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Research Article

More Evidences for the Possible Role of Err Beta (ERRβ) as a Tumor Suppressor in Estrogen Receptor Positive and Negative Breast Cancer

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Abstract

Estrogen-related receptors (ERRs) are a group of nuclear receptors that share sequence homology with ER whereas do not bind to Estrogen. In this article, studies were conducted with ERRβ, which is considered to have antiproliferative capabilities, to summarize its effects upon overexpression and knockdown. Clonogenic assay, and vivo zebra fish model tumour xenograft assay was used to detect the tumour growth and suppression. Wound healing and trans well migration as well as invasion assays were used to detect the metastasis in breast cancer. In this study, we established ERRβ as a possible tumour suppressor. Furthermore, epithelial-mesenchymal transition (EMT) pathway was checked with respective markers and it was observed that ERRβ overexpression in MCF7 and MDAMB -231 cells reduced the expression of mesenchymal markers where in promoting an upregulation of epithelial markers. Using triple negative MDAMB 231 cell lines, the zebra fish model was also utilized to check for tumor growth and migration. It was discovered that overexpression of the ERRβ resulted in a considerable reduction in the size of the tumor and its metastasis. Overall, our research showed that ERRβ interferes with breast cancer cell ability to proliferate and metastasize. Therefore, emphasizing on the function of ERRβ and, in turn, the process of EMT, may prevent the oncogenesis and metastasis in breast cancer cells.

Keywords: Breast Cancer; Estrogen Related Receptor Beta (ERRβ); Estrogen Receptor; Estrogen Related Receptor

Abbreviations

BC: Breast Cancer; ER: Estrogen Receptor; ERR: Estrogen Related Receptor; ERRβ: Estrogen Related Receptor Beta

Introduction

Breast cancer is the most common malignancy affecting women worldwide and as per 2020 GLOBOCON data the reported number of new cases is around 2.3 million world-wide. It is the most prevalently diagnosed cancer and taking the 5th position for cancer related deaths [22]. Based on the expression of Estrogen, Progesterone and Human Epidermal growth factor breast cancer is categorized into 4 major molecular subtypes such as luminal A (ER+/HER^{-}), luminal B (ER+/HER2-or HER2+), triple negative/basal type and HER2 type. Among the subtypes luminal subtype comprise of 60-70% having high potential towards endocrine therapy. But as of now treatment for ER negative breast cancer is yet to be explored and this demands intensive research to unravel potential therapeutic molecular targets.

BC accounts for the second leading cause of cancer related deaths in women worldwide [15]. However, due to lack of therapy, Estrogen receptors (ERs) play an important role in breast cancer progression and treatment. Estrogen related receptors (ERRs) are a group of orphan nuclear receptors, which have sequence homology with ERs and share target genes whereas binding of any natural ligand to it is yet to be reported. Here, we will be investigating the possible roles of ERRβ in breast cancer. Recent studies from our group suggest the tumor suppressor role of ERRβ in breast cancer cells. Tamoxifen-resistant ER-negative breast cancer cell lines as well as ER-positive breast cancer cell lines cannot proliferate because of diethylstilbestrol (DES), an ERR ligand. These behaviours imply that possible treatment targets for ERR target genes should be examined [20]. While the expression of ERRα and ERRγ is positively connected [11] with the emergence of steroid receptor-positive breast cancer and tamoxifen resistance, the oestrogen-related receptor β controls tumorigenesis differently (20). Breast tumour cell fraction in S-phase is inversely linked with ERRβ mRNA expression levels, which may indicate that ERRβ inhibits cellular growth [15]. The ligand binding domain (LBD) and DNA binding domain (DBD) of estrogen-related receptors (ERRs) share a great deal of sequence homology with estrogen receptor.

The SF-1 response element (SFRE/ERRE: TnAAGGTCA), [10] which is an extended half ERE site, and full oestrogen response elements (traditional EREs: AGGTCAnnnTGACCT) are two types of response elements that ERRβ binds to as a homodimer (Sengupta D., *et al*.). In prostate cancer cells, ERRβ may activate the promoter of p21WAF1/CIP1, which is a universal inhibitor of cyclin dependent kinases (CDKs), and this receptor may have anti-proliferative characteristics [5] in breast cancer cells. Estrogen-related receptor-beta (ERRβ) may have anti-proliferative properties [1] in breast cancer cells, and this receptor activate the $p21^{WAF1/CIP1}$ promoter [26] in prostate cancer cells, which is a universal inhibitor of cyclin dependent kinases.

