (n=9/group) and treated with ERβ agonist (LY500307 and S-equol). Mammary growth (ductal elongation and ductal branching) was examined after 4 months of continuous treatment. As shown in Figure 1, mammary ductal branching and hyperplasia in ductus and mammary labulo-alveolar structures were significantly affected with treatment when compared with control. There was no significant difference in the ductal elongation and also there was no change in the body weight (data not shown).

Studies described here show that treatment with ERβ agonists affects mammary growth. Specifically, significant decrease in the ductal hyperplasia and lateral ductal branching (labulo-alveolar growth) an indication of mammary growth and initiation of neoplastic changes clearly suggest ERβ agonists are very effective in preventing breast cancer initiation in experimental models studied. Studies that examines effect of these compounds on prevention and the tumor incidence with other models is in progress (ongoing study).

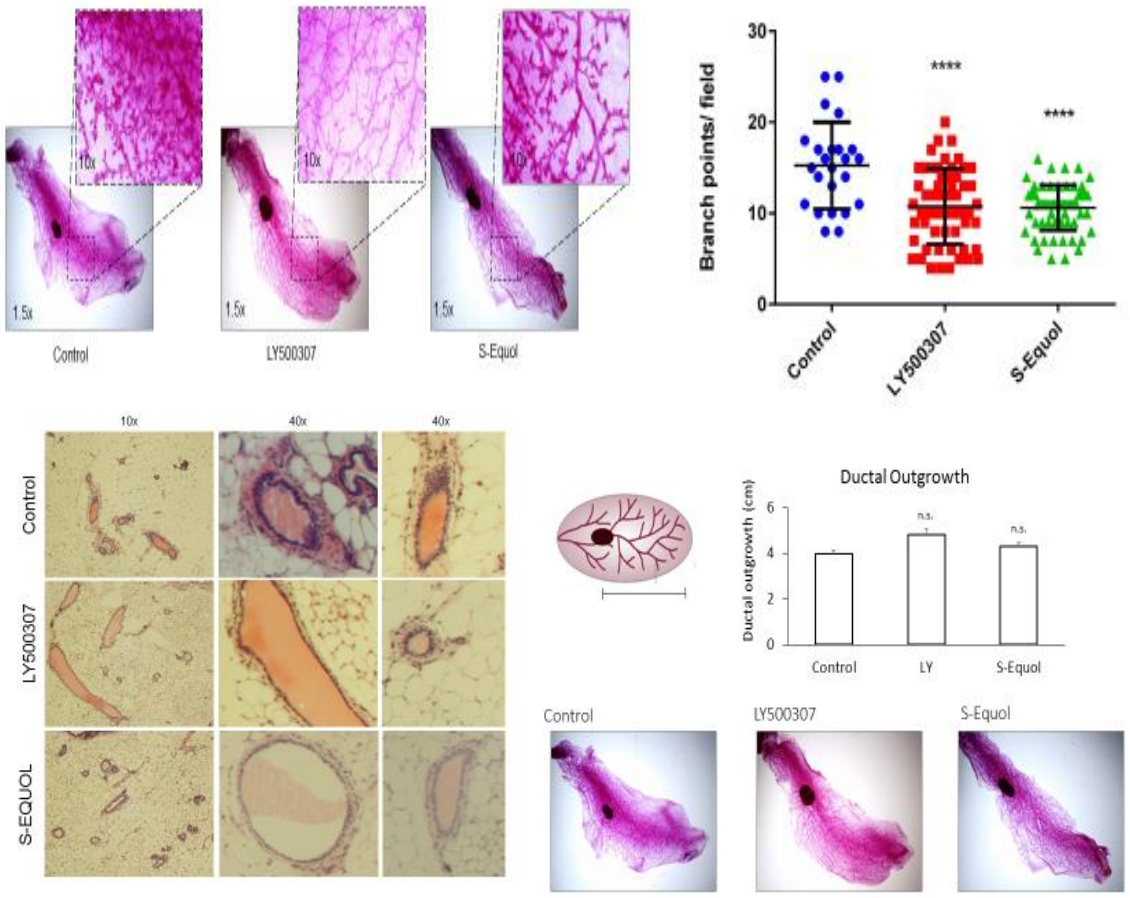


Figure 1. *Effect of ERβ agonists on the mammary growth/ prevention of pre/neoplastic changes (initiation of tumorigenesis): While there is no significant difference on the ductal elongation (lower left) with treatment with ERβ agonists, there was a significant reduction on mammary growth (labulo-alveolar/branching) ($p<0.001$) (upper right and left) compared to controls. There was also decrease or lack of devolvement hyperplasia in treated animals (lower left).*

Results for objective 2: *Establish that ERβ agonists block progression and recurrence of breast cancer immune-component tumor models.* To test the therapeutic efficacy of ERβ agonists to block the tumor progression using immune-component breast cancer tumor models. We have used the D2A1 (BALB/c) and MM51 (FVB) murine model cells and tested their growth in the presence and absence of ERβ agonists employing cell growth and colony formation assays. In addition, we have tested effect of LY on tumor growth using both D2A1 and MM5I syngeneic mice tumor models. As shown in Figure 2, ERβ agonists (LY500307 and S-equol) were effective in blocking the both cell growth and colony formation. In addition, LY was very effective in reducing tumor growth in syngeneic mice compared to control. When combined with aromatase inhibitor (letrozole) the tumor growth was further reduced significantly.

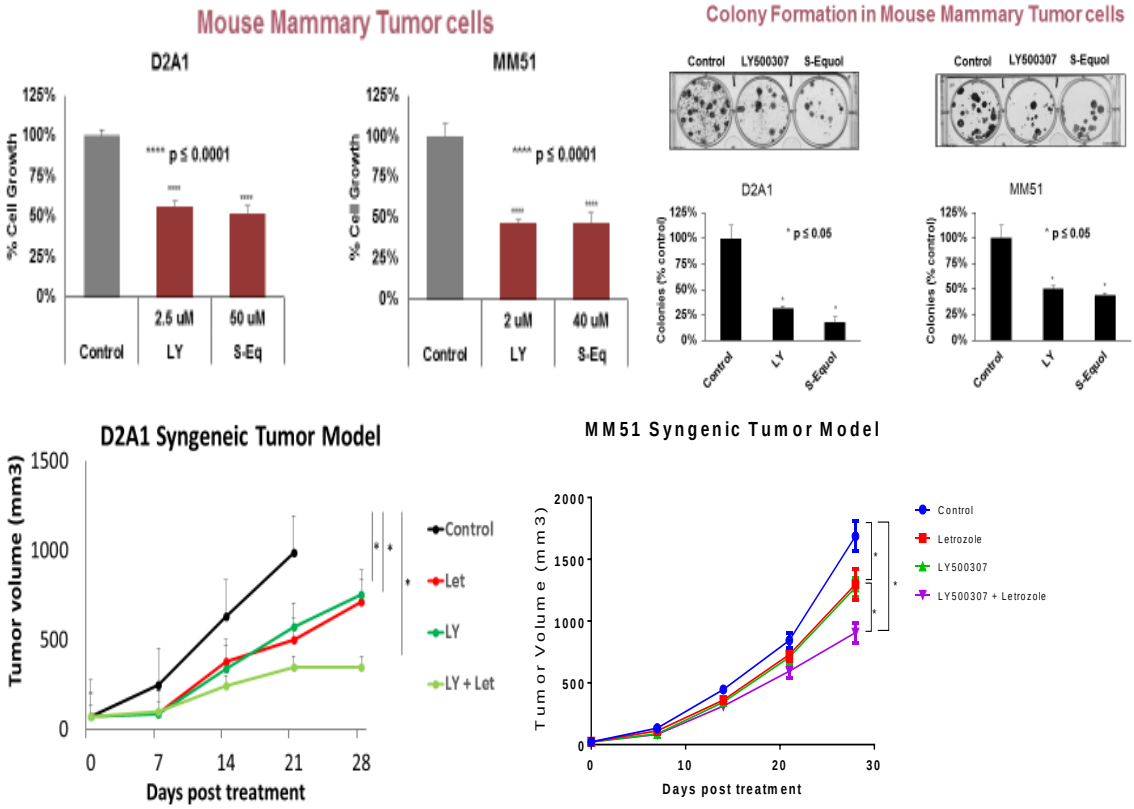


Figure 2. *Effect of ERδ agonists on in vitro and in vivo growth. LY500307 and S-Equol were effective in blocking the growth in culture (top left panels) and colony formation (top right panels). LY500307 was very effective in block tumor growth (p<0.001) in both syngeneic tumor models (bottom panels).*

Results for objective 3: *Test whether ERβ agonists restore sensitivity to AE/AI endocrine therapy using various endocrine therapy sensitive and resistant cells.*

To test the ability of ERβ agonists to affect the growth of both endocrine therapy sensitive and resistant breast cancers, we tested their growth using cell growth and colony formation assays.

As shown in figure 3, ERβ agonists (LY500307 and S-Equol) significantly affected the growth hormone sensitive breast cancer cells. We also tested the effect of these compounds using MCF7-LTLT an aromatase inhibitor (letrozole) resistant breast cancer cell line and a tamoxifen resistant breast cancer cell line (MCF7-TAM).

Data presented in figure 4 shows that both ERβ agonists affected the growth of aromatase inhibitor resistant breast cancer cells. Like, S-equol, LY500307 was very effective in blocking the growth of tamoxifen resistant breast cancer cells in culture, but its effect on anchorage independent growth (colony formation) was not significant. These observations suggest the unlike in letrozole resistant breast cancer cells the mechanism of action of these agents in tamoxifen resistant cells appears to be different. More studies are planned to help to learn the mode of action of these compounds in hormone therapy sensitive versus resistant tumors.

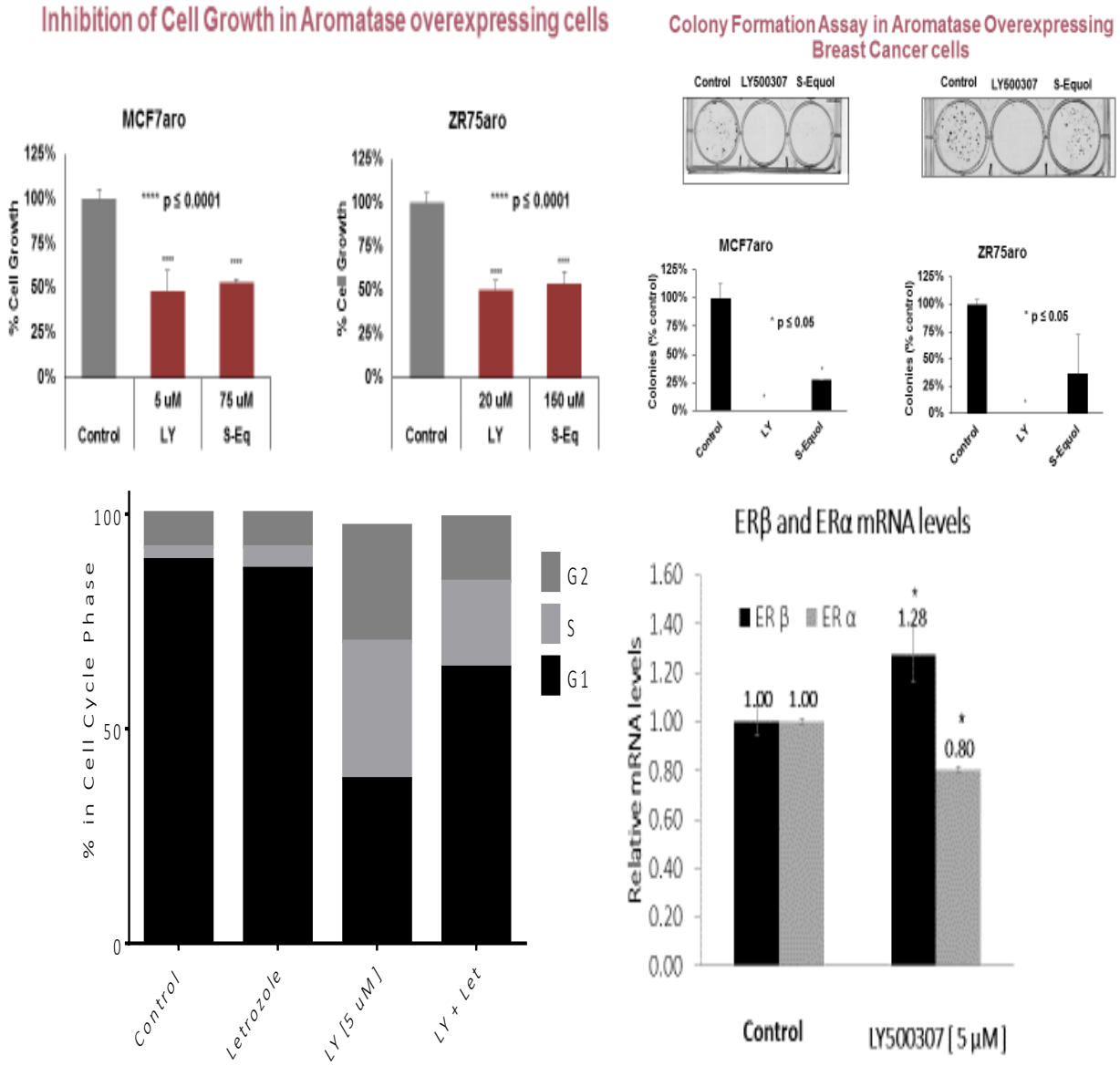


Figure 3. *Effect of ERβ agonists on the growth of hormone responsive breast cancer cells. LY500307 and S-Equol were effective in blocking the growth in culture (top left panel) and colony formation (top right panel). Unlike with letrozole, LY500307 appears to restrict the growth by affecting S and G1 phase of cell cycle (bottom left panel) and also affected the levels of ERα contributing change in the ratio of ERα/ERβ (bottom right panel).*

Results for objective 4: *Establish the mechanisms of prevention/tumor suppressive functions of ERβ*

To examine the molecular mechanisms that are involved in the prevention of initiation of neoplastic changes in response to ERβ agonists, we used mammary tissues from studies described (Major goal 1, sub task 1) 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 prevention in transgenic animals.

