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TITLE: Defining the Molecular Actions of Dietary Fatty Acids in Breast Cancer:
Selective Modulation of Peroxisome Proliferator-Activated Receptor Gamma

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14. ABSTRACT Stark differences in the actions of linoleic acid (LAA), an omega-6 fatty acid, and eicosapentaenoic acid (EPA), an omega-3 fatty acid, on breast cancer tumors have been described. We propose that transactivation of peroxisome proliferators-activated receptor gamma (PPAR.) mediates the physiological effects of different dietary fatty acids on breast cancer. PPAR. plays a role in the development and progression of breast cancer tumors. We have shown that individual ligands of PPAR. can selectively activate PPAR. in three different ways. Selective activation of PPAR. by a single ligand occurs between tissue types and between individual breast cancer cell lines. Also, unique ligands selectively activate PPAR. within a single cell type. We propose that fatty acids will elicit their effects on breast cancer cells in a similar manner. Using both pharmaceutical and molecular approaches we have demonstrated that PPAR. serves as a molecular target for both LAA and EPA. Our data shows that fatty acids utilize PPAR. to activate a PPAR response element reporter system and that the receptor is both sufficient and necessary to observe this response. Also, EPA treatment increases the ability of PPAR. to bind to DNA. Furthermore, through multiple approaches we have determined that fatty acids do not need to be converted to prostaglandins but themselves can function as PPAR. ligands. To date, we have determined that both LAA and EPA act as PPAR. agonists. The objective of future studies will be to demonstrate that LAA and EPA act as selective PPAR. modulators (SPARMs) in breast cancer cells. We hypothesize that, though both LAA and EPA are PPAR. agonists, they function as SPARMs by causing unique gene expression and that this is in part the mechanism responsible for the different physiological actions of these fatty acids. We have also demonstrated that rosiglitazone, a PPAR. ligand, also serves as an agonist of the estrogen receptor in breast cancer cells. Finally, we have determined that changes in expression of myc-associated zinc finger protein (MAZ) drives elevated PPAR. levels in breast cancer cells.					
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Introduction

Breast cancer remains one of the leading forms of cancer in American women. One in every eight women in the United States will be diagnosed with breast cancer, a 5-fold higher rate than that observed in women living in Japan and China. Studies examining women that emigrate from Asian countries to the United States have found similar breast cancer risk as American women within 40 years suggesting that genetics alone can not account for differences in breast cancer incidence between these populations (1). A number of environmental aspects are being explored to elucidate factors that might influence breast cancer risk. Though controversial, it has been estimated that diet influences the development of up to 50% of all breast cancer cases in American women (2). A growing body of literature indicates that the type of dietary fat consumed (diets high in omega-3 fatty acids versus diets high in omega-6 fatty acids) influences breast cancer (3, 4) suggesting that consumption of specific fatty acids may impact breast cancer differently. Animal studies, have also provided convincing evidence of a correlation between types of fats ingested and mammary tumor development and growth (5-8). The focus of this proposal is to define the molecular link between specific fatty acids and the progression of breast cancer. We are exploring the possibility that fatty acids may elicit their effects in breast cancer cells by acting as ligands of the peroxisome proliferator-activated receptor gamma (PPAR γ). Our laboratory has previously demonstrated that individual fatty acids can activate a PPAR-response element (PPRE), but whether this effect was the direct result of PPAR γ activation has been left unexplored (9). Evidence suggests PPAR γ is involved in the initiation (10, 11) and progression (12-14) stages of breast cancer. The objective of this proposal is to determine the mechanism of action that individual fatty acids use to either positively (increase cellular differentiation and/or decrease cellular proliferation) or negatively (increase cellular proliferation and/or tumor metastases) impact breast cancer cells. We propose that PPAR γ is the molecular target responsible for the physiological effects of different dietary fatty acids on breast cancer.

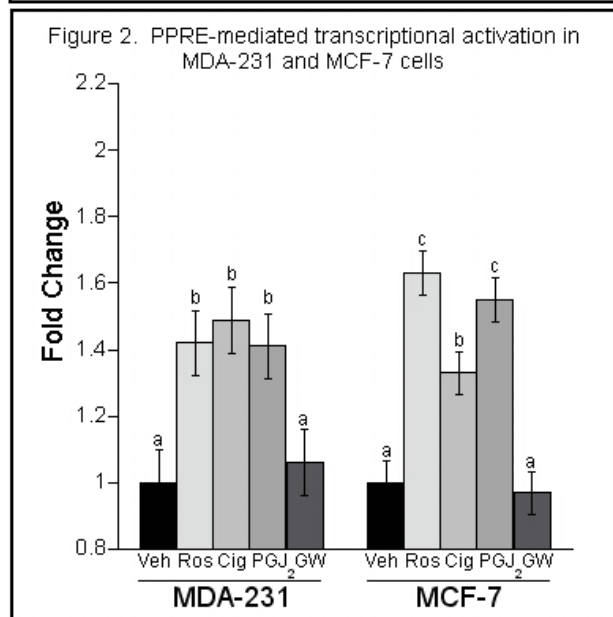
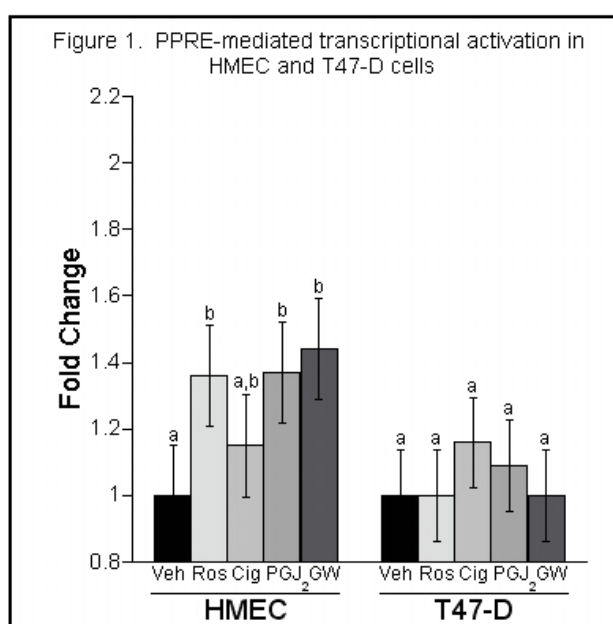
Body

The orphan nuclear receptor, PPAR γ is one of three in a family of receptors (PPAR α , β , and γ) (15-17). It is expressed in numerous cell types including adipocytes, epithelial cells of the breast, colon, and lung, and macrophages among others (18-22). Several ligands of PPAR γ have been identified including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂, linoleic acid (LAA), lysophosphatidic acid, and the thiazolidinedione class of anti-diabetic drugs such as ciglitazone and rosiglitazone (Ros) (9, 23-26). Transactivation of the receptor requires ligand binding, heterodimerization with retinoid X receptor alpha (RXR α), and binding of this complex to PPAR-specific response elements (PPREs) in the promoter regions of target genes (22, 27).

To begin to explore the role that individual fatty acids might play in the progression of breast cancer we wanted to first demonstrate that individual ligands of PPAR γ could selectively modulate the receptor. The ability of individual ligands to selectively mediate the activity of a nuclear receptor dependent on the tissue type examined has been used to develop compounds that act as selective estrogen-receptor modulators (SERMs). Tamoxifen, which was originally described as an estrogen-receptor antagonist, has been found to act as an agonist in several different tissue types (28-30). It has been proposed that individual ligands may be able to act as selective PPAR γ modulators (SPARMs) in a manner similar to the way other compounds function as SERMs (31).