P21 expression decreases with estrogen treatment and the development of anti-estrogen resistance [16], which supports the importance of ERRβ as a therapeutic agent in breast cancer. Previous report from our laboratory demonstrated the abrogated expression of ERRβ in breast cancer cells. Ectopic expression of ERRβ inhibits cell proliferation through Breast cancer amplified sequence 2 (BCAS2) and Follistatin (FST) and also induces apoptosis in breast cancer cells [20]. Further, studies demonstrated that ERα regulates the expression of ERRβ through estrogen in breast cancer. There were elevated levels of ERRβ in normal breast tissues and ER+ve breast tumors compared to breast carcinoma and ER-ve breast tumors respectively [20]. Keeping in view the possible role of ERRβ as tumor suppressor gene, or a negative regulator of cell cycle and cancer cell proliferation, our study aims [15] to investigate the function of ERRβ in various pathways which leads to cancer progression.

Materials and Methods

Human cancer cell lines and Cell culture treatments

MCF7 and MDA-MB-231 cells were procured from cell repository of National Centre for Cell Sciences (NCCS, Pune, India). The cells were cultured and maintained in DMEM supplemented with 10% foetal bovine serum (FBS) along with 1% penicillin/streptomycin antibiotic solution (Sigma-Aldrich, P4333). Cell lines were maintained for 48hrs with changing of media in every 12 hours followed by transfection.

Plasmids and transfection

All the plasmids were purchased from Add gene and transient transfections were performed using Takara Bio's Xfect Polymer transfection reagent (21112359A), as recommended by the manufacturer. Plasmid-driven protein expression was allowed to proceed for 48hrs before further experimental procedures.

Transfection

MCF 7 and MDAMB 231 cells were grown in 6 well plates containing DMEM supplemented with 10% (v/v) foetal bovine serum. 24 hours post plating, cells were transfected with ERRβ YFP plasmid and YFP plasmid (Addgene) using Xfect Polymer transfection reagent (Takara bio, 21112359A) according to manufacture protocol. Similarly, shRNA of respective samples was also used for knockdown using lipofectamine RNAiMAX reagent (Invitrogen) and were maintained for 48 hours. 48 hours post transfection, the cells were taken for whole cell lysate prea\paration.

Preparation of whole cell extracts and Western blotting

Whole cell lysates were isolated from breast cancer cells using 1X RIPA buffer supplemented with 1X Protease inhibitor and were separated in 10% and 8% SDS-PAGE gel, transferred onto PVDF membrane (Merck- Millipore, BM9MA5648A). Blots were incubated with blocking buffer (5% nonfat dry milk in TBST) for

1 hour and were further incubated with 1 µg each of primary antibodies i.e. , Anti-ERRβ (Novus), Anti-GAPDH (Sc-47724, Santa Cruz), EMT markers antibodies (CST) followed by respective HRP conjugated secondary antibodies (HRP conjugated anti-rabbit and anti- mouse). The blots were further incubated with enhanced chemiluminescence solution (ECL, Takara bio) and were visualized in Chemi Doc XRS+ imager (Bio-Rad, Hercules, CA, USA). GAPDH levels were considered as a loading control for each whole cell extract and images were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Colony-forming assay (Clonogenic assay)

Around 1000 cells in 2ml culture medium (control and ERRβ over expressed stable cells) were seeded in triplicates on 6 – well plates and allowed to grow for 2 weeks until colonies were observed. Cells were fixed in 1% methanol for 15 minutes at room temperature and stained with 0.5% crystal violet for 15 minutes and washed with PBS. Images were viewed in gel doc and also captured using a digital camera, colonies were counted manually and the graph was plotted.