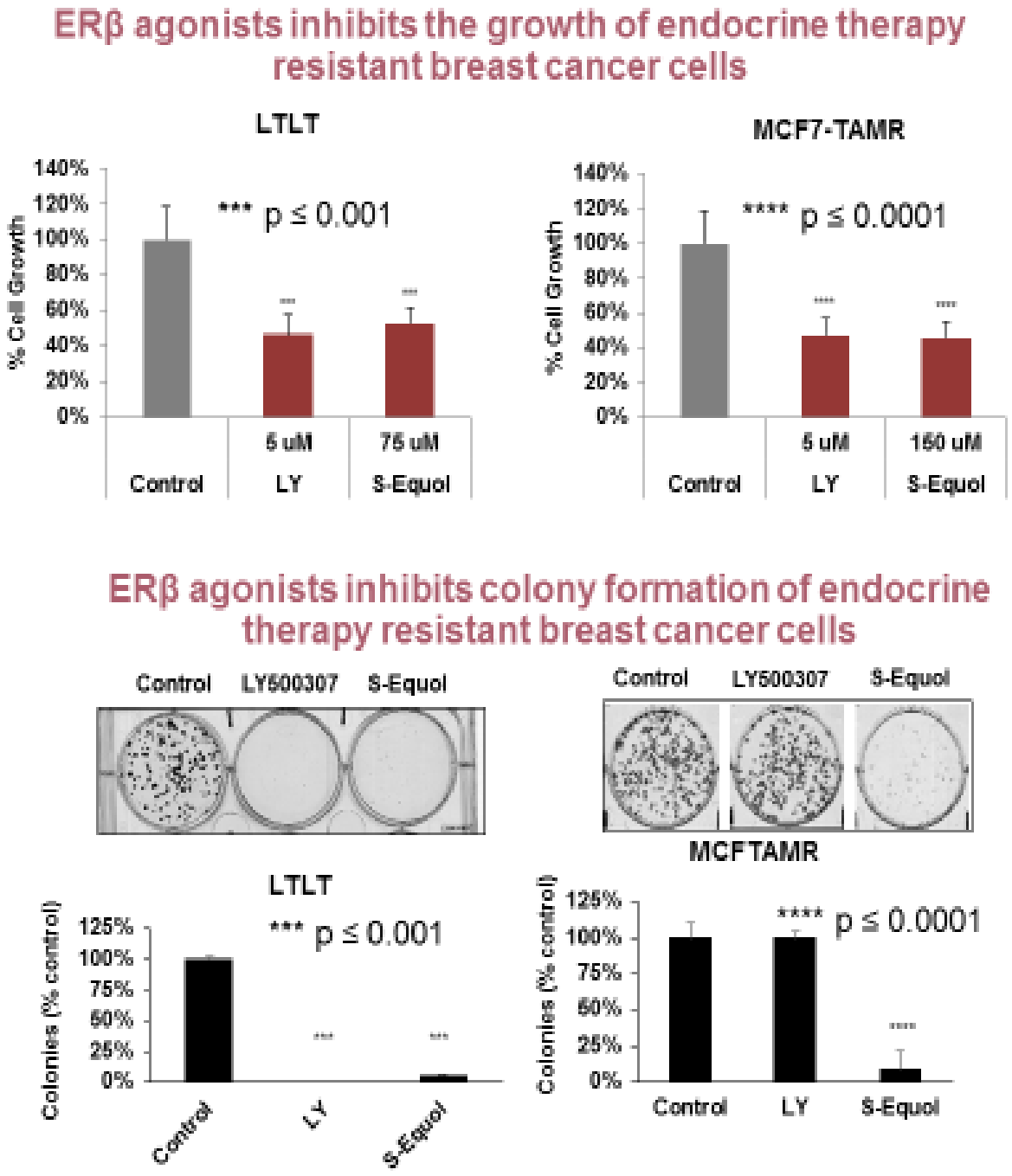


Figure 4. *Effect of ERβ agonists on the growth of hormone therapy resistant breast cancer cells. LY500307 and S-Equol were effective in blocking the growth of letrozole resistant breast cancer cells (top and bottom left panels). Both LY500307 and S-equol were effective in blocking the growth tamoxifen resistant cells (top right panel) but LY500307 not very effective in blocking colony formation (bottom right panel).*

As shown in figure 5, treatment with ERβ agonist resulted significant changes in the expression of various genes. While both ERβ agonists affected change in the expression of various genes and distinct set genes are modulated by these compounds. As shown in figure 5 we have also identified pathways that are affected ERβ agonists.

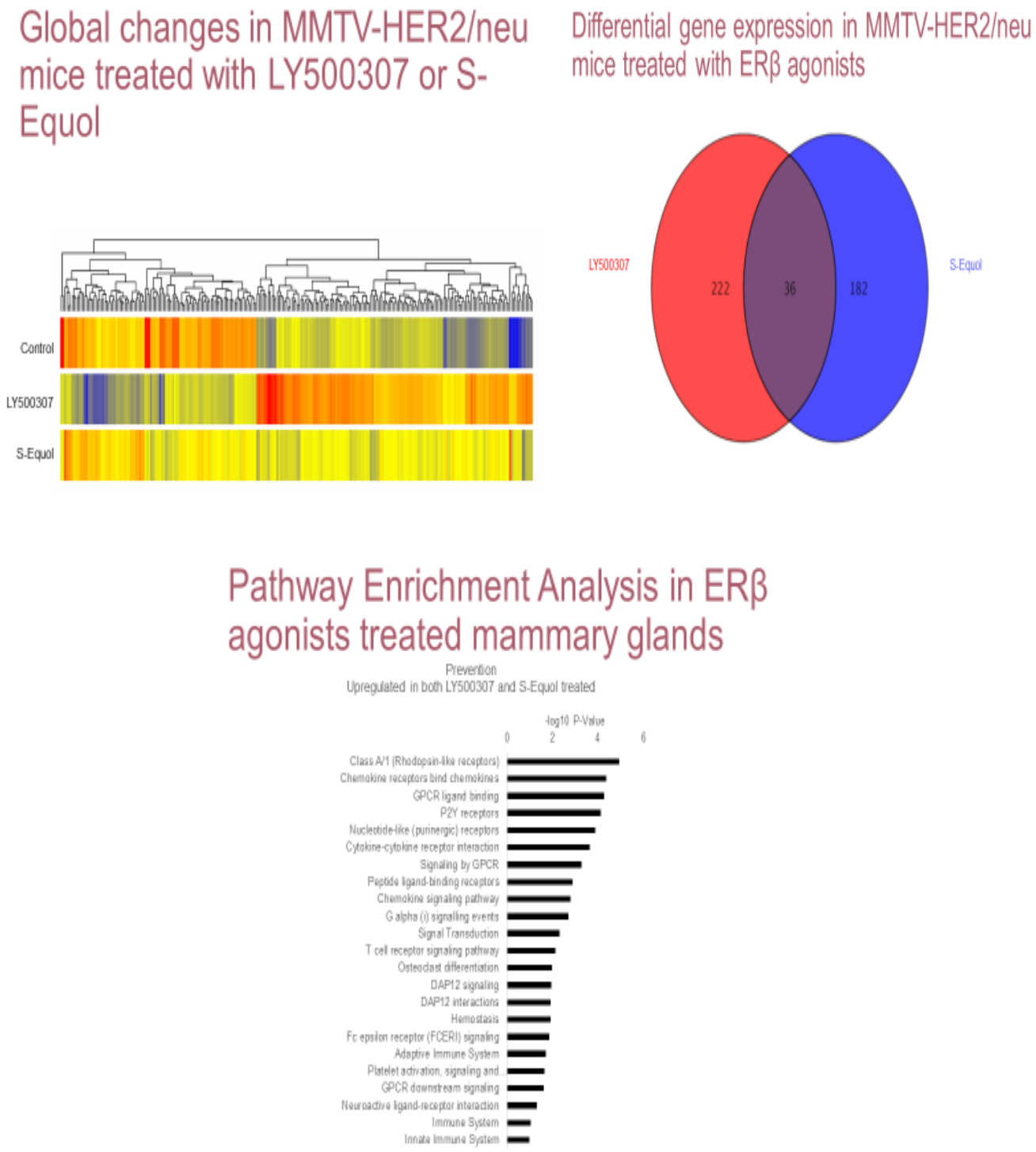


Figure 5: *Change in the expression of various genes involved in ERβ agonists-mediated prevention of neoplastic changes in transgenic mice.*

To further validate these observations, we have examined top few genes whose expression is significantly affected (either increased or decreased) using qRT-PCR analysis. Data presented in figure 6 confirms the observations seen with microarray analysis. These observations will be further expanded using tissues from tumor reduction and other studies as planned to learn the mechanism of action of ERβ agonists in preventing and blocking tumor progression.

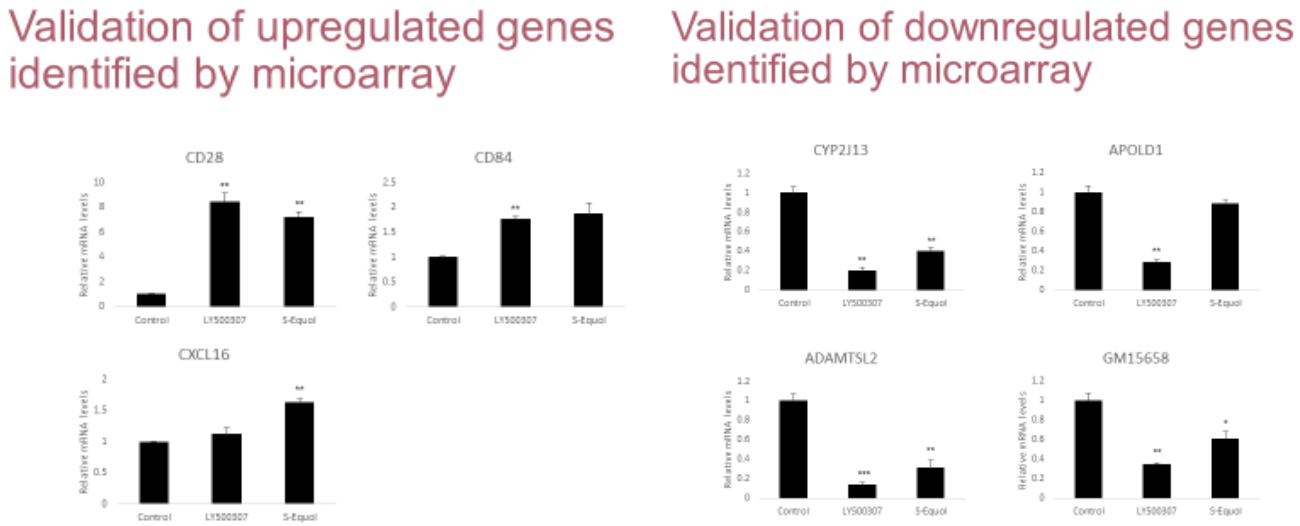


Figure 6: Validation of change in the expression of genes *involved in ERδ agonists-mediated prevention of neoplastic changes in transgenic mice.*

Second Year Grant Period from: 07/01/2017-06/30/2018

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.

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

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. 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).