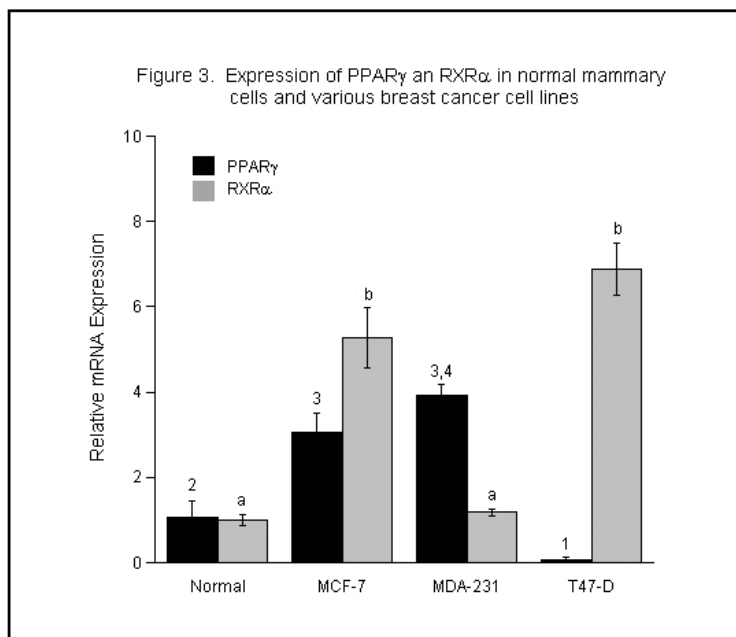
To address these fundamental questions we have conducted a series of experiments that tested the ability of individual PPAR γ ligands to selectively activate PPAR γ . To this end we have utilized a PPRE-reporter construct transfected into the cells prior to ligand treatment. The data from these studies has been collected, analyzed, and the resulting manuscript was published in *Molecular and Cellular Endocrinology* (see appendix).

In these studies, we first tested the ability of several different PPAR γ ligands to activate the PPRE-reporter in either normal mammary epithelial (HMEC), estrogen-dependent breast cancer cells (T47-D and MCF-7), or estrogen-independent breast cancer cells (MDA-MB-231). Following transfection with a PPRE reporter plasmid, HMEC, T47-D, MDA-MB-231, and MCF-7 cells were treated with either vehicle control or PPAR γ ligands for 18 hours. For the four cell lines, differences in ligand activity were observed. In the HMEC, Ros and PGJ₂ both significantly increased reporter activity over control (Fig. 1). Interestingly, GW, a known antagonist of PPAR γ , also significantly stimulated reporter activity in HMECs. GW treatment did not change reporter activity compared



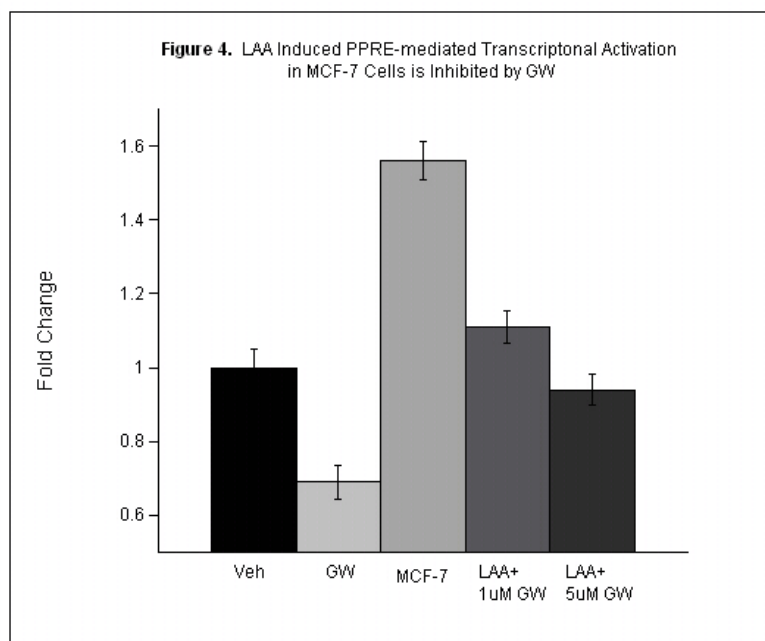
to control in any of the other breast cancer cell lines. No treatments significantly increased reporter activity in the T47-D cells (Fig. 1). In MDA-MB-231 cells Ros, Cig, and PGJ₂ all significantly enhanced PPAR γ activation over control, while these same three treatments also increased reporter activation in MCF-7 cells when compared to control (Fig. 2). Both Ros and PGJ₂ treatments resulted in significantly higher activity than Cig in MCF-7 cells.

Next we wanted to determine if mRNA levels of PPAR γ and its heterodimeric partner, RXR α , in cells were predictive of how the breast tumor cells would respond to PPAR γ ligands. To do this, mRNA concentrations of PPAR γ 1 and RXR α were measured in all four cell lines (HMEC, T47-D, MDA-MB-231, and MCF-7) used previously in the transfection assays in the absence of ligand treatment. Data is presented as fold change in expression compared to the HMECs. T47-D cells had significantly lower levels of PPAR γ than all of the cell lines (Fig. 3). However, these cells had the highest expression of RXR α among all cell lines. MCF-7 cells express significantly higher levels of PPAR γ expression than the HMECs or T47-Ds and higher RXR α than all of the cells tested except T47-Ds. MDA-MB-231 cells had PPAR γ mRNA levels similar to MCF-7 cells, but had lower RXR α expression.



Data from these experiments demonstrated that selective activation of PPAR γ occurs in multiple ways. Distinct ligands selectively activate PPAR γ dependent on the tissue type from which the cell line was derived (data not shown, see MCE manuscript for full description). SPARM activity was also observed between different cell lines of the same tissue origin. Specifically, normal mammary cells and breast cancer cells responded differently to individual ligands and differences were observed between unique breast cancer cell lines. Also, individual ligands selectively activated the PPARE reporter within single breast cancer cell lines.