Matrigel invasion assay

Trypsinized cells were washed with PBS and resuspended in serum- free medium. For the assay, inserts were coated with 100 microlitre Matrigel (BD Biosciences) a day prior plating cell. Control and ERRβ overexpressed cells were plated in the inserts (Costar, 8.0 µm) placed in 24 well plates containing 500 microlitre DMEM with FBS (chemoattractant). After 24 hours of incubation, the cells inside the inserts were removed by wiping with a cotton bud, and the insert base were fixed with 1% methanol followed by crystal violet staining. Invasion was quantified manually by counting using microscope.

In vitro scratch assay

YFP and ERRβ YFP overexpressed stable cells were grown in 6-well plates with 70% confluency and were subjected to a uniform scratch with 10-µL pipette tip across the well. Leica inverted fluorescence microscope was used for photomicrograph and checking the cell migration at regular intervals of 24 hrs (0,24, and 48 hrs). Quantification was done using Image J software.

MTT assay

YFP and ERRβ YFP overexpressed stable MCF7 cells (1 × 10^3 cells/well) were grown in 96-well plates in DMEM, supplemented with 5-10% heat inactivated FBS at 37°C for 72 hours. After 72 hours of incubation, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed by adding 10 µL MTT and further dissolving the crystals with 100µL DMSO followed by incubation in rocker for 15-20 minutes. Using a Varioskan Flash Multimode Reader, absorbance was measured at 570 and 630 nm (Thermo Scientific, Waltham, MA, USA). Each experiment was run in duplicate at least three times.

Zebrafish xenograft

The International Animal Care and Use Committee, ILS, approved the protocol that was used for the animal study. In Zebrafish, a tumour was created utilising control (YFP) and ERRβ YFP stable overexpressed MCF 7 cells (*Danio rerio*). The detailed procedure is mentioned in the results section.

Statistical analysis

One-way analysis of variance with Tukey's multiple comparison test were used with GraphPad Prism 6.0 to evaluate statistical differences. Differences were seemed statistically significant for all experiments when p values were < 0.05.

Results and Discussion

ERRβ is downregulated in ER positive and negative breast cancer cells

TCGA samples suggest low expression of ERRβ in ER positive and negative breast cancer patients as compared to the normal ones. (Figure 1 A and B). Further, western blot analysis also shown similar results, that is low expression of ERRβ in MCF 7 and MDAMB-231 cells as compared to normal breast cell lines. (Figure C).

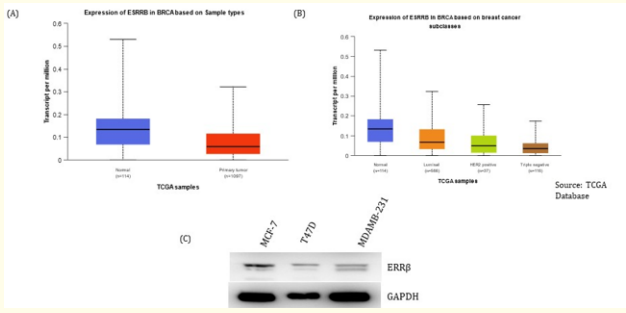

Figure 1: Figure 1 (A) and(B) is the TCGA data base analysis showing lower ESRRβ(ERRβ) expression in Breast Cancer tumor samples as compared to the normal tissue samples. Figure 1 (c) depicts lower expression of ERRβ in triple negative and positive samples as compared to normal ones.

ERRβ plays significant role in epithelial to mesenchymal transition in triple negative cells as well as estrogen positive breast cancer cell line. (MDAMB-231 and MCF-7)

We observed that the expression of mesenchymal markers such as N Cadherin, Vimentin, Snail etc.is decreased in ERRβ overexpressed MCF-7 and MDA-MB-231 cells. Epithelial markers like E cadherin are upregulated in ERRβ overexpressed MCF-7 and MDA-MB-231 breast cancer cell lines (Figure 2 A I, and 2 BI). Similarly, the mesenchymal markers are upregulated and epithelial markers are downregulated upon ERRβ knockdown (Figure 2 C 1). This indicates ERRβ might play role in EMT.