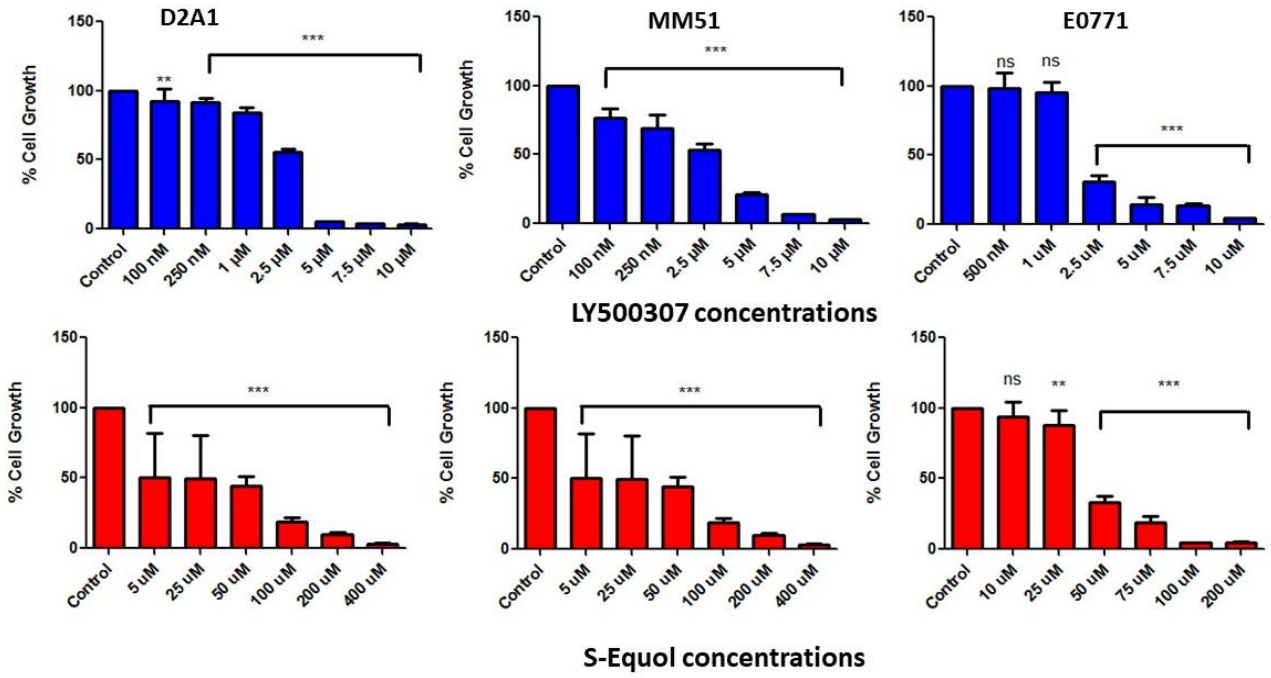


Figure 1. *Effect of ERβ agonists on in vitro growth of mammary cancer cells. LY500307 and S-Equol were effective in blocking the growth in dose dependent manner (p<0.01- p<0.001) in all three tumor models.*

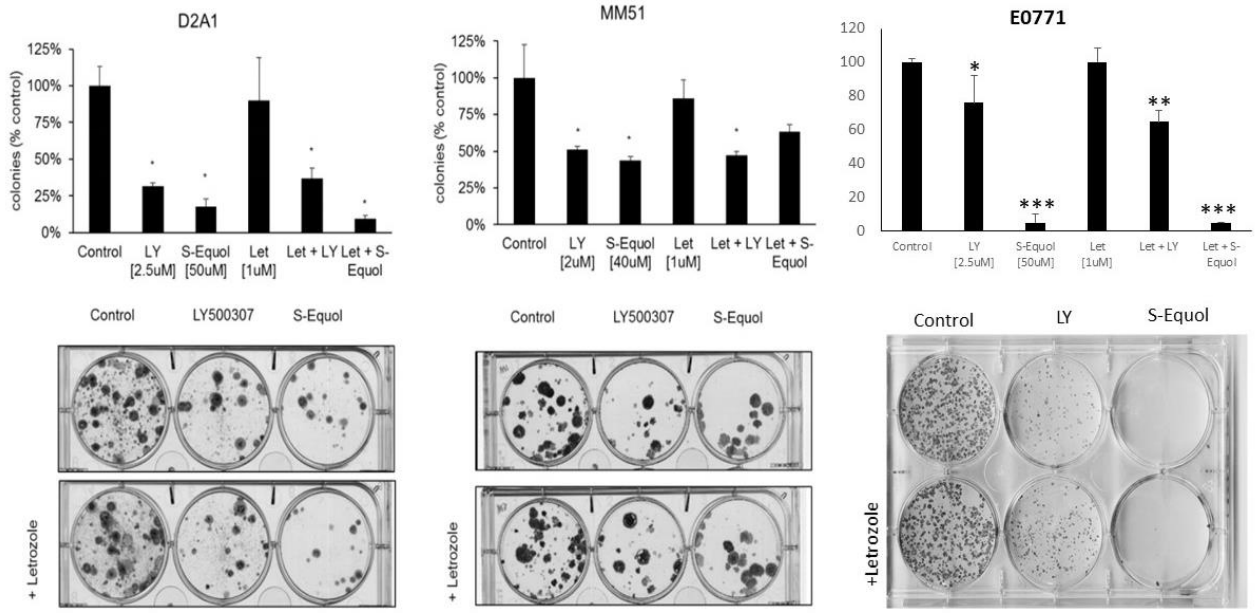
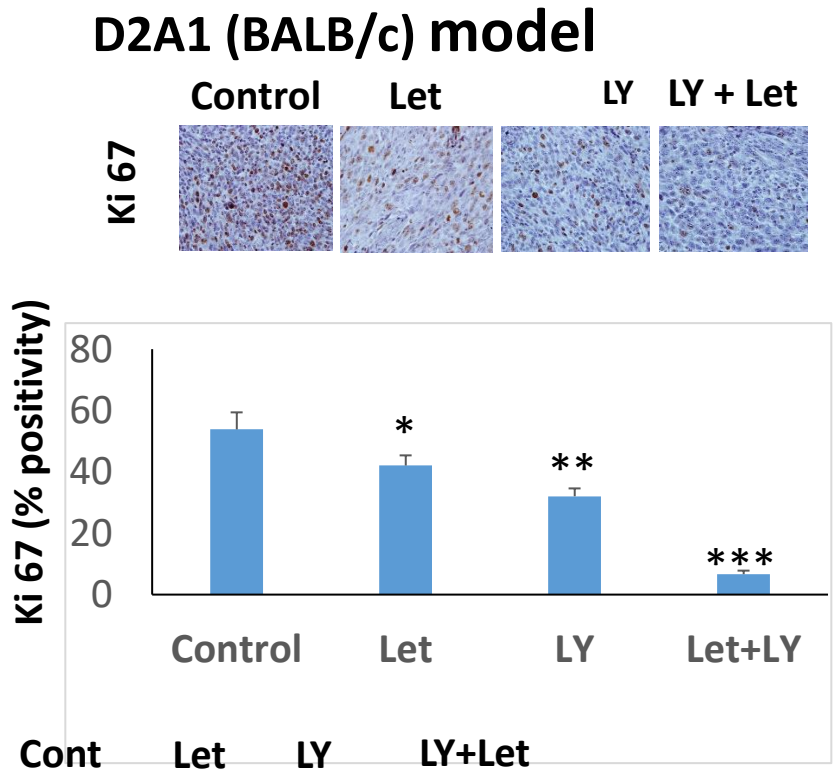


Figure 2. *Effect of ERβ agonists on colony formation of mammary tumor cells. LY500307 and S-Equol were effective in blocking the colony formation (p<0.01). Unlike these compounds alone, the effect of blocking colony formation was even more effective in combination with Letrozole (Let) p<0.001) in all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

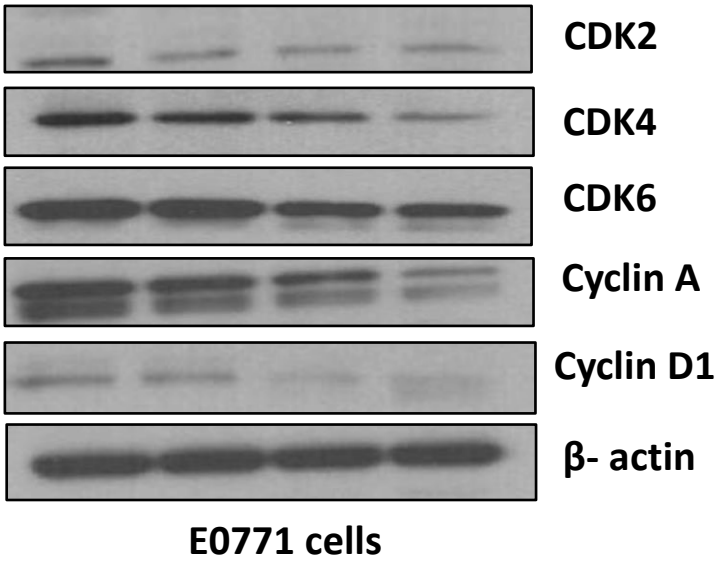
As shown in figure 2, ERβ agonists (LY500307 and S-equol) were effective in blocking the colony formation. Unlike ERβ agonists alone, these compounds in combination with AI were even more effective in blocking the formation of colony formation. In addition, the

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).

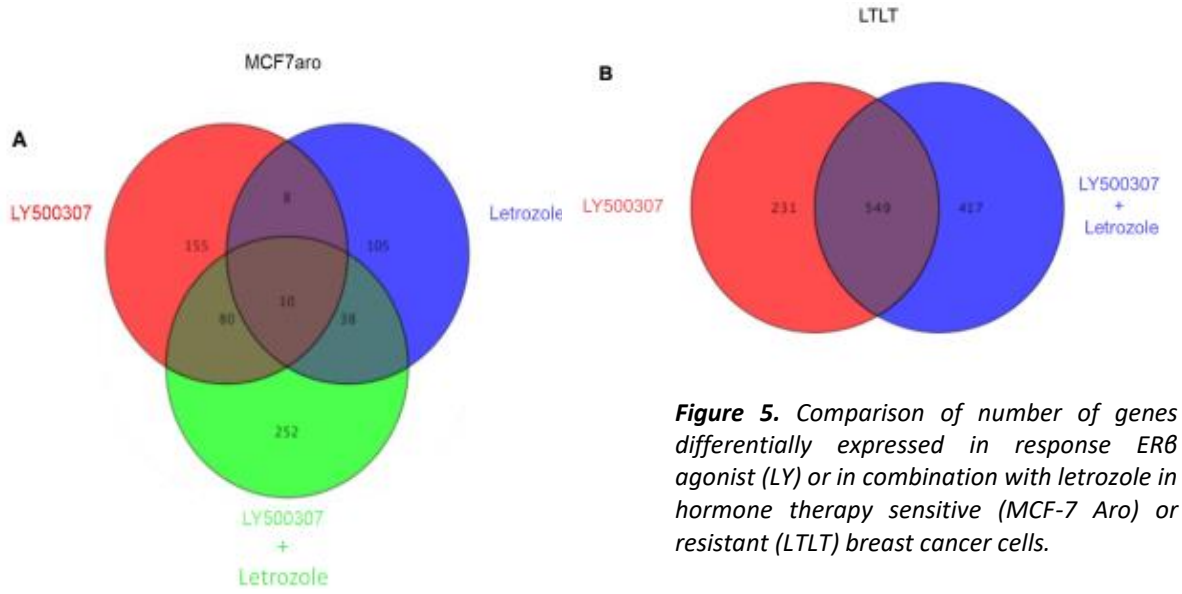


Figure 5. *Comparison of number of genes differentially expressed in response ERβ agonist (LY) or in combination with letrozole in hormone therapy sensitive (MCF-7 Aro) or resistant (LTLT) breast cancer cells.*

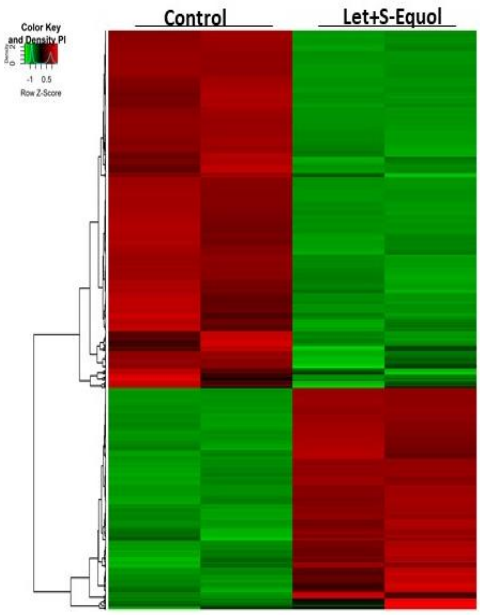
As shown in figure 5, treatment with ERβ agonist (LY) resulted significant changes in the expression of various genes. While ERβ agonist affected change in the expression of various genes and distinct set genes are modulated by this compound.