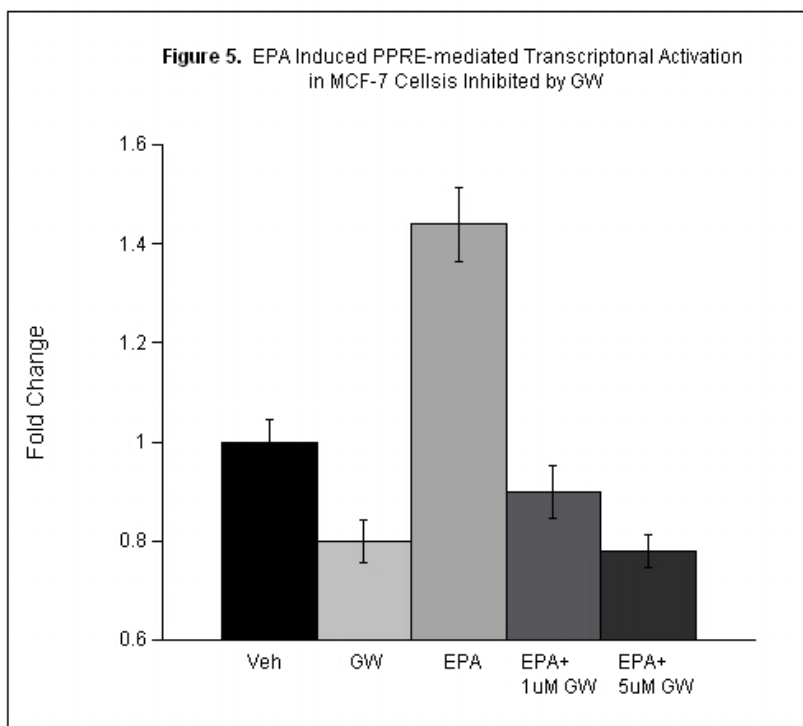
We have now begun to determine if individual fatty acids utilize PPAR γ as a molecular target in breast cancer cells and if like other ligands they can function as SPARMs resulting in diverse physiological effects. Although fatty acids have been shown to activate PPARE-reporter assays in a number of different cell types (9, 17, 32), left unexplored was whether PPAR γ serves as a mediator of these responses. Defining this mechanism is critical to the greater understanding of how and if fatty acids function directly through PPAR γ in breast cancer cells. To study this, we have utilized both pharmaceutical and molecular approaches. In the first approach, we used GW, the PPAR γ specific antagonist, to block the ability of individual fatty acids to increase PPARE-reporter activity. We have previously established the antagonistic actions of GW in this system by inhibiting the PPARE reporter activation induced by Ros, a selective PPAR γ agonist (data not shown). We then wanted to determine if the PPARE activation which results from treatment of either LAA, an *n*-6 fatty acid, or eicosapentaenoic acid (EPA), an *n*-3 fatty acid, could be inhibited by GW. For these experiments, MCF-7 cells were transiently transfected with a PPARE reporter construct and subsequently treated with either LAA or EPA in combination with GW for 18 hrs. Treatment with 150 μ M LAA significantly

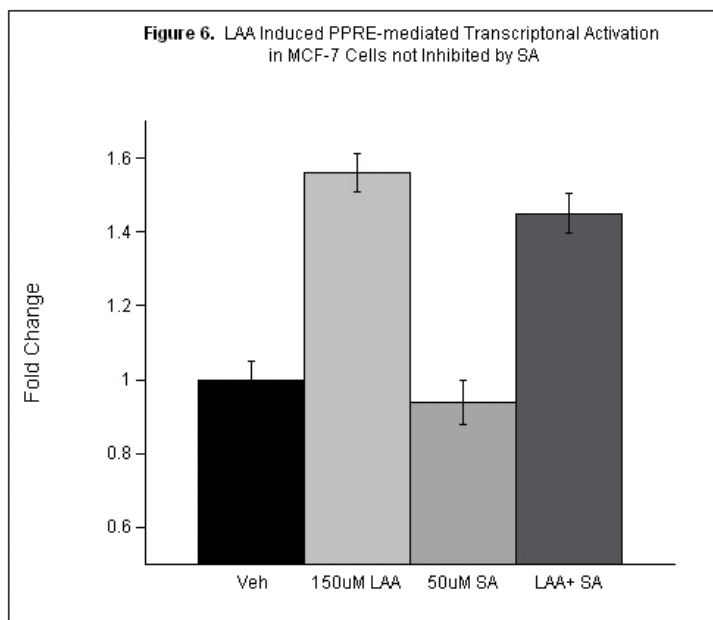


formed at the cellular level (33) and individual prostaglandins have been shown to be ligands of PPAR γ (23, 34). Therefore, we sought to determine whether LAA itself or a prostaglandin formed from LAA was responsible for the increased activity in the PPRE-reporter assay in MCF-7 cells. Cyclooxygenase (COX) enzymes utilize LAA and other fatty acids as substrates in the formation of prostaglandins (35, 36). In order to determine whether LAA is itself a ligand of PPAR γ rather than an upstream metabolic precursor of the ligand, we co-treated MCF-7 cells with LAA and salicylic acid (SA). Salicylic acid has been shown to effectively inhibit COX activity (37, 38). In these studies, cells treated with SA alone, at an optimal dose for inhibiting COX activity, had no increased PPRE-reporter activation (Fig. 6). MCF-7 cells co-treated with LAA and SA had increased reporter activity similar to that of cells treated with LAA alone. We have confirmed this data in other systems in which LAA and EPA enhance PPRE reporter activity (data not shown). For the EPA studies, aspirin (acetyl salicylic acid) which has also been shown to inhibit COX activity was used to inhibit the enzyme. Collectively these studies indicate that the conversion of fatty acids to prostaglandins is not required for PPRE activation.

increased PPRE reporter activity and this effect was inhibited in a dose response pattern when the cells were co-treated with GW (Fig. 4). Addition of 100 μ M EPA also significantly enhanced reporter activity in the MCF-7 cells compared to vehicle control (Fig. 5). Co-treatment of these cells with EPA plus GW significantly decreased reporter activity compared to EPA treatment alone to levels not significantly different from control.

In addition to examining the molecular pathways of LAA and EPA in MCF-7 cells, we wanted to determine if these fatty acids were themselves the functional ligand of PPAR γ . Fatty acids are metabolic precursors of prostaglandins that are

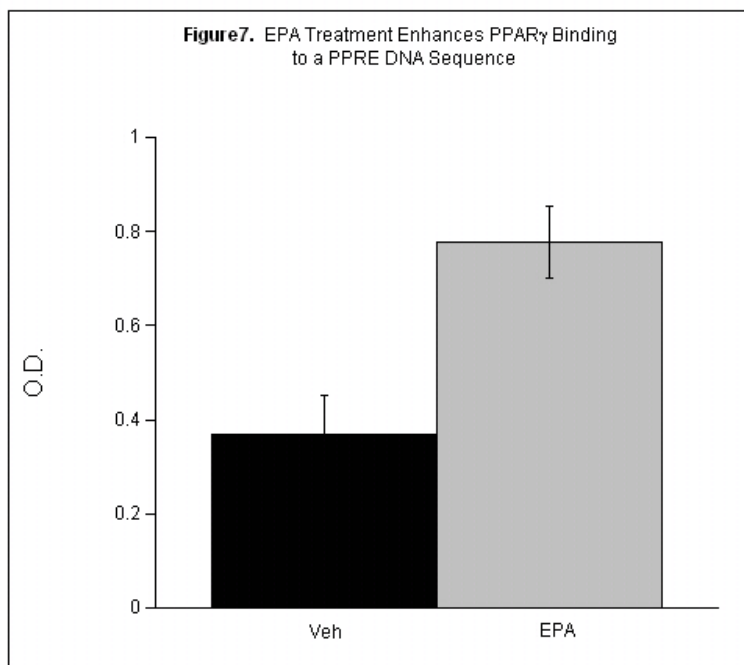




To further evaluate the ability of LAA and EPA to influence PPAR γ 's activation of a PPRE, we then employed a PPAR γ negative cell line (22Rv1). In these studies, we co-transfected 22Rv1 cells with both the PPRE-reporter construct and a PPAR γ 1 expression plasmid and then treated the cells with either LAA or EPA for 18 hrs. In the absence of PPAR γ expression, neither LAA nor EPA influenced the PPRE-reporter, but activity was enhanced in cells co-transfected with PPAR γ (data not shown). This activation was inhibited by the addition of GW. Taken together, these data demonstrate the molecular consequence of either LAA or EPA exposure in these cells results

in the direct transactivation of PPAR γ and the upregulation of the PPRE-reporter. However, this data falls short of identifying PPAR γ as the only molecular target of these ligands. Individual fatty acids have been shown to bind the other isoforms of PPAR (α and δ) (32). So, while we have not ruled out the possibility that LAA and/or EPA may also elicit responses through PPAR α or δ , we have, through both pharmacological and molecular approaches, clearly identified PPAR γ as a molecular target of these fatty acids in MCF-7 cells.