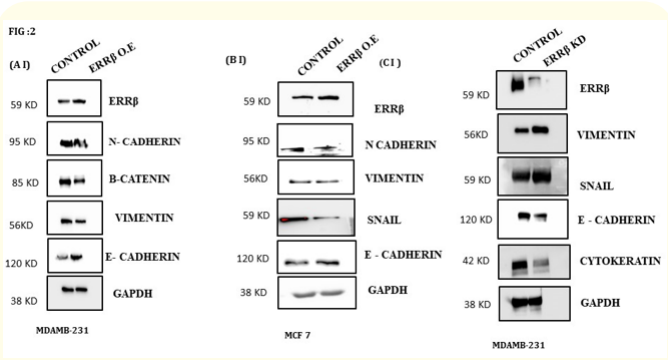

Figure 2: Figure 2 (A I) and (B I) shows the downregulation of mesenchymal markers (N Cadherin, Vimentin etc.) and upregulation of Epithelial markers (E Cadherin) in MDAMB-231 and MCF-7 cells respectively upon ERRβ overexpression. Figure 2 (C I) confirms the upregulated mesenchymal markers and downregulated epithelial markers upon ERRβ knock down.

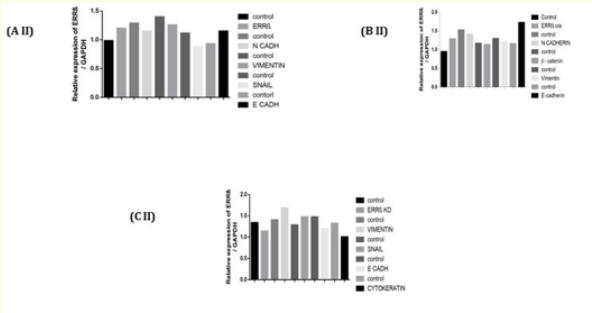

Figure 2: Figure 2.1 (A II), (B II) and (C II) depicts the relative densitometry analysis with respect to Figure 2 (A I), (BI) and (C I) using Image J and Graph Pad Prism Software.

ERRβ leads to suppression of cancer cell growth, invasion and migration

To understand the cellular functional regulation of ERRβ, ERRβ YFP overexpressed MCF 7 and MDAMB 231 cell lines were taken for clonogenic, migration and invasion assays. We tested the growth of cells and it was found that transient overexpression of ERRβ in both MCF 7 and MDAMB 231 cell lines resulted in decreased cell growth and considerably decreased capability to form colonies in clonogenic assays (Figure 3 C and 3 D). Further wound healing assays (scratch assays) were performed to evaluate the migration capacity of ERRβ overexpressed cells. Both MCF 7 and MDAMB 231 cells with overexpressed ERRβshowed a reduction in migration capability and wound closure as compared to the control cells (Figure 3 A and 3 B). In migration and trans well Matrigel invasion assays and, significant reduction of migration and invasion and was observed in ERRβ over expressed cells as compared to control YFP cells, and further increased migration in ERRβ knockdown cells (Figure 3 E and 3 F). MTT assay also depicted decrease in cell viability in ERRβ overexpressed MCF 7 cells (Figure 4 B). Taken together, these results indicate that ERRβ suppresses cancer cell growth, invasion and migration and cell viability.

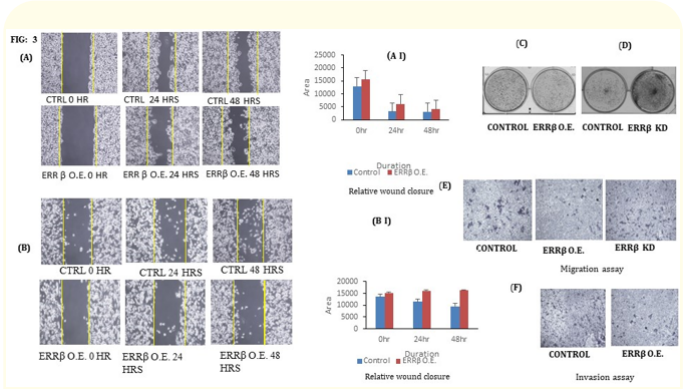

Figure 3: Wound healing/ Scratch assay was performed in both MCF 7 (A) and MDAMB 231 (B) cell lines in a duration of 0, 24, and 48 hours and respective closure area was measured using Image J software. Figure 3 (A I) and (B I) shows the relative expression of wound closure in both cell lines as mentioned. Figure 3 (C) and (D) shows clonogenic assay in ERRβ overexpressed and knockdown cells respectively. Figure 3 (E) represents migration assay in control, ERRβ overexpressed and knockdown cells .Figure 3 (F) shows the Matrigel Invasion assay for control and ERRβ overexpression.