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

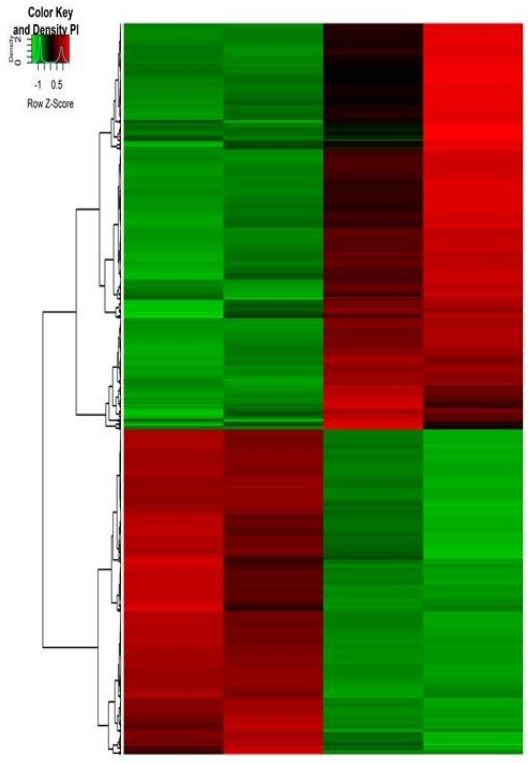


Molecular and cellular Functions

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.*

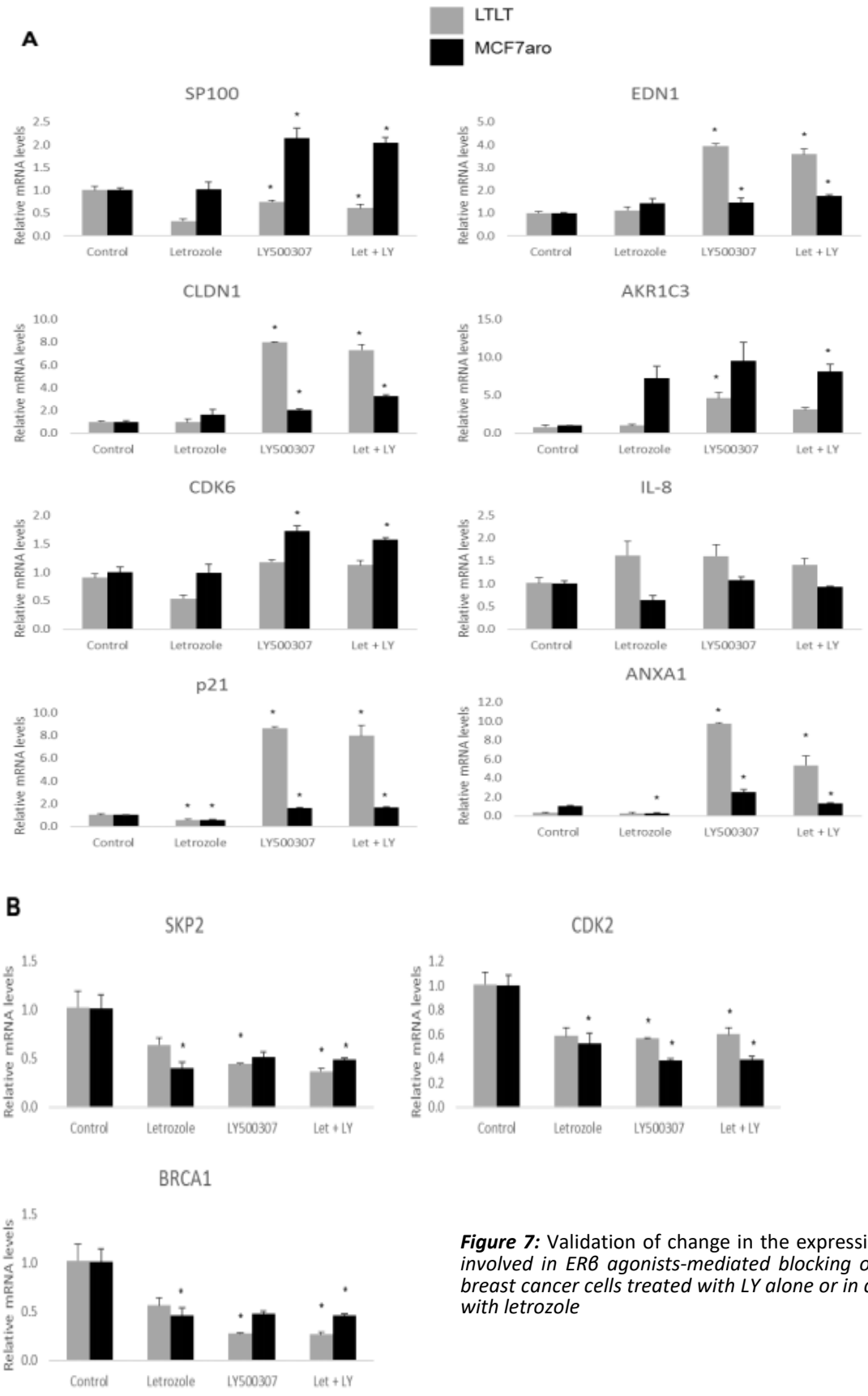


Figure 7: Validation of change in the expression of genes *involved in ERβ agonists-mediated blocking of growth of breast cancer cells treated with LY alone or in combination with letrozole*

Results for Object 4: *Establish the role of 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. 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 confirmed with sequencing the presence or required mutation in the clones was confirmed by sequencing. Work is in progress using these clones.

Third Year Grant Period from: 07/01/2018-06/30/2019

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.

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)***.

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.

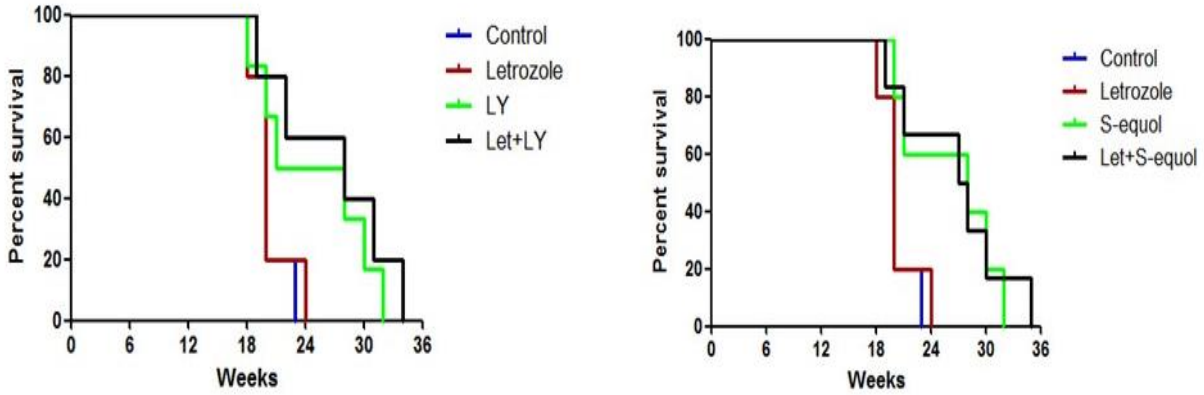


Figure 1. *Effect of ERβ agonists on tumor incidence in HER-2/neu transgenic mice. LY500307 (left panel) and S-equol (right panel) were effective in delaying the formation mammary tumors.*

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.

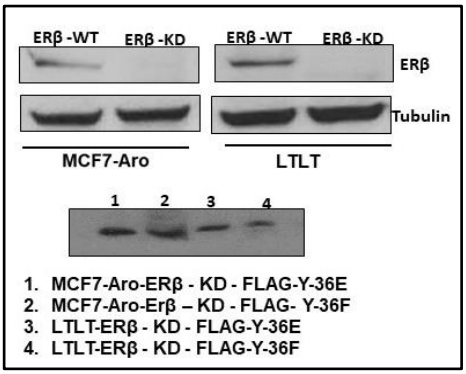


Figure 2: Demonstration of lack of ERβ expression: After knocking down ERβ expression using CRISPR approach, modified ERβ proteins were expressed to test the importance of phosphotyrosine switch

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.

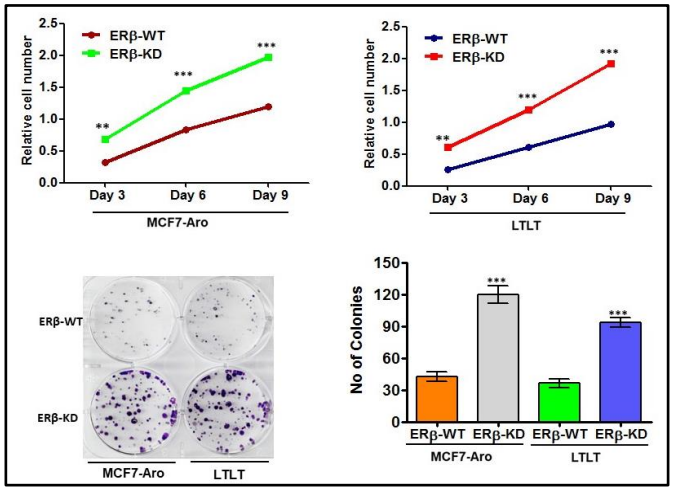


Figure 3: Lack of ERβ (knockdown) affects the growth of breast cancer cells in culture and colony formation

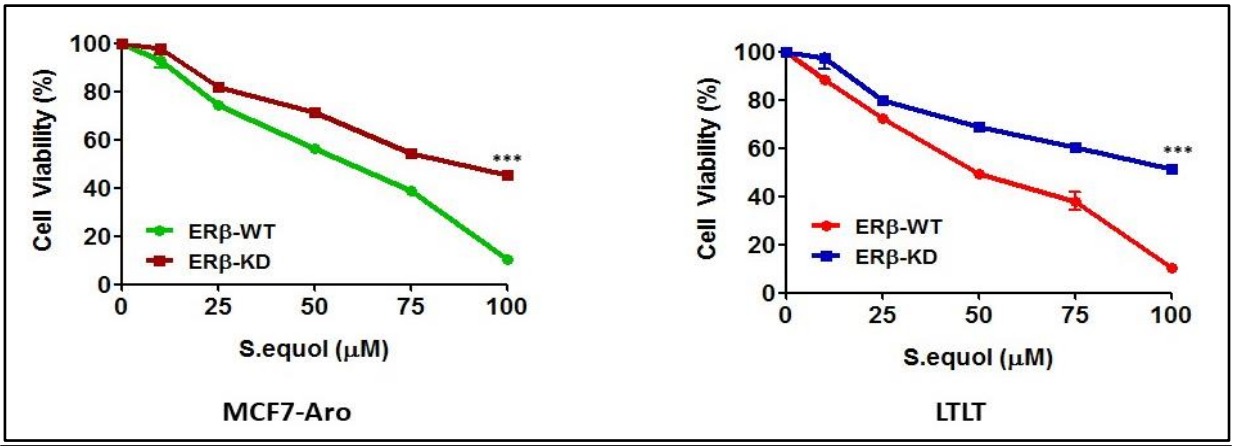


Figure 4: ERβ knockdown abrogates ERβ agonist-mediated growth suppression of breast cancer cells.