In addition to PPRE activation, we have shown that EPA increases binding of PPAR γ to a DNA. For these studies, recombinant PPAR γ and RXR α proteins were incubated with either vehicle or EPA. An ELISA-based methodology was used to quantify PPAR γ binding to a PPRE following EPA treatment. Either vehicle or 100 μ M EPA was incubated with recombinant PPAR γ and RXR α proteins *in vitro*. These reaction mixtures were then added to 96-well plates seeded with an oligonucleotide containing a PPRE. PPAR γ specificity and DNA binding was quantified by incubating with a primary antibody for PPAR γ and colorimetric analysis, respectively. EPA significantly increased the ability of PPAR γ to bind the PPRE when compared to vehicle control (Fig. 7). This data is critical in demonstrating that EPA can influence the actions of un-ligand bound PPAR γ . To our knowledge, this is the first data to show that addition of a ligand enhances the ability of PPAR γ to bind DNA. Furthermore, since this is a cell-free



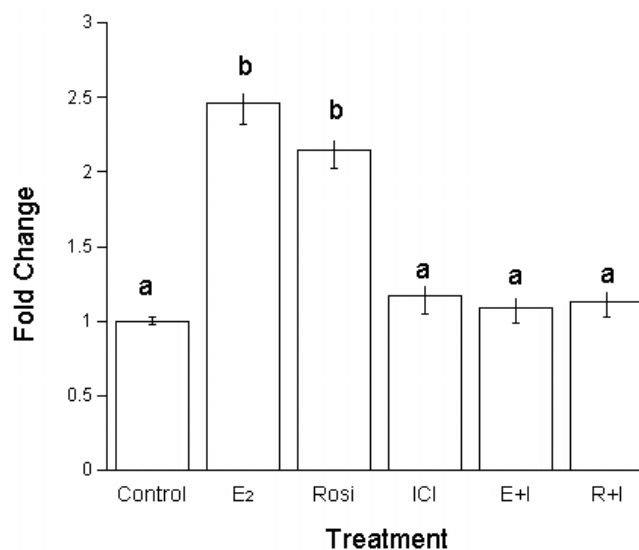
system, these data further support the notion that fatty acids themselves are functional ligands of PPAR γ .

To date, we have demonstrated that PPAR γ can serve as a molecular target of both *n*-3 and *n*-6 polyunsaturated fatty acids. In fact, both LAA and EPA enhance PPRE-reporter activity. However, epidemiology, animal, and cell line data all suggests that these two fatty acids result in opposing effects in breast cancer cells. Therefore, we hypothesize that while both compounds activate PPAR γ that the molecular and cellular changes that occur in response to PPAR γ activation is quite different for each compound. To begin to explore this, we have collected RNA from MCF-7 cells treated with vehicle, 150 μ M LAA, or 100 μ M EPA and then analyzed changes in gene expression utilizing microarray technology. We have begun to analyze this vast collection of data. Preliminary information suggests that the two treatments result in over 5,000 genes that are significantly changed dependent on treatment. The next step in this process will be to identify PPAR γ response genes. Once this analysis is complete, genes will be verified using rt-PCR. We anticipate that the data will enable us to determine how two ligands that activate the same receptor result in drastically different physiological responses. We hypothesize that, though both LAA and EPA are PPAR γ agonists, they function as SPARMs by causing unique gene expression and that this is in part the mechanism responsible for the different physiological actions of these fatty acids.

In recent studies, we have begun to explore the possibility that some compounds known to bind PPAR γ may also act as ligands of estrogen-receptor alpha (ER α). Approximately 65% of all breast cancers are ER positive and are diagnosed as estrogen-dependent cancers. As such, it is critical to identify compounds that stimulate ER activity. Ros is a thiazolidinedione drug that we have studied in the past with regards to its ability to function as a SPARM. However, recently we have explored the ability of Ros to activate an ER response element (ERE) in MCF-7 cells. To do this, MCF-7 cells were transiently transfected with a luciferase

reporter under the control of an ERE. Cells were then treated with estradiol (E₂) or Ros for 18h (Fig. 8). E₂ treatment significantly increased ERE reporter activity. Interestingly, Ros treatment also resulted in a significant increase in ERE reporter activity. ICI 182,780 (ICI), a pure ER α antagonist, was used to determine whether Ros activation of an ERE reporter is dependent on the ER signal transduction pathway. ICI treatment alone did not alter ERE reporter activity but ICI completely blocked ERE reporter activation by both E₂ and Ros. On going experiments are exploring the ability of Ros to influence cellular proliferation of MCF-7 cells and the molecular pathways responsible for these actions. The ability of Ros to activate both PPAR γ and ER α could have important implications for the use of these drugs in the treatment of diabetes in patients at risk of developing breast cancer.

Figure 8. ERE-Mediated Reporter Activity in MCF-7 Cells



Methods:

Cells and cell culture

HMEC, MDA-MB-231, T-47D and MCF-7 were obtained from the ATCC (Rockville, MD) and maintained as described in the attached manuscript. Two cell lines were used in experiments investigating fatty acids. Breast epithelial adenocarcinoma cells (MCF-7) were used in most experiments. Human prostate carcinoma cells (22Rv1) were used as PPAR γ negative cells. Both cell lines were cultured in medium that provided optimal conditions for their growth. Cells were maintained in medium containing 10% fetal bovine serum (FBS) and were grown in medium lacking phenol red at 37°C in a 5% CO₂ atmosphere. Cells were grown in T-75 flasks before being transferred to 24-well plates in preparation for transfection.

Fatty acid preparations

LAA and EPA were purchased in pure fatty acid form and then dissolved in hexane to create a fatty acid stock solution. The stock solutions were maintained under nitrogen gas at all times and fresh fatty acid preparations were made before every experiment. For transfection assays, appropriate volumes of the stock solutions were then combined with calculated volumes of 6N NaOH to form fatty acid salt complexes. The preparations were then dried under nitrogen gas until no fluid remained. The fatty acid salt was then dissolved in cell culture media containing 10% FBS. It has been reported that the availability of free fatty acids in the body is dependent on the presence of albumin and therefore depends on albumin concentration (39). As a result, the chosen FBS conditions were necessary to form fatty acid/ albumin complexes. Once the fatty acid was completely dissolved in the media, hydrochloric acid was used to balance the pH and the media was filter sterilized through a 0.2 μ m syringe filter. For the DNA binding studies, EPA was purchased and used in sodium salt form.

Plasmids

The PPRE-reporter construct, 3XPPRE-TK-pGL3, contains three copies of a PPRE sequence (AGGACAAAGGTCA) upstream of the mTK promoter between the Xho I and Hind III restriction enzyme sites of the pGL3 basic vector. CMV promoter controlled β -Galactosidase (β -GAL) expression vector was a kind gift from Dr. Melinda Wilson. pBluescript cloning vector plasmid was purchased from Stratagene.