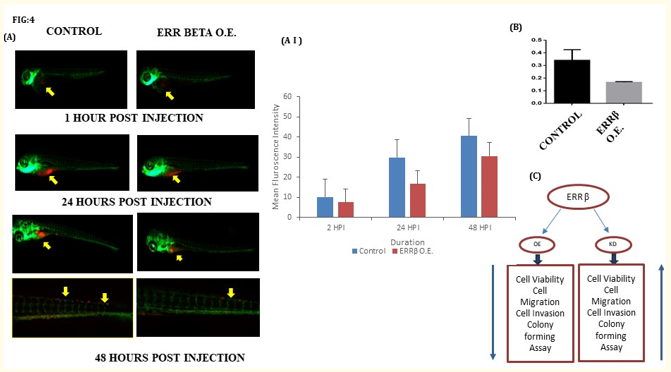

Figure 3: Wound healing/ Scratch assay was performed in both MCF 7 (A) and MDAMB 231 (B) cell lines in a duration of 0, 24, and 48 hours and respective closure area was measured using Image J software. Figure 3 (A I) and (B I) shows the relative expression of wound closure in both cell lines as mentioned. Figure 3 (C) and (D) shows clonogenic assay in ERRβ overexpressed and knockdown cells respectively. Figure 3 (E) represents migration assay in control, ERRβ overexpressed and knockdown cells .Figure 3 (F) shows the Matrigel Invasion assay for control and ERRβ overexpression.

ERRβ suppresses tumor growth and metastasis in zebrafish

To validate the tumor regression properties of ERRβ, we used Zebrafish (*Danio rerio*) tumor xenograft model to further justify the results. An equal number of control and ERRβ over expressed MDAMB-231cells were stained with tracker CM-Dil dye (1,1'- dioctadecyl-b3,3,3',3'- tetramethyl indocarbocyanine perchlorate) and these were injected in to perivitelline space of 48 – hours post fertilized embryos. Tumor growth and metastasis was measured as fluorescence intensity at 2 hours, 24 hours, and 48 hours post injection. Tumor growth and metastasis decreased 24 hours post injection in the ERRβ overexpressed zebrafish. Further it was also found that 48 hours post injection, there was a reduction in tumor size and minimal migration in ERRβ overexpressed cells injected Zebrafish as compared to the control (Figure 4 A I and A II).

Conclusion

Our work intends to look into how ERR functions in numerous pathways that contribute to the development of cancer, keeping in mind that ERR may serve as a tumour suppressor gene or a negative regulator of cell cycle and cancer cell proliferation. We examined a number of factors in relation to our earlier research in order to clarify this and identify the molecular mechanism involving the apoptotic activity of ERR in breast cancer cells, as well as the function of ERR in the epithelial to mesenchymal transition. Additionally, we verified the regression characteristics of the zebrafish model. Since breast cancer cells express ERR less than non-cancerous immortalized cells, it is important to examine how down-regulated ERR expression in cancer cells relates to many characteristics of cancer. Its role in EMT is well marked. Different molecules associated to ERR BETA through various pathways are yet to be studied.

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Ethical Approval

Materials, cell lines and Zebrafish used for the research work do not need an ethical approval. The Zebrafish was procured bearing the Registration ID: ILS/IAEC-214-AH/APR-21.

Bibliography

1. Ariazi Eric A., *et al*. "Estrogen-related receptor alpha and Estrogen-related receptor gamma associate with unfavourable and favourable biomarkers, respectively, in human breast cancer". *Cancer Research* 62.22 (2002): 6510.

2. Cooper J A., *et al*. "Risk factors for breast cancer by oestrogen receptor status: a population-based case-control study". *British Journal of Cancer* 59.1 (1989): 119-125.

3. Cowley S M., *et al*. "Estrogen receptors alpha and beta form heterodimers on DNA". *The Journal of Biological Chemistry* 272.32 (1997): 19858-19862.