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

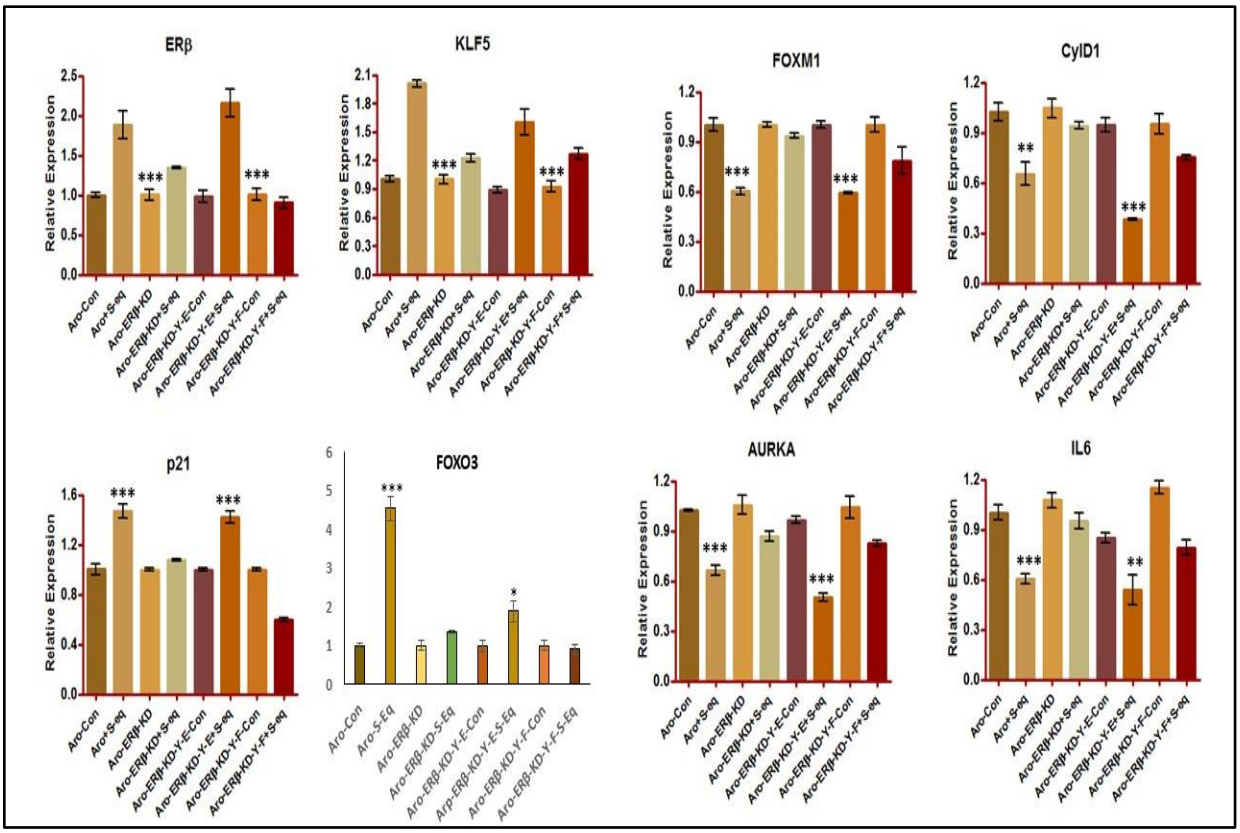


Figure 5: Constitutive activation of ERβ (phosphorylation) affects the regulation of a number of ERβ dependent genes in the presence of its agonist S-equol.

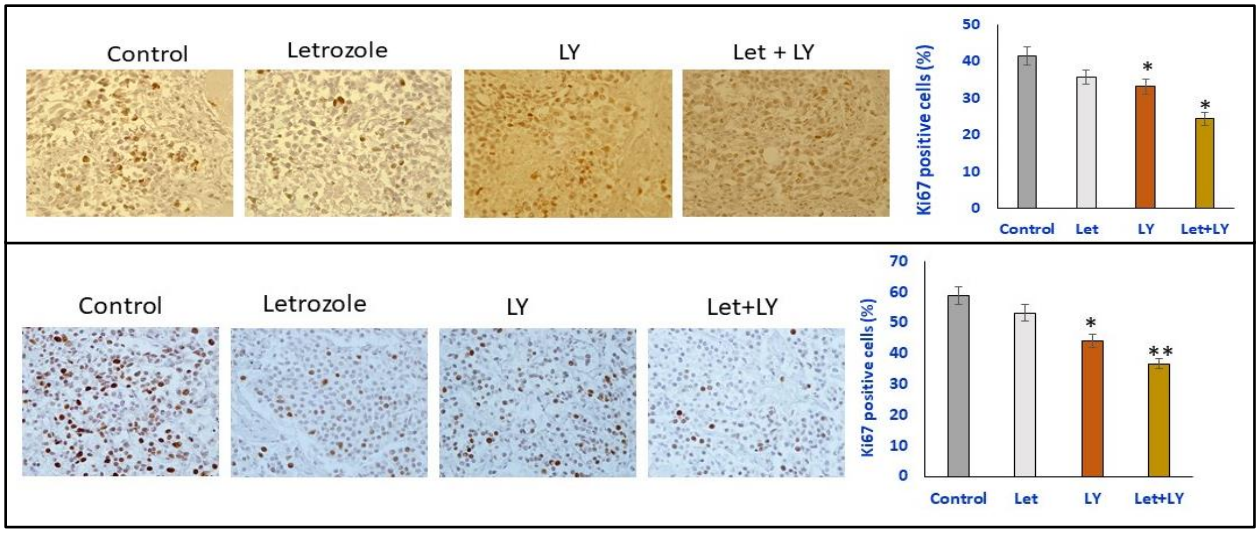


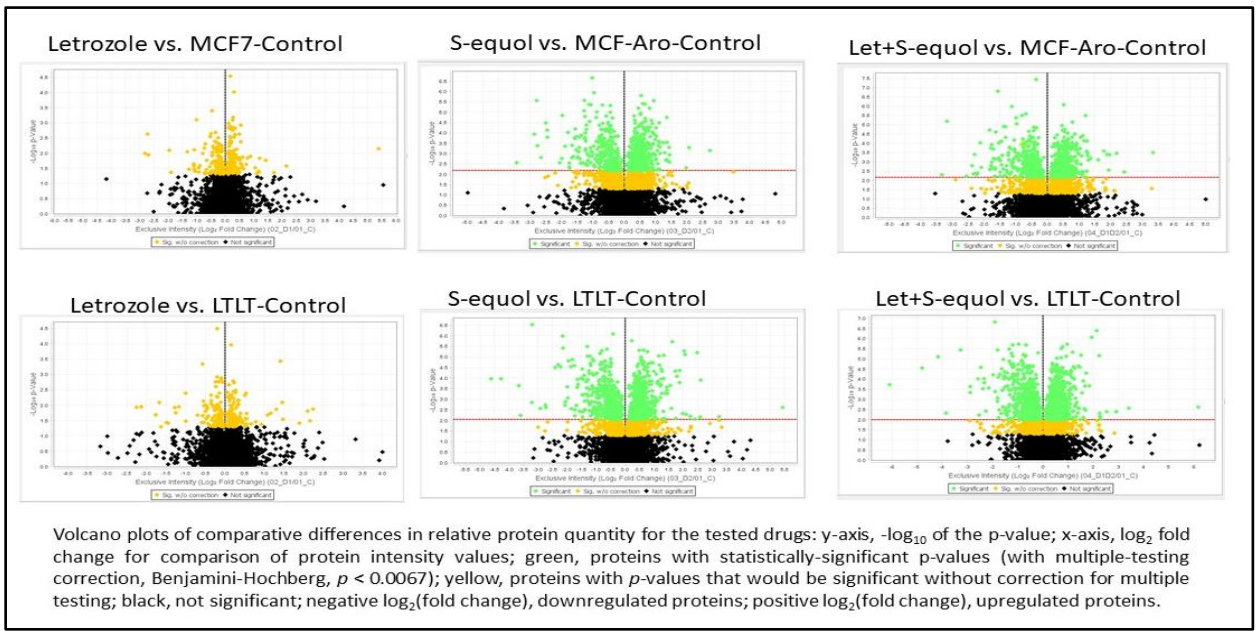
Figure 6: Effect of ERβ agonists on the growth PDX tumors. PDX model tumor COHSC-31 (top panel). PDX model TM00107 (lower panel). ERβ agonist, LY500307 (LY) was very effective in blocking the proliferation of PDX tumor alone or in combination with aromatase inhibitor letrozole (Let).

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.

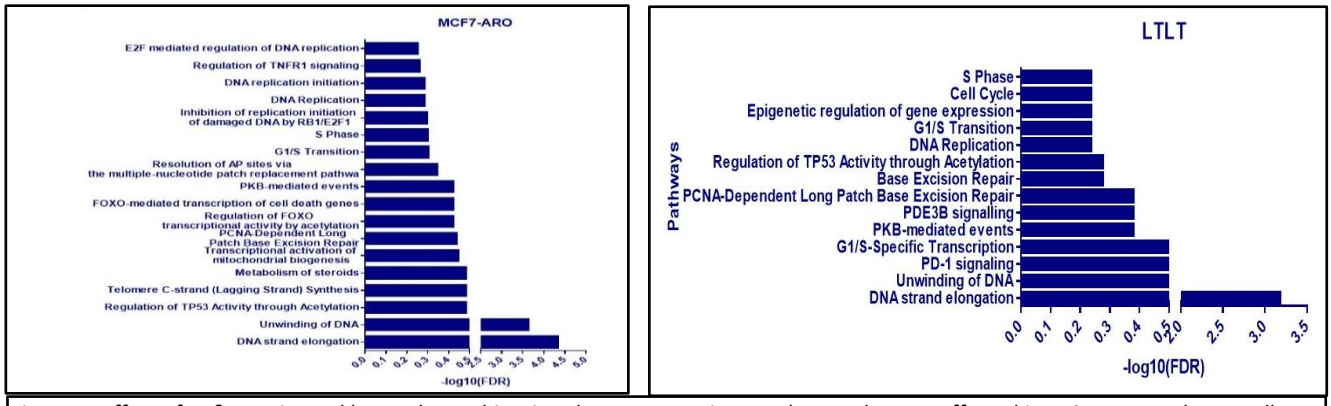


Figure 7: Effect of ERβ agonist and letrozole combination therapy on various pathways that are affected in MCF7-Aro and LTLT cells.

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.

No-Cost Extension (Fourth Year) 07/01/2018-06/30/2019 Period

Major Activities:

During the no-cost extension period we have continued work with sub-tasks

1. Established the effect of ERβ agonists in blocking the tumor growth in genetically engineered mice models.
2. Tested whether ERβ agonists affect the growth of ER positive breast PDX models
3. Identified mechanistic pathways that contributes to tumor suppressive actions of ERβ in human hormone therapy sensitive and resistant models.
4. Tested the importance of ER mutations in the therapy resistance

Specific Objectives:

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

Work on Objectives continued during no-cost extension period:

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***). *Completed and reported in previous report periods. Continued work with third tumor model during this period (reported below)*

Objective 2: Test the effects of ERβ agonists effect on the growth of ER positive breast PDX models (***Major Task 2 - Subtask 3: 13-36 months). Tested the effects of two other ERβ agonists during this period and finding are reported below.***

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 3-Subtask 2:13-36)***. ***Continued investigations using both genomic and proteomic approaches and findings are reported below.***

Significant Results (outcomes):

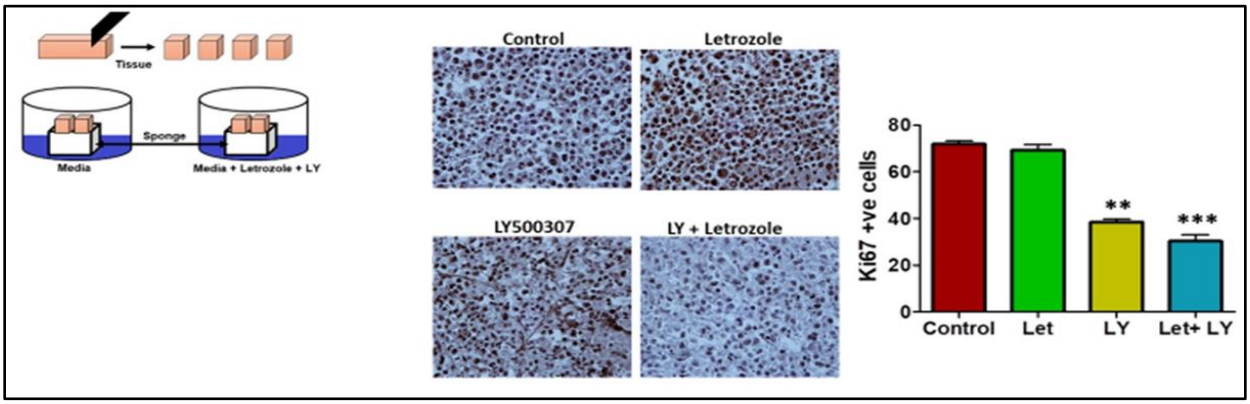


Figure 1. Effect of ERβ agonist on EO771 tumor growth assed using ex vivo approach. Growth was quantified using Ki67 staining. Growth was compared between control and treatment groups

Results for objective 1: To test the therapeutic efficacy of ERβ agonists to block the tumor growth, we used *ex vivo* approach employing EO771 (C57/B6 genetic background) tumor model. We also examined change in the expression of genes involved in cell cycle that are known to affected with and without treatment in all three models. EO771 mouse mammary tumor grown in C57/B6 syngenic host. Similar in size tumor tissues collected from freshly harvested tumor was cultured using ex vivo approach and tested for tissue growth in the presence or absence of treatment along with appropriate controls. As shown in **figure 1**, there was no significance difference in the growth between untreated control or letrozole (Let) treatment alone. Whereas treatment with ERβ agonist LY50037 (LY) or combined with letrozole there was significant decrease in the tumor growth as measured using proliferation marker Ki67. Results presented here and combined with previously reported findings clearly suggest that combination therapy with letrozole and with any one of ERβ agonists is very effective in blocking tumor growth in syngenic mouse mammary