Transfection assays

In most cases, cells were transiently transfected with 3 μ g of PPRE-reporter and 1 μ g of β -GAL plasmid per 24 well plate. For 22Rv1 experiments, cells were transfected with 3 μ g PPRE-reporter, 1 μ g β -GAL, and either 1 μ g bluescript or 1 μ g PPAR γ 1 per 24 well plate. For studies evaluating the ability of Ros to act as a ligand of ER we utilized and ERE-reporter instead of the PPRE-reporter. Plasmids were transfected into cells using ESCORT transfection reagent over a four hour period. Cells were subsequently treated with 150 μ M LAA, 100 μ M EPA and/or other compounds (GW9662, Ros, ICI, etc...) for 18 hrs. Following treatment, cells were lysed in 50 μ l passive lysis buffer. The quantification of induced Firefly (*Phontius pyralis*) luciferase protein was performed using the reagent found in the Luciferase Assay System Kit according to the manufacturer's instructions. Luminometry was performed on a Berthold Lumat 9507. β -GAL activity was utilized as a constitutively active reporter. β -GAL activity was measured using a β -GAL Enzyme Assay System according to manufacturer's instructions. Mean fold induction was obtained by dividing the RLU/ β -GAL ratio data from each treatment well by the mean values of the vehicle control appropriate for each treatment. Each set of treatments were performed in replicates of 6 in 3 separate experiments.

RT-PCR Analysis

Real-time PCR was performed on total RNA using the TaqMan One-Step RT-PCR Master Mix Kit purchased from Applied Biosystems and used according to manufacturers instructions. Commercial FAM labeled probe/primer pairs constructed by Applied Biosystems using the Celera genomic database were used to assess PPAR γ and RXR α mRNA levels. Quantitation of mRNA was performed using an ABI Prism 7700 Sequence Detection System and the TaqMan methodology, which uses the 5' nuclease activity of the Taq DNA polymerase to generate a real-time quantitative DNA assay. Data were analyzed using a C_t cycle method. At the completion of the amplification (40 cycles), the amount of target message in each reaction was recorded as a threshold cycle number (C_t), which is inversely correlated to the abundance of the initial message level. C_t measures the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The amount of target was normalized to the endogenous reference target, human GAPDH, again using a FAM labeled Taqman probe/primer solution available from Applied Biosystems. This normalized target C_t value was then set relative to a normalized calibrator sample (i.e. untreated normal cell type) as given by the equation $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t$ represents ΔC_t , target sample minus ΔC_t , calibrator. Finally, this value was then used to produce a relative quantity by comparison to an appropriate control sample.

Quantification of PPAR γ binding to DNA

To determine if EPA influenced PPAR γ 's ability to bind to a PPRE an enzyme-linked immunosorbent assay (ELISA)-based protocol was used. Prior to using this TransAM PPAR γ kit an *in vitro* reaction was performed. For this reaction, either vehicle (methanol) or 100 μ M EPA sodium salt (final concentration) was incubated with 100ng each of PPAR γ and RXR α recombinant proteins at room temperature for 20 min. The TransAM PPAR γ kit was then performed according to kit instructions. Briefly, 3 μ l aliquots of each reaction were added to the 96-well ELISA plate in triplicate. The wells of the ELISA plates were coated with an immobilized oligonucleotide that contains a PPRE (5'-AACTAGGTCAAAGGTCA-3'). After incubation with the ligand/protein reaction mixture, the wells were washed and a primary antibody recognizing an accessible epitope on PPAR γ protein upon DNA binding was added. Incubation with an appropriate secondary antibody conjugated to horseradish peroxidase was then performed following another wash. This step was followed by a colorimetric reaction which was quantified using spectrophotometry.

Key Research Accomplishments

- We have demonstrated that individual PPAR γ ligands can selectively activate the receptor in cancer cell lines derived from different tissues.
- We have shown that selective modulation of PPAR γ occurs between normal mammary epithelial cells as well as different breast cancer cell lines when the cells are treated with PPAR γ ligands. These data suggest that breast tumors in individual patients may respond to PPAR γ differently.
- We have shown that individual PPAR γ ligands can selectively modulate the receptor within a single cell line.
- We have demonstrated that distinct expression patterns of RXR α and PPAR γ mRNA in tumor cells may be predictive of how they will respond to PPAR γ ligand treatment, but further investigation is necessary to better define this approach.
- We have demonstrated that both LAA and EPA can induce PPRE-reporter activity in breast cancer (MCF-7) cells and that this effect is inhibited when cells are co-treated with a PPAR γ specific antagonist.
- We have shown that inhibiting prostaglandin formation in MCF-7 cells does not significantly change the ability of fatty acids (LAA) to induce a PPRE-reporter. This data suggests that the fatty acids themselves are ligands of PPAR γ and need not be metabolized to elicit their response on the receptor.
- Using a PPAR γ negative cell line (22Rv1), we have confirmed that expression of PPAR γ is required for either LAA or EPA to induce PPRE-reporter activity clearly defining that PPAR γ can serve as a molecular target of fatty acids.
- We have demonstrated that EPA treatment increases the ability of PPAR γ to bind to DNA. This is the first time a ligand has been shown to enhance binding of PPAR γ to its response element. Furthermore, because this is a cell-free system, these data support the conclusion that metabolism of fatty acids to other compounds is not required for them to interact with PPAR γ .
- We have completed microarray studies in which MCF-7 cells were treated with either LAA or EPA. Ongoing analysis of these data will identify genes that are selectively modulated by PPAR γ following treatment with the different fatty acids.
- We have determined that Ros, like E₂, can activate an ERE-reporter. Our data indicates that this activation is the result of Ros serving as a ligand of ER α .
- Ros induces growth of MCF-7 cells at low concentrations and inhibits cell growth at higher concentrations (see manuscript)
- We have demonstrated that changes in expression of myc-associated zinc finger protein (MAZ) results in elevated expression of PPAR γ 1 that is commonly seen in breast cancer cells (see manuscript)

Reportable Outcomes

Training and Employment

- In addition to completing studies outlined in this grant, supporting funds have enabled the PI to participate in scientific endeavors that are beyond the scope the original grant. The PI has participated in collaborative projects related to breast cancer research which resulted in presentations and eventual manuscripts (see details below). Additional manuscripts are in early stages of preparation and as such are not listed below, but will be credited to this grant.
- In January 2006, Dr. Allred was invited to interview for a tenure-track faculty position at Texas A&M University. Since that time, Dr. Allred has accepted that faculty position in the Department of Nutrition and Food Science and has relocated to College Station, Texas. In this position he will develop an independent research program.

Manuscripts

- **Allred, C.D.** and Kilgore, M.W. Selective Activation of PPAR γ in Breast, Colon, and Lung Cancer Cell Lines. *Molecular and Cellular Endocrinology*. 235: 21-29. 2005.
- Wang, X., **Allred, C.D.**, Southard, R.C., Wilson, M.E., and Kilgore, M.W. Myc-Associated Zinc Finger Protein Mediates the Overexpression of PPAR gamma in Human Breast Cancer Cells by Driving Promoter Switching. *Breast Cancer Research and Treatment*, 2007
- Talbert, D.R., **Allred, C.D.**, Zaytseva, Y.Y., and Kilgore, M.W. Transactivation of ER α by Rosiglitazone induces proliferation in breast cancer cells. *Breast Cancer Research and Treatment*, 2007
- **Allred, C.D.**, Talbert, D.R., Southard, R.C, and Kilgore, M.W. PPAR γ 1 as a molecular target of eicosapentaenoic acid in colon cancer (HT-29) cells. *Journal of Nutrition*. 138: 250-256. 2008.
- **Allred, C.D.**, Talbert, D.R., and Kilgore, M.W. Modulation of Peroxisome Proliferator-Activated Receptor Gamma by Linoleic Acid in MCF-7 Cells. Manuscript in Preparation.