4. Divekar Shailaja D., *et al*. "Estrogen-related receptor β (ERRβ) - renaissance receptor or receptor renaissance?". *Nuclear Receptor Signaling* 14 (2016): e002.

5. Giguère V. "Orphan nuclear receptors: from gene to function". *Endocrine Reviews* 20.5 (1999): 689-725.

6. Gronemeyer H. "Transcription activation by estrogen and progesterone receptors". *Annual Review of Genetics* 25 (1991): 89-123.

7. Hawkins M B., *et al*. "Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts". *Proceedings of the National Academy of Sciences of the United States of America* 97.20 (2000): 10751-10756.

8. Horard B and J-M Vanacker. "Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand". *Journal of Molecular Endocrinology* 31.3 (2003): 349-357.

9. Huss Janice M., *et al*. "Constitutive activities of estrogen-related receptors: Transcriptional regulation of metabolism by the ERR pathways in health and disease". *Biochimica et Biophysica Acta* 1852.9 (2015): 1912-1927.

10. Klinge C M. "Estrogen receptor interaction with estrogen response elements". *Nucleic Acids Research* 29.14 (2001): 2905-2919.

11. Kumari Kanchan., *et al*. "Estrogen-related receptors alpha, beta and gamma expression and function is associated with transcriptional repressor EZH2 in breast carcinoma". *BMC cancer* 18.1 (2018): 690.

12. Lazennec G., *et al*. "ER beta inhibits proliferation and invasion of breast cancer cells". Endocrinology 142.9 (2001): 4120-4130.

13. Lewis-Wambi Joan S and V Craig Jordan. "Treatment of Postmenopausal Breast Cancer with Selective Estrogen Receptor Modulators (SERMs)". *Breast disease* 24 (2005): 93-105.

14. Lu D., *et al*. "Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors". *Cancer Research* 61.18 (2001): 6755-6761.

15. Madhu Krishna B., *et al*. "Estrogen receptor α dependent regulation of estrogen related receptor β and its role in cell cycle in breast cancer". *BMC cancer* 18.1 (2018): 607.

16. Mukherjee Shibani and Susan E Conrad. "c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells". *The Journal of Biological Chemistry* 280.18 (2005): 17617-17625.

17. Paruthiyil Sreenivasan., *et al*. "Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest". *Cancer Research* 64.1 (2004): 423-428.

18. Perou C M., *et al*. "Molecular portraits of human breast tumours". *Nature* 406.6797 (2000): 747-752.

19. Pettersson K., *et al*. "Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha". *Molecular Endocrinology (Baltimore, Md.)* 11.10 (1997): 1486-1496.

20. Sengupta D., *et al*. "ERRβ signalling through FST and BCAS2 inhibits cellular proliferation in breast cancer cells". *British Journal of Cancer* 110.8 (2014): 2144-2158.

21. Ström Anders., *et al*. "Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D". *Proceedings of the National Academy of Sciences of the United States of America* 101.6 (2004): 1566-1571.

22. Sung Hyuna., *et al*. "Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries". *CA: A Cancer Journal for Clinicians* 71.3 (2021): 209-249.

23. Tremblay G B., *et al*. "Dominant activity of activation function 1 (AF-1) and differential stoichiometric requirements for AF-1 and -2 in the estrogen receptor alpha-beta heterodimeric complex". *Molecular and Cellular Biology* 19.3 (1999): 1919-1927.

24. Wang Xia and Yong Lin. "Tumor necrosis factor and cancer, buddies or foes?". *Acta pharmacologica Sinica* 29.11 (2008): 1275-1288.

25. Wu Wan-fu., *et al*. "Targeting estrogen receptor β in microglia and T cells to treat experimental autoimmune encephalomyelitis". *Proceedings of the National Academy of Sciences of the United States of America* 110.9 (2013): 3543-3548.

26. Yu S., *et al*. "Orphan nuclear receptor estrogen-related receptor-beta suppresses in vitro and in vivo growth of prostate cancer cells via p21 (WAF1/CIP1) induction and as a potential therapeutic target in prostate cancer". *Oncogene* 27.23 (2008): 3313-3328.