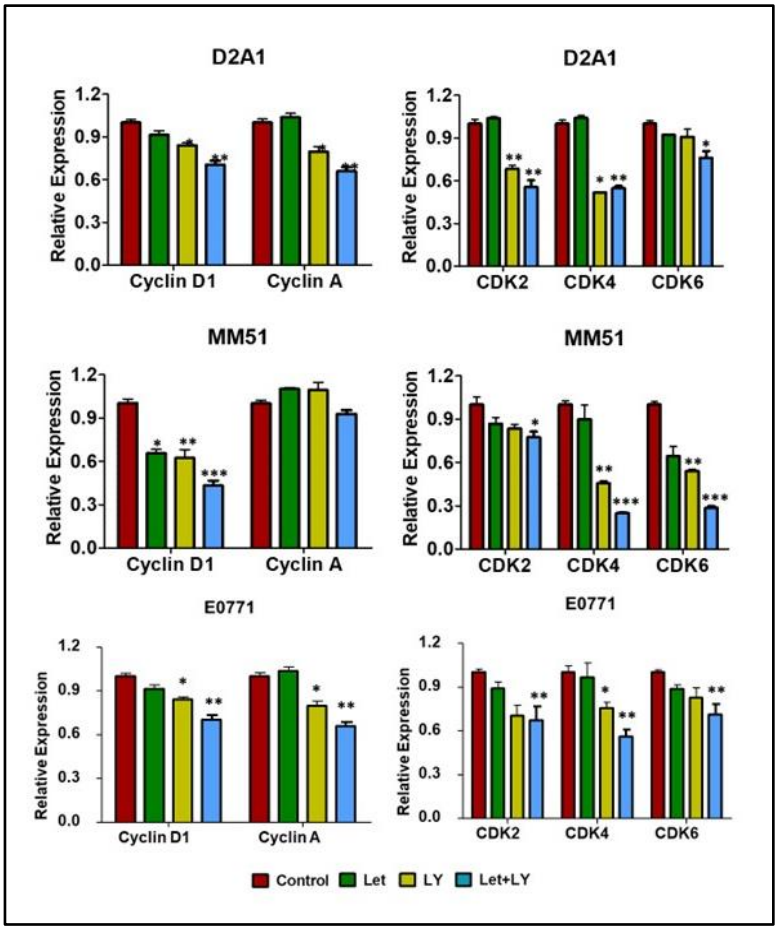


Figure 2: Effect of ERβ agonist on genes involved in cell cycle in syngenic mouse mammary tumor models. P<0.05 or less in comparison between controls and experimental groups.

tumor models. One of the main pathways that appears to modulate ERβ agonists antitumor inhibitory activity appears to be mediated by cell cycle regulation in all three mouse syngenic tumors models (**Fig. 2**).

Results for Objective 2: To evaluate the antitumor activity of ERβ agonists, in a biologically relevant *in vivo* models, we used two different PDX models. Mice were engrafted with an ER+ PDX, COHSC-31 (kind gift from Dr. Chen, City of Hope) and other 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.3**) is shown with PDX models COHSC-31 and TM00107 using liquiritigenin and S-equol, letrozole alone or in combination with both. Compared control, Liq or S-equol decreased the proliferation (as determined using Ki 67 staining pattern) of PDX tumors and effect was highly significant when combined with letrozole. We have seen similar effect with LY other ERβ agonist (reported before).

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.

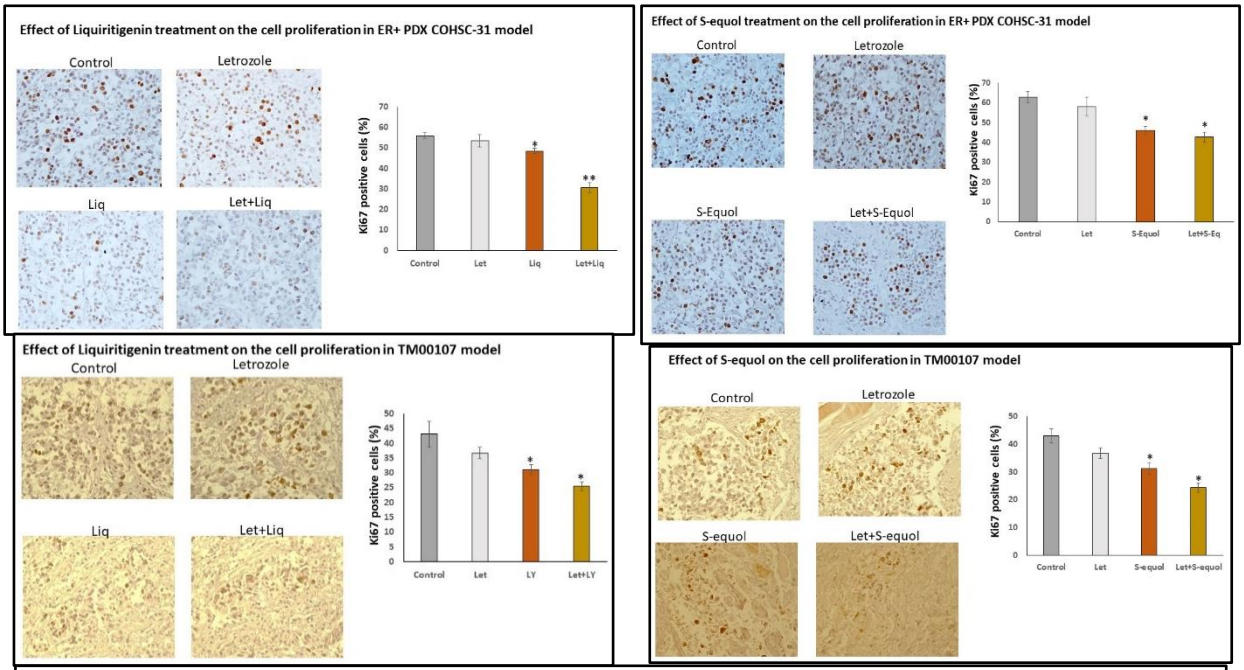


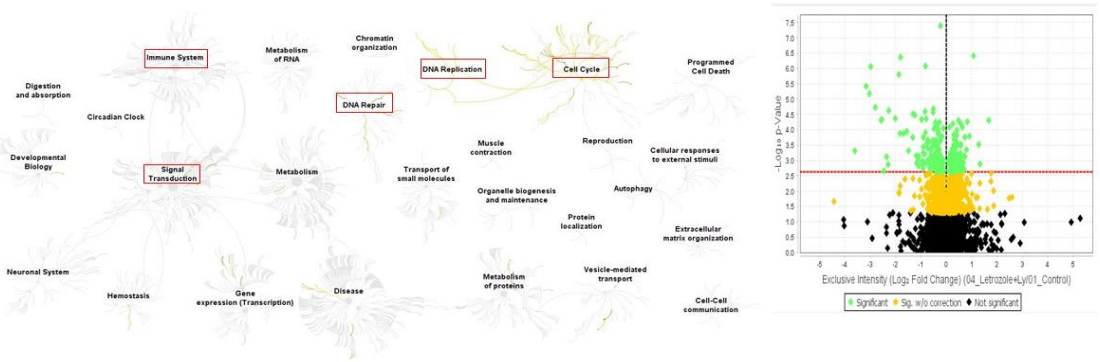
Figure 3: Effect of ERβ agonists on the PDX tumors. Effect of both liquiritigenin and S-equol were tested on COHSC-31 (top row) and TM00107 (bottom row). Decreased proliferation was significant in both models due to ERβ agonists treatment ($p<0.05$).

Results for Objective 3: To study what mechanisms regulates the tumor suppressive functions of ERβ, we have used both genomic and proteomic approaches. During the previous years, we have reported the findings using genomic approach. During no-cost extension period, we have continued our studies that 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 and ERβ agonists LY50037 or liquiritigenin processed for mass spectrometric and pathway analyses. As shown in **figure 4** 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 agonists LY50037 or liquiritigenin alone or in combination modulates a number of different pathways both in MCF-7-ARO (hormone therapy sensitive) and letrozole resistant (LTLT) cells as showed by Reactome pathway analysis.

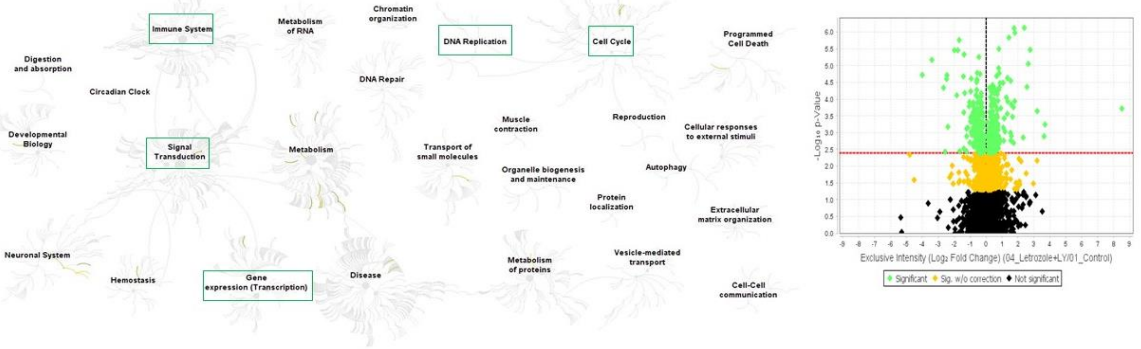
These findings suggest ERβ agonists affects the regulation of ERβ-dependent actions by regulating different pathways both in hormone therapy sensitive and resistant breast cancer cells. Biomarkers will help to design specific therapeutic approaches and evaluate therapeutic efficacy using identified biomarkers in future studies.

Effect of ERβ agonists on the growth and genes that are modulated by ERβ in ERα wild type and mutant breast cancer cells: Emerging evidence suggest that one of the mechanism that affects the response to hormone antagonist such as tamoxifen or aromatase inhibitor therapy that leads to development of resistance to these agents is due to specific mutants that arise in ERα. To test how mutations in ERα that affects the tumor suppressive/growth inhibiting effects ERβ and its agonists action, we have tested the effect of ERβ agonists on the growth of both wild type and ERα mutant (D538G and Y537S) ZR-75 breast cancer cells. We have determined the effect of ERβ agonists, LY50037, liquiritigenin or S-equol on the growth of both. ERα–WT and two ERα mutant ZR-75 cells. Using RT-qPCR analyses, we also tested how these agonists affected the expression of selected genes, including cell cycle modulators, cells.

Global Mass Spec based DIA analyses of whole cell lysates identified unique pathways modulated by Let+LY500307 in MCF7-Aro cells compared to controls



LY500307 and in combination with letrozole treatment modulated different pathways analyzed by Global Mass Spec in LTLT cells



LY500307 and in combination with letrozole treatment modulated different pathways analyzed by reactome pathway analysis in both MCF7-Aro and LTLT cells

MCF7-Aro-Let+LY

LTLT-Let+LY

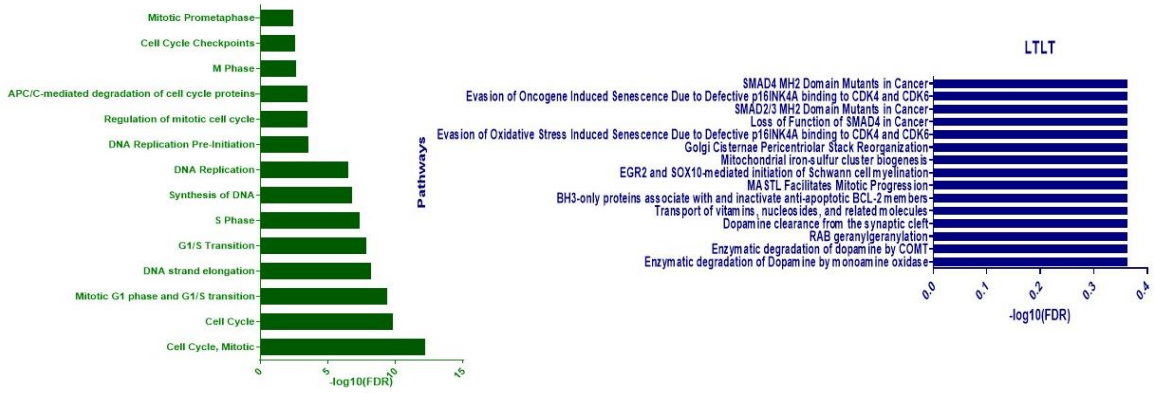


Figure 4: Effect of ERβ agonist and letrozole combination therapy on various pathways affected in MCF-7-Aro and LTTL cells.