Abstracts presented

- **Allred, C.D.** and Kilgore, M.W. Selective Activation of PPAR α Demonstrates Tissue Specificity Between Tumor Cell Lines. Presented at the Keystone Research Conference: Orphan Nuclear Receptors in 2004 in Keystone Colorado.
- **Allred, C.D.**, Talbert, D.R, and Kilgore, M.W. Selective activation of PPAR α in Cancers of the Breast, Colon, and Lung. Presented at the Twenty Fourth Annual University of Kentucky Symposium in Reproductive Sciences in 2005 in Lexington, Ky.
- **Allred, C.D.**, Talbert, D.R., and Kilgore, M.W. Defining the Molecular Actions of Linoleic Acid in Colon Cancer: Modulation of Peroxisome Proliferator-Activated Receptor Gamma. Presented at the Endocrine Society's Eighty Seventh Annual Meeting in 2005 in San Diego, CA.
- **Allred, C.D.**, Talbert, D.R., and Kilgore, M.W. Defining the Molecular Actions of Linoleic Acid in Breast Cancer: Modulation of Peroxisome Proliferator-Activated Receptor Gamma. Presented at the Era of Hope Meeting in 2005 in Philadelphia, PA.
- Talbert, D.R., **Allred, C.D.**, and Kilgore, M.W. The Antidiabetic Thiazolidinedione Rosiglitazone Transactivates ER and Induces Proliferation in Breast Cancer Cells. Presented at the American Association for Cancer Research annual meeting in 2006 in Washington, D.C.

- **Allred, C.D.**, Talbert, D.R., and Kilgore, M.W. Peroxisome proliferator-activated receptor gamma as a molecular target of polyunsaturated fatty acids in breast cancer cells. Presented at the Experimental Biology Meetings in 2006 in San Francisco, CA.
- Talbert, D.R., **Allred, C.D.**, and Kilgore, M.W. Rosiglitazone Activation of ER α induces proliferation in MCF-7 breast cancer cells and is dependent on the ERK-MEK pathway. Presented at the Endocrine Society's Eighty Eighth Annual Meeting in 2006 in Boston, MA.
- **Allred, C.D.**, Talbert, D.R., Southard, R.C., and Kilgore, M.W. Defining the Molecular Actions of Eicosapentaenoic Acid in Colon Cancer: Modulation of Peroxisome Proliferator-activated Receptor Gamma. Presented at the Food, Nutrition, Physical Activity and the Prevention of Cancer: A Global Perspective: A Launch Conference in 2007 in Washington D.C.
- Allred, K.F., Vanamala, J., Murano, P., and **Allred, C.D.** Identification of a Novel Phytoestrogen in Coffee Beans. Presented at the Experimental Biology Meetings in 2008 in San Diego, CA

Invited Lectures

- Invited oral presentation at Twenty Fourth Annual University of Kentucky Symposium in Reproductive Sciences May 2005
- Invited oral presentation at Texas A&M University January 2006
- Gave the Sonia Wolf/Wilson Lectureship seminar at the University of Texas, Division of Nutritional Science- September 2007

Conclusions

PPAR γ is highly expressed in breast cancer tumors and treatment of these cells with known PPAR agonists *in vitro* have been shown to suppress tumor cell growth. This has led to the possibility that PPAR γ may be utilized as a therapeutic target in the treatment and prevention of breast cancer. Through a combination of pharmacological and molecular approaches we have now demonstrated that PPAR γ serves as a molecular target of both *n*-6 (LAA) and *n*-3 (EPA) fatty acids. Future studies, beginning with microarray analysis, will test the hypothesis that these compounds act as SPARMs. We propose that both LAA and EPA stimulate PPAR γ mediated gene transcription, but that each compound induces unique gene expression patterns that result in very diverse physiological responses in breast cancer cells. This mechanism in part would explain how consumption of diets high in either *n*-6 or *n*-3 fatty acids have opposing effects on breast cancer development and progression. The end result of these studies will be a stepping stone toward developing dietary recommendations for fatty acid consumption for patients with breast cancer as well as those at high risk of developing the disease.

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Abbreviations

- PPAR γ - peroxisome proliferator-activated receptor gamma
- ER α - estrogen receptor alpha
- PPRE- PPAR γ response element
- ERE- ER response element
- RXR α - retinoid X receptor alpha
- TZD- thiazolidinedione
- Ros- rosiglitazone
- Cig- ciglitazone
- PGJ₂- 15-deoxy-delta 12,14-PGJ₂
- GW- GW9662
- LAA- linoleic acid
- EPA- eicosapentaenoic acid
- E₂- estradiol
- RLU- renilla units
- rt-PCR- real-time polymerase chain reaction
- C_t- cycle number

Selective activation of PPAR γ in breast, colon, and lung cancer cell lines

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Abstract

Peroxisome proliferator-activated receptor gamma (PPAR γ) plays a critical albeit poorly defined role in the development and progression of several cancer types including those of the breast, colon, and lung. A PPAR response element (PPRE) reporter assay was utilized to evaluate the selective transactivation of PPAR γ in 10 different cell lines including normal mammary epithelial, breast, lung, and colon cancer cells. Cells were treated with one of four compounds including rosiglitazone (Ros), ciglitazone (Cig), 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), or GW 9662 (GW). We observed differences in transactivation between cell lines from different tissue origin, across cell lines from a single tissue type, and selective modulation of PPAR γ within a single cell line by different ligands. Interestingly, GW, a PPAR γ antagonist in adipocytes, enhanced PPRE reporter activation in normal mammary epithelial cells while it had virtually no effect in any of the cancer cell lines tested. Within each cancer type, individual cell lines were found to respond differently to distinct PPAR γ ligands. For instance, Ros, Cig, and PGJ₂ were all potent agonist of PPAR γ transactivation in lung adenocarcinoma cell lines while these same ligands had no effect in squamous cell or large cell carcinomas of the lung.

Message levels of PPAR γ and retinoid X receptor alpha (RXR α) in the individual cell lines were quantitated by real time-polymerase chain reaction (RT-PCR). The ratio of PPAR γ to RXR α was predictive of how cells responded to co-treatment of Ros and 9-*cis*-retinoic acid, an RXR α agonist, in two out of three cell lines tested. These data indicate that PPAR γ can be selectively modulated and suggests that it may be used as a therapeutic target for individual tumors.

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Keywords: PPAR; Thiazolidinediones; Breast cancer; Colon cancer; Lung cancer

1. Introduction

The American Cancer Society estimated that collectively cancers of the breast, colon and lung accounted for 42% of all cancer deaths in men and 50% of all cancer deaths in women in 2004. In fact, breast, lung, and colon cancer rank as the top three types of malignancies identified in women today and one out of every eight women will develop breast cancer. In men, lung cancer is the most prevalent cause of cancer related death with malignancies of the prostate and colon following as next most common. A wide variety of chemotherapeutic options are being explored to treat these diseases. Novel therapeutic targets are being developed in an effort to identify

endogenous, hormonal targets to either suppress cancer cell growth or induce apoptosis. One of the emerging targets for such treatments is peroxisome proliferator-activated receptor gamma (PPAR γ).

The orphan nuclear receptor, PPAR γ , is one of three of a family of receptors (PPAR α , β , and γ) (Dreyer et al., 1992; Issemann and Green, 1990; Kliewer et al., 1994). It is expressed in numerous cell types including adipocytes, epithelial cells of the breast, colon, and lung, and macrophages among others (Braissant et al., 1996; Kilgore et al., 1997; Lemberger et al., 1996; Nagy et al., 1998; Tontonoz et al., 1994). Several ligands of PPAR γ have been identified including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), linoleic acid, lysophosphatidic acid, and the thiazolidinedione class of anti-diabetic drugs such as ciglitazone (Cig) and rosiglitazone (Ros) (Forman et al., 1995; Kliewer et al., 1997; Larsen

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et al., 2003; McIntyre et al., 2003; Thoennes et al., 2000). Transactivation of the receptor requires ligand binding, heterodimerization with retinoid X receptor alpha (RXR α), and binding of this complex to PPAR-specific response elements (PPREs) in the promoter regions of target genes (Kliwer et al., 1992; Tontonoz et al., 1994).

Recent evidence demonstrates that PPAR γ is overexpressed in many different tumor types (DuBois et al., 1998; Tontonoz et al., 1997). In the breast, adenocarcinoma cells from patients expressed higher levels of PPAR γ than normal epithelial cells from the surrounding mammary gland (Elstner et al., 1998). Similarly, in the colon, expression of PPAR γ protein is significantly higher in human colon cancer sections when compared with non-tumor tissue (Chen et al., 2002). PPAR γ has also been identified in both adenocarcinoma and squamous cell carcinomas of the lung (Theocharis et al., 2002). Exposing cancer cells to PPAR γ ligands produces physiological effects that may be exploited for treatment purposes. In culture, synthetic PPAR γ ligands have been shown to inhibit growth of several tumor cell lines (Brockman et al., 1998; Elstner et al., 1998; Mueller et al., 1998). A number of studies have determined that PPAR γ ligands induce cellular differentiation and/or apoptosis in breast, colon, and lung cancer cells (Chang and Szabo, 2002; Elstner et al., 1998; Mueller et al., 1998; Sarraf et al., 1998). The combination of receptor overexpression in tumors and known physiological effects of its ligands on cancer cells makes PPAR γ a viable target of future chemotherapeutic agents.

The ability of individual ligands to selectively mediate the activity of a nuclear receptor dependent on the tissue type examined has been used to develop compounds that act as selective estrogen-receptor modulators (SERMs). Tamoxifen, which was originally described as an estrogen-receptor antagonist, has been found to act as an agonist in several different tissue types (Fisher et al., 1998; Jordan and Morrow, 1999; Levenson and Jordan, 1999). It has been proposed that individual ligands may be able to act as selective PPAR γ modulators (SPARMs) in a manner similar to the way other compounds function as SERMs (Sporn et al., 2001). We previously demonstrated that individual fatty acids can selectively activate a PPRE-reporter assay in estrogen-dependent breast cancer (MCF-7) cells (Thoennes et al., 2000). Specifically, omega-3 fatty acids inhibited transactivation of PPAR γ to levels below control while omega-6, monounsaturated and saturated fatty acids stimulated the activity of the PPRE reporter. These data demonstrated that individual compounds can selectively activate PPAR γ within the context of a single breast cancer cell line. However, compounds have yet to be identified that act as PPAR γ agonists in one tissue while functioning as antagonists of the receptor in other tissues.

In the studies presented here, we sought to determine if distinct ligands could selectively activate PPAR γ across different cell lines of mammary, colon, and lung origin. To this end we have utilized a PPRE-reporter construct transfected into the cells prior to ligand treatment. Data from these experiments demonstrated that selective activation of PPAR γ

occurs in multiple ways. Distinct ligands selectively activate PPAR γ dependent on the tissue type from which the cell line was derived. SPARM activity was also observed between different cell lines of the same tissue origin and individual ligands selectively activated the PPRE reporter within single cell lines. These data indicate that it may be possible to design PPAR γ ligands that can be used to selectively mediate receptor activity and thus customize treatment regimens against specific cancers.

2. Materials and methods

2.1. Reagents

All PPAR γ ligands were purchased from Cayman Chemical Company (Ann Arbor, MI). Ciglitazone (Cig) and GW9662 (GW) were solubilized in ethanol purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY). Rosiglitazone (Ros) was dissolved in dimethyl sulfoxide (DMSO) and PGJ₂ was solubilized in methyl acetate purchased from Sigma (St. Louis, MO).

2.2. Cells and cell culture

Ten individual cell lines were used in these experiments. Four mammary cell lines including normal mammary epithelial (HMEC) and three breast cancer cell lines (MCF-7, T47-D, and MDA-MB-231). Two colon cancer cell lines (Caco-2 and HT-29) and four lung cancer (A549, H358, H520, and H1299) were utilized. HMEC were purchased from Cambrex (Rockville, MD) while the MCF-7, T47-D, MDA-MB-231, H358, H520, and H1299 cells were all purchased from American Type Culture Collection (Bethesda, MD). The HT-29 and A549 cells were generously provided by Dr. David Kaetzel (University of Kentucky, College of Medicine) and the Caco-2 cells were a gift from Dr. Charlotte Kaetzel (University of Kentucky, College of Medicine). All cells were cultured in medium previously described to provide optimal conditions for their growth. When possible multiple cell lines were maintained in the same medium to reduce error when comparing across cell types. Cells were maintained in medium containing 10% FBS. All cell types were grown in medium lacking phenol red at 37 °C in a 5% CO₂ atmosphere. Cells were grown in T-75 flasks before being transferred to 12-well plates in preparation for transfection.

2.3. PPRE reporter plasmid

The reporter construct, 3XPPRE-TK-pGL3, contains three copies of a PPRE sequence (AGGACAAAGGTCA) upstream of the mTK promoter between the *Xho*I and *Hind*III restriction enzyme sites of the pGL3 basic vector (Promega, Madison, WI). *Bam*HI and *Bgl*II were then used to release the 2.2 kb fragment containing the 3XPPRE-mTK-Luciferase. This fragment was ligated into the *Bam*H I receptor site

of pRL-TK plasmid (Promega) completing the new reporter which contains both Luciferase and Renilla in a single expression plasmid. Renilla expression was used as a transfection efficiency control.

2.4. Transfection assays

Cells were transiently transfected with 5 μ g of PPRE reporter plasmid per 12-well plate. Cells were transfected with ESCORT transfection reagent for 4 h. Cells were subsequently treated with either 10 μ M Ros, 10 μ M Cig, 1 μ M PGJ₂, or 1 μ M GW for 18 h. In all cases, PPAR γ ligand concentrations for each compound used were those shown to be maximally effective following dose response studies (data not shown). Proper vehicle controls including ethanol, DMSO, and methyl acetate were run for each treatment group. Following treatment, cells were lysed in 50 μ l passive lysis buffer and treated according to manufacturer's instructions (Promega dual luciferase assay kit). Luminometry was performed on a Berthold Lumat 9507 and data calculated as raw Luciferase Units (RLUs) divided by raw Renilla units. Mean fold induction was obtained by dividing the RLU data from each treatment well by the mean values of the vehicle control appropriate for each treatment. Each set of treatments were performed in replicates of six in three separate experiments.

2.5. RNA preparation

Total RNA was isolated from cultured cells utilizing an RNeasy[®] Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Untreated cells from each cell line were used for RNA isolation. All cell lines were maintained in optimal growth conditions prior to RNA collection. RNA was stored at -80°C and concentration was measured at by spectrophotometry.

2.6. Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time PCR was performed on total RNA using the TaqMan One-Step RT-PCR Master Mix Kit purchased from Applied Biosystems (Foster City, CA) and used according to manufacturer's instructions. Commercial FAM labeled probe/primer pairs constructed by Applied Biosystems using the Celera genomic database were used to assess PPAR γ (cat#: Hs00234592_m1) and RXR α (cat#: Hs00172565_m1) mRNA levels. Quantitation of mRNA was performed using an ABI Prism 7700 Sequence Detection System and the TaqMan methodology, which uses the 5'-nuclease activity of the Taq DNA polymerase to generate a real-time quantitative DNA assay. Data were analyzed using a C_t cycle method.