As shown in **figure 5**, ERβ agonists are we effective in blocking the growth of ERα wild type and mutant ZR-75 breast cancer cells suggesting these agents are very efficacious in blocking the growth of therapy resistant breast cancers. We further examined how these agents affected the expression of genes that are modulated by ERβ agonists and that block the growth of therapy resistant breast cancer cells

As shown in **figure 6** all three ERβ agonists have shown similar effect on the expression of genes that are modulated by these agents suggesting that tumor suppressive actions of ERβ are not affected due to mutations in ERα. These findings suggest ERβ agonists are very effective in controlling the growth of both breast tumors that express wild type ERα or mutant forms of this receptor. These findings further imply that ERβ agonists provide effective tumor suppressive benefit both types of tumors that is which are sensitive to aromatase inhibitor or resistant to AI therapy.

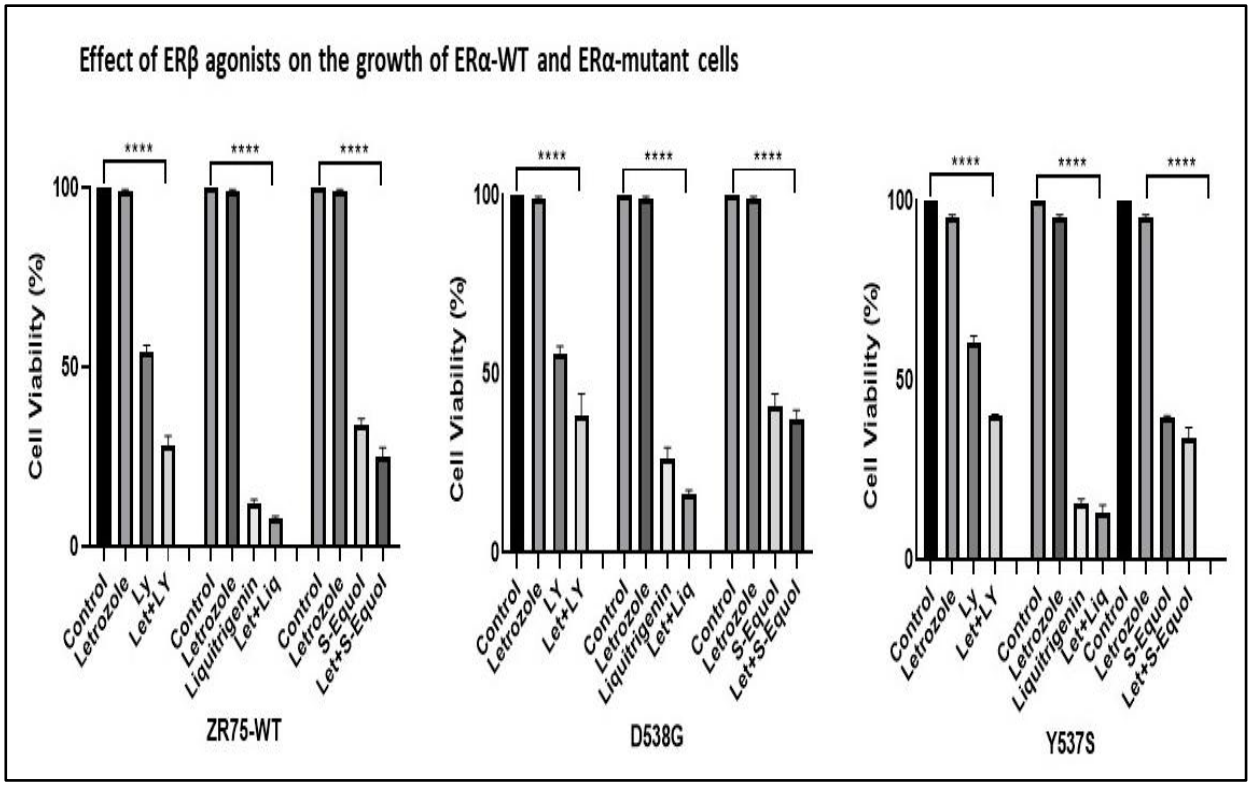


Figure 5: Effect of ERβ agonists on the growth of breast cancer cells (ZR-75) that express either ERα wild type or mutant form that contributes resistance to hormonal therapies.

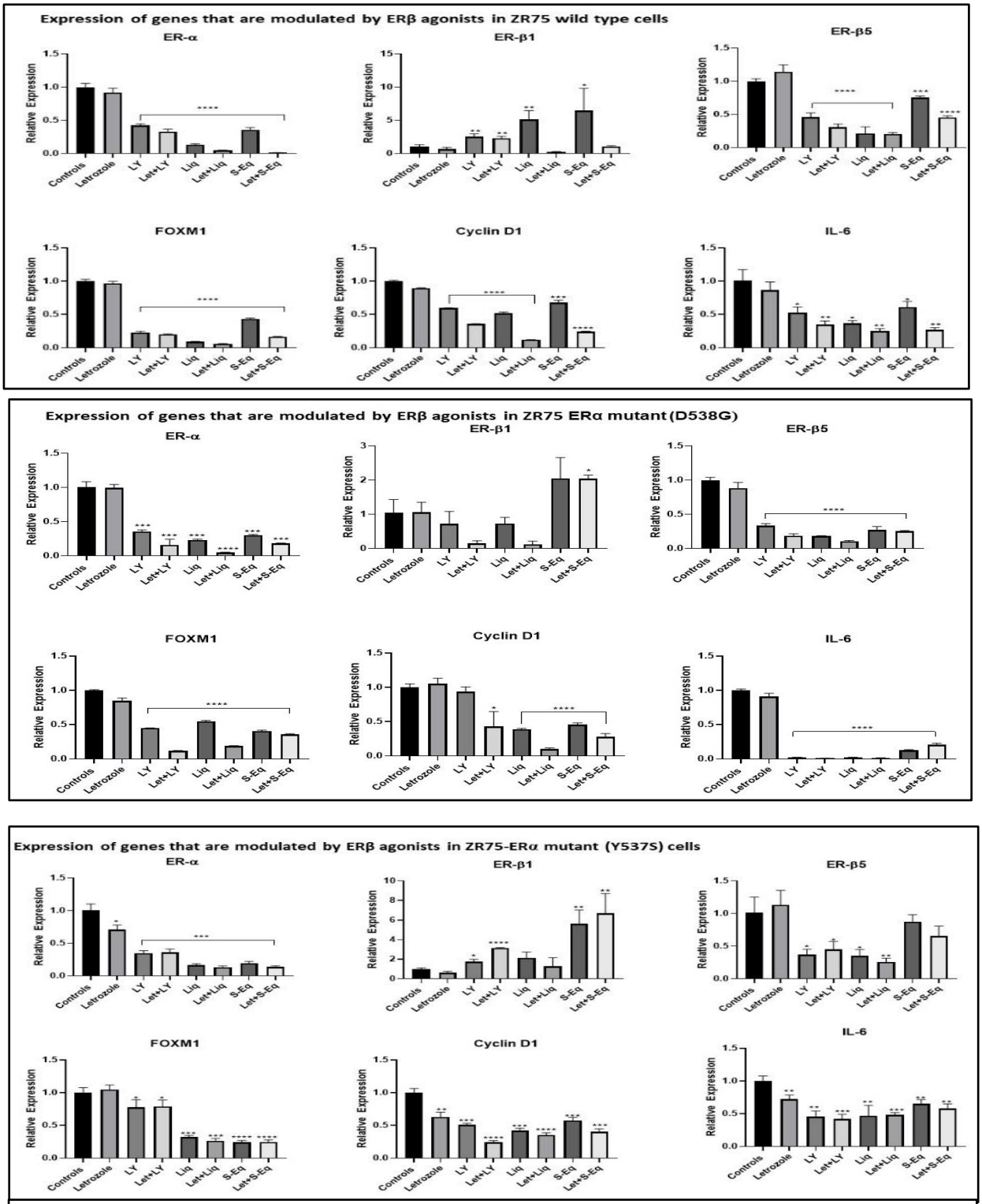


Figure 6: Effect of ERβ agonists on the genes modulated by ERβ in ZR-75 breast cancer cells that express either ERα wild type or mutant form that contributes resistance to hormonal therapies

Major Findings/Conclusions for the entire funding period:

- *Using genetic models, we have shown that ERβ/its agonists reduced mammary growth and initiation of malignant changes.*
- *Ours was first study that demonstrated ERβ/its agonists prevents/delays the mammary cancer initiation and its progression.*
- *ERβ/its agonists were able to restore sensitivity to endocrine therapy resistant cells to hormone therapies.*
- *Using immune-competent mammary cancer models, we have shown that ERβ/its agonists reduced tumor growth by altering the mechanisms involved in cell cycle and other pathways.*
- *Alteration of pathways that contribute to tumor suppressive functions of ERβ appears to different based on the type of ligand (agonist) that induced this receptor based on the expression of known affected genes and other identified using genomic approach.*
- *Activated ERβ (phosphorylated form of ERβ) is critical for its action that is 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 positive and negative 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).*
- *ERβ/its agonists exert similar effect on blocking the growth and modulating the expression genes involved in tumor suppressive functions of ERβ in breast tumors that express either wild type ERα or its mutant forms that contribute resistance to endocrine therapies.*

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

This project provided training opportunities for 15 students (2 medical,1 graduate and 12 high school) and three post-doctoral fellows (one MD and two 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 UTHealth San Antonio cancer center workshops, and attendance of grand rounds, and attendance of ethics seminar series. Further, postdoctoral fellow attended AACR-SABCS and AACR annual meeting to present our research findings.

How were the results disseminated to communities of interest?

Findings was presented to scientific community at four San Antonio Breast Cancer Symposium (international meeting attended ~7500 from 80 countries) and four American Association of Cancer Research (AACR) annual meetings during the grant period.

3

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

We also are in the process of preparing manuscripts to be published in a peer reviewed scientific journals using findings from these studies. COVID-19 impaired completion of manuscript submission. Several manuscripts are at the final stage of submission.

4. IMPACT:

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

These studies provided evidence for therapeutic efficacy of estrogen Receptor B 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. Based on the observations from this study, a phone 0/proof principle clinical study was initiated by UTHSCSA NCI-designated cancer center. Under the Leadership of one of the investigators from this project and a medical onconlogist completed these studies. Findings from this neoadjuvant trial was reported at 2020 ASCO meeting by the study PI (Dr. Lathrop, Kate I) of this study (https://meetinglibrary.asco.org/record/190852/abstract).

What was the 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. Therapeutic use of ERβ agonists on delaying the progression of glioblastomas of the brain are very significant in preclinical models and human PDX models. UTHSCSA NCI-designated Cancer center is exploring to bring this to clinical trial once the funding is identified. 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. In addition, provided an opportunity use concepts to study the regulation of ERβ levels using specific ligands to test the therapeutic efficacy to glioblastomas in rodent models. Efforts are in progress to have UTHealth San Antonio own version of ERβ agonist to initiate early phase clinical trials to treat glioblastoma patients.

What was the impact on technology transfer?

The results of this study provided rationale to examine the efficacy of ERβ agonists blocking the growth of ovarian and endometrial cancers along with endometriosis. Some of these findings also 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?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No changes and there are nothing to report

Actual or anticipated problems or delays 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 overcome this problem and accomplished set objective.

Nothing to Report

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

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:

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

Journal publications.

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

Covid-19 disruption slowed the completion and submission of the manuscripts, we are catching up with communicating all findings with other manuscripts along with the following two.

- 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)

Concepts based these studies have resulted in publication of other manuscripts focusing ER β agonists (PubMed 29661831; 30992459; 33470499)

Books or other non-periodical, one-time publications.

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. Published 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.

1. Samayoa, C., Krishnegowda, N.K., Vadlamudi, R.K., and **Tekmal R.R**.: Pre-clinical investigation of estrogen receptor β agonists for the treatment of breast cancer. 39th Annual San Antonio Breast Cancer Symposium, P6-11-15, 2016. Cancer Res 2017;77(4 Suppl): Abstract nr p6-11-15
2. Ramasamy, K., Samayoa, C., Krishnegowda, N.K., Vadlamudi, R.K., and **Tekmal R.R.**: Potential therapeutic use of Estrogen Receptor β agonist in the prevention and progression of breast cancer using HER-2/neu mouse model. Proc. Am. Assn. Cancer Res., 108th Annual Meeting, Abstract 3616, 2017. Cancer Res 2017;77(13 Suppl): Abstract nr3616.doi:101158/1538-745-AM2017-3016
3. 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.
4. Ramasamy, K., 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
5. 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.
6. 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
7. Ramasamy K, Chen, S., Suryavathi, V., Li, R., Vadlamudi RK, and **Tekmal RR.**: Estrogen receptor β agonist S-Equol promotes letrozole sensitivity of endocrine therapy resistant breast cancer cells by upregulating FOXO3 expression 42nd Annual San Antonio Breast Cancer Symposium, P6-04-19, 2019.
8. Ramasamy, K., Suryavathi, V., Suresh Kumar, M.A., Weintraub, S., Vadlamudi, R.K., and **Tekmal, R.R**.: Estrogen receptor β agonists reverse letrozole therapy resistance of breast cancer through FOXO3 and FOXM1 mediated actions. Proc. Am. Assn. Cancer Res., 112th Annual Meeting, Abstr. 4365, 2020

Website(s) or other Internet site(s)

None

- **Technologies or techniques**

Concepts on Potential use ERβ agonists as therapeutic agents to treat glioblastomas using *in vitro* and *in vivo* models

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

substitute for any other invention reporting required under the terms and conditions of an award.

None

- **Other Products**

Modified cell lines and *in vitro* as well as *in vivo* models will be available once the studies are published with scientific community

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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 phosphorylation using ERβ knock in and knock out models. **Rong Li, Ph.D. Separated before no-cost extension period from UTHSCSA. He served as collaborator. His separation did 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?

No change.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

See the attached copy of the abstracts presented at national and international meetings based on this study.

Cancer Research

Poster Session Abstracts

Abstract P6-11-15: Pre-clinical investigation of estrogen receptor β agonists for the treatment of breast cancer

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

DOI: 10.1158/1538-7445.SABCS16-P6-11-15 Published February 2017

Abstracts: 2016 San Antonio Breast Cancer Symposium; December 6-10, 2016; San Antonio, Texas

Abstract

Breast Cancer is the primary cause of cancer-associated mortality worldwide, and in United States alone, more than 250,000 women are diagnosed every year. Current breast cancer treatment strategies focus on Estrogen Receptor α signaling, given that the majority of cases diagnosed are ERα positive. These treatment strategies include endocrine therapies; such as anti-estrogens or aromatase inhibitors. Although, endocrine therapy has been demonstrated to be successful and effective, therapy resistance commonly arises and results in relapse. While current endocrine therapies focus on ERα signaling, emerging studies highlight the importance of Estrogen Receptor β.Unlike ERα, ERβ has been shown to have tumor-suppressive function in various cancers, including breast cancer. Recent studies have identified, synthesized, and tested the clinical safety of ERβ-selective agonists. The objective of this study was to investigate the utility of using ERβ agonists in the treatment of breast cancer.

To investigate the utility of ERβ agonists in the treatment of breast cancer, we used in-vitro and in-vivo pre-clinical models systems. Our results demonstrated that treatment with ERβ agonists, S-Equol and LY500307, was able to inhibit the short-term and long-term growth of both endocrine therapy sensitive and resistant breast cancer cells. Progression through the cell cycle, cell migration and cell invasion was also abrogated upon treatment. In-vivo, our syngeneic tumor mouse model demonstrates a decline in tumor growth rate after treating with a combination of letrozole and ERβ agonist. Gene expression array analysis reveal that treatment with ERβ agonist elicits changes in key signaling molecules involved in cell death and cell cycle pathways. In Letrozole resistant cells, Letrozole treatment had not effect on gene expression, while LY500307 treatment resulted in the modulation of 780 genes. Interestingly, combining Letrozole with LY500307 resulted in the modulation of 966 genes, of which 417 were unique to the combination treatment. Our studies suggest that activation of ERβ signaling is a valuable strategy in the treatment of breast cancer, even in cases which have developed resistance to current endocrine therapies.

Citation Format: Samayoa C, Krishnegowda NK, Vadlamudi RK, Tekmal RR. Pre-clinical investigation of estrogen receptor β agonists for the treatment of breast cancer [abstract]. In: Proceedings of the 2016 San Antonio Breast Cancer Symposium; 2016 Dec 6-10; San Antonio, TX. Philadelphia (PA): AACR; Cancer Res 2017;77(4 Suppl):Abstract nr P6-11-15.

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Endocrinology

Abstract 3616: Potential therapeutic use of Estrogen Receptor β agonist in the prevention and progression of breast cancer using HER-2/neu mouse model

Kumaraguruparan Ramasamy, Cathy Samayoa, Naveen K. Krishnegowda, Ratna K. Vadlamudi, and Rajeshwar R. Tekmal

DOI: 10.1158/1538-7445.AM2017-3616 Published July 2017 Check for updates

Proceedings: AACR Annual Meeting 2017; April 1-5, 2017; Washington, DC

Abstract

Breast cancer is the most common cancer among women worldwide. Hormone-mediated therapy to treat estrogen receptor alpha (ERα) positive breast cancers include the use of ER antagonist, Tamoxifen, aromatase inhibitors and other compounds that degrade ERα. Unlike ERα, ERβ has been shown to have tumor-suppressive function in various cancers, including breast cancer. Recent studies have identified, synthesized, and tested the clinical safety of ERβ-selective agonists. Given the tumor-suppressive properties of ERβ, it may be possible to use these compounds to induce or activate the ERβ and test their role in the chemoprevention and blocking the progression of breast cancer. We have investigated the therapeutic utility of ERβ agonists in the prevention and progression of breast cancer using MMTV-HER2/neu mice transgenic mouse model. MMTV-HER2/neu mice develop premalignant lesions at 4-5 months, and tumors starting at month 7 due to overexpression of the HER2/neu proto-oncogene. MMTV-HER2/neu mice were treated with an ERβ agonist, LY500307 examined the prevention and progression of mammary cancers in these mice. When compared to controls, ERβ agonist-treated mice exhibited a significant decrease in the development of preneoplastic changes. Differential gene expression analysis revealed a significant change in the expression of a number of genes in response to LY500307 treatment. Pathway analysis identified an enrichment for chemokines signaling pathways, particularly TNF, in blocking the development of preneoplastic changes resulting from treatment with ERβ agonists. Our studies also show a decrease in the formation of mammary tumors in HER-2/neu mice with preexisting preneoplastic changes when treated with LY500307. This study suggests that ERβ agonist treatment may be a valuable therapeutic option for the prevention in women at increased risk of breast cancer and in blocking the progression of hormone receptor positive breast tumors.

Citation Format: Kumaraguruparan Ramasamy, Cathy Samayoa, Naveen K. Krishnegowda, Ratna K. Vadlamudi, Rajeshwar R. Tekmal. Potential therapeutic use of Estrogen Receptor β agonist in the prevention and progression of breast cancer using HER-2/neu mouse model [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2017; 2017 Apr 1-5; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2017;77(13 Suppl):Abstract nr 3616. doi:10.1158/1538-7445.AM2017-3616

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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

Article Info & Metrics

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

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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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

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.

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P6-04-19. Estrogen receptor β agonist S-Equol promotes letrozole sensitivity of endocrine therapy resistant breast cancer cells by upregulating FOXO3 expression

December 14, 2019, 7:00 AM - 9:00 AM

Authors

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Disclosures

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

Abstract

Background: Breast cancer (BC) is the most frequently diagnosed cancer and the primary leading cause of cancer deaths in women worldwide. Although the clinical outcome of BC patients has been considerably improved, resistance to endocrine and chemotherapy treatments contributes to BC relapse and mortality. Understanding the molecular pathways that contribute to BC resistance will enable to develop new treatments for improving the efficacy of endocrine therapy. Recent studies suggested that estrogen receptor beta (ERβ) and its ligand S-Equol promote suppression of BC progression. However, the mechanisms by which S-equol inhibits growth of BC cells and enhance therapy response remains unknown. Here, we examined the mechanisms by which S-equol contribute to BC growth inhibition in hormone therapy sensitive and resistant BC cells.

Methods: We evaluated the therapeutic efficacy of S-equol in therapy sensitive (MCF7-Aro) and letrozole resistant (MCF7-Aro-LTLT) cells. Expression levels of target genes were evaluated in both cell lines by global RNA-seq analysis, data-independent acquisition (DIA) mass spectrometry analyses, qRT-PCR and Western blot. ERβ knock down cell lines were generated by CRISPR-Cas9 technology. Xenograft and patient derived BC explants (PDEX) were used for preclinical evaluation of the antiproliferative activity of S-equol.

Results: S-equol reduced the growth of endocrine sensitive and resistant BC models ***in vitro*** and ***in vivo***. Treatment of ER-positive PDEX with S-equol decreased the proliferation compared to untreated tumors. RNA-seq analysis of S-equol treated BC cells showed modulation of ERβ target genes involved in tumor progression and resistance to hormone therapies. Mass spectrometry based DIA analysis showed that S-equol treatment upregulated 438 proteins and downregulated 429 proteins in MCF7-Aro cells compared to controls. Whereas, DIA analysis in Letrozole-resistant cells revealed that S-equol upregulated 572 proteins and downregulated 481 proteins compared to controls. Reactome pathway analysis revealed that S-equol treatment modulated the proteins involved in DNA replication, cell cycle, proteins regulating p53 activity and VEGF-VEGFR2 pathway. Our mechanistic studies confirmed that treatment with S-equol upregulated FOXO3 and downregulated FOXM1. Using CRISPR-Cas9 mediated ERβ knockout cells, we confirmed that S-equol could not modulate forkhead box transcription factors in BC cells lacking ERβ compared to parental cells.

Conclusions: Collectively, our results suggest that S-equol exhibits its antitumor activity by targeting forkhead box transcription factors and that S-equol might be an effective treatment strategy in treating / preventing breast cancer recurrence. (Supported by BCRP 151884/DOD grant W81XWH-16-1-0294).

Session PO.EN02.01 - Molecular, Preclinical, and Clinical Endocrinology 2

Add to My Itinerary

4365 / 8 - Estrogen receptor β agonists reverses letrozole therapy resistance of breast cancer through FOXO3 and FOXM1 mediated actions

June 22, 2020, 9:00 AM - 6:00 PM

Virtual Meeting II: E-Posters

Presenter/Authors

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Disclosures

K. Ramasamy: None. **S. Viswanadhapalli:** None. **S. M.a.:** None. **S. Weintraub:** None. **R.K. Vadlamudi:** None. **R.R. Tekmal:** None.

Abstract

Introduction: Breast cancer (BC) is the most common cancer in women worldwide and leading cause of cancer death. More than 70% of cancers are estrogen receptor alpha (ERα) positive. Despite effective therapeutic strategies for treating hormone receptor-positive (HR+) breast cancer, resistance to endocrine therapy that is either de novo or acquired still occurs. Understanding the molecular pathways that contribute to BC resistance will enable to develop new treatments for improving the efficacy of endocrine therapy. Studies have shown that estrogen receptor beta (ERβ) and its agonists inhibits the different types of cancers including BC. Herein, we investigated mechanism(s) for therapeutic efficacies of ERβ agonists in breast cancer.

Methods: To investigate the mechanisms of ERβ agonists (S-Equol and LY500307), in the treatment of breast cancer, we used therapy sensitive (MCF7-Aro) and letrozole resistant (MCF7-Aro-LTLT) cells and ERβ knock down cell lines, generated by CRISPR-Cas9 technology. The wild type and ERβ knock down cells with or without treatment of ERβ agonists were analyzed, for cell proliferation, protein (Western), Gene expression array analysis, RNA-seq analysis, data-independent acquisition (DIA) mass spectrometry analyses and RNA (RT-qPCR) analysis. Xenograft and patient derived BC explants (PDEX) were used to test the antiproliferative activity of ERβ agonists.

Results: Our results demonstrated that treatment with ERβ agonists, S-Equol and LY500307 inhibited the growth of both endocrine therapy sensitive and resistant breast cancer cells. Gene expression array analysis of LY500307 treated breast cancer cells showed the modulation of signaling molecules involved in cell death and cell cycle pathways. RNA-seq analysis showed that treatment with S-equol modulated the ERβ target genes involved in tumor progression and resistance to hormone therapies. Mass spectrometry based DIA analysis and Reactome pathway analysis revealed that S-equol treatment modulated the key proteins involved in DNA replication and cell cycle. Both ERβ agonists S-Equol and LY500307 inhibited the cell proliferation of parental cells whereas they could not elicits the inhibitory effect on the knock out cells. By using ERα positive PDX tumors, we have demonstrated that both the ERβ agonists inhibited the proliferation of human BC tumors. Our mechanistic studies confirmed that increased FOXO3 activation by S-equol in LTLT cells can reverse letrozole resistance. Our results showed that inhibiting FOXM1 by ERβ