At the completion of the amplification (40 cycles), the amount of target message in each reaction was recorded as a threshold cycle number (C_t), which is inversely correlated to the abundance of the initial message level. C_t measures the fractional cycle number at which the amount of amplified

target reaches a fixed threshold. The amount of target was normalized to the endogenous reference target, human GAPDH (cat#: Hs99999905_m1), again using a FAM labeled Taqman probe/primer solution available from Applied Biosystems. This normalized target C_t value was then set relative to a normalized calibrator sample (i.e. untreated normal cell type) as given by the equation $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ represents ΔC_t , target sample minus ΔC_t , calibrator. Finally, this value was then used to produce a relative quantity by comparison to an appropriate control sample.

2.7. Statistical analysis

As previously described (Thoennes et al., 2000), fold changes in luciferase to renilla ratios were subject to a two-factor analysis of variance (ANOVA) hypothesis testing ($\alpha = 0.05$) based on the two nominal variables of treatment and experimental date using a custom designed program running on the StatServer 6.1 (Insightful, Seattle, WA) server housed in the University of Kentucky's Department of Statistics. In every case, the post-hoc test, Tukey's pair-wise comparison, was performed to identify significant differences between the various treatments within a cell line. Briefly, the Tukey methodology simultaneously determined the presence of significant differences between individual treatment mean estimations across the entire balanced set of pairwise comparisons using the studentized range distribution, q . Mean fold changes in luciferase/renilla ratios of treatments compared to vehicle controls were displayed by column graph with one-half of the critical value for comparison from the Tukey's comparison as an estimation of error. Significant differences within those comparisons for a single cell line are designated by an alpha-numeric system.

For RT-PCR analysis, cycle threshold measurements, C_t , for the mRNA targets of both PPAR γ and RXR α were repeated in triplicate within each cell line. The average C_t value for both PPAR γ and RXR α in all 10 cell lines were subjected to analysis of variance hypothesis testing (ANOVA) using Microsoft Excel v10.0 at $\alpha = 0.05$ significance threshold. Following ANOVA, Fisher's least significant difference, LSD, pair-wise comparison was implemented post-hoc. Briefly, the LSD test determines a single critical value based on the mean squared error within groups and a critical value ($\alpha = 0.05$) found in the t distribution. If the average absolute difference between any two groups was greater than the LSD critical value, then the pair-wise comparison for those two groups were found to be significantly different at ($p < 0.05$).

3. Results

3.1. Effect of PPAR γ ligands on reporter activation in breast cancer cells

Following transfection with a PPRE reporter plasmid, HMEC, T47-D, MDA-MB-231, and MCF-7 cells were

treated with either vehicle control or PPAR γ ligands for 18 h. For the four cell lines, differences in ligand activity were observed. In the HMEC, Ros and PGJ₂ both significantly increased reporter activity over control (Fig. 1A). Interestingly, GW, a known antagonist of PPAR γ , also significantly stimulated reporter activity. GW treatment did not change reporter activity compared to control in any of the other breast cancer cell lines. No treatments significantly increased reporter activity in the T47-D cells (Fig. 1A). In MDA-MB-231 cells Ros, Cig, and PGJ₂ all significantly enhanced PPAR γ activation over control, while these same three treatments

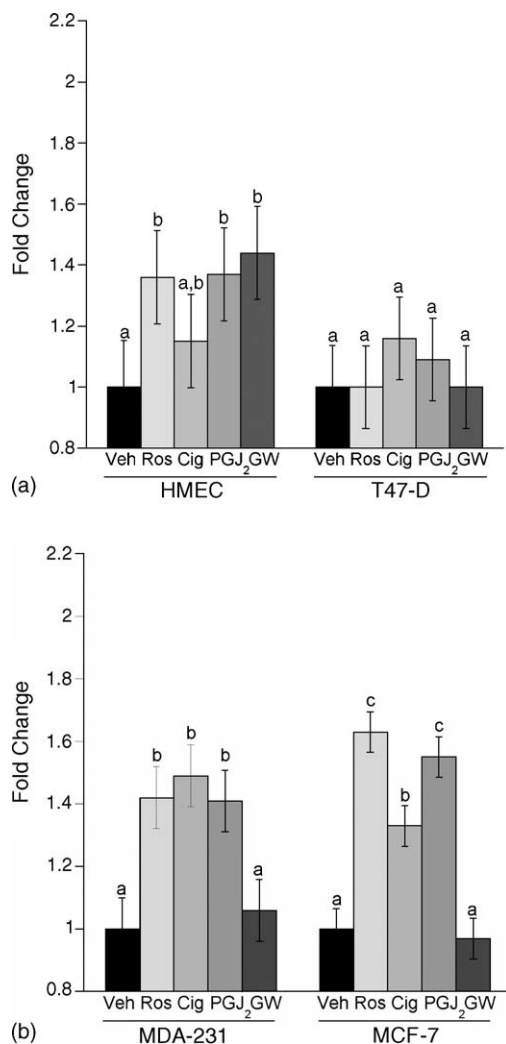


Fig. 1. (A and B) Effect of PPAR γ ligands on reporter activation in breast cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPAR γ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

also increased reporter activation in MCF-7 cells when compared to control (Fig. 1B). Both Ros and PGJ₂ treatments resulted in significantly higher activity than Cig in MCF-7 cells.

3.2. Effect of PPAR γ ligands on reporter activation in colon cancer cells

Two colon cancer cell lines (HT-29 and Caco-2) were also tested in the same manner described for the mammary cells (Fig. 2). In general, HT-29 cells were more responsive to the PPAR γ agonist than the Caco-2 cells. In the Caco-2 cells, only the Ros treatment caused significant increases in PPRE reporter activity when compared to control. Alternatively, in HT-29 cells Ros, Cig, and PGJ₂ treatments all resulted in significantly higher reporter activation when compared to vehicle control whereas, GW treatment was not significantly different from control in either colon cell line.

3.3. Effect of PPAR γ ligands on reporter activation in lung cancer cells

To examine PPAR γ activation in lung cancer cells, four cell lines were chosen. A549 and H358 cells were derived from adenocarcinoma lung tumors while H520 and H1299 cell lines are non-adenocarcinoma derived cell lines. H520 cells are lung squamous carcinoma cells and H1299 cells

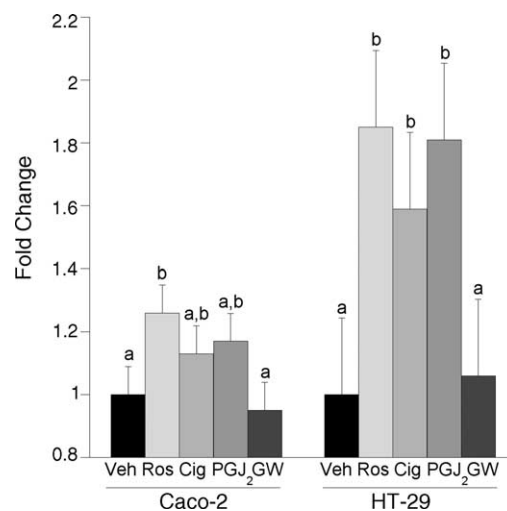


Fig. 2. Effect of PPAR γ ligands on reporter activation in colon cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPAR γ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

were derived from a metastatic site of a patient with large cell carcinoma of the lung. Cells were transfected and treated in the same manner as the other cancer cell lines. Similar to the breast and colon differences in reporter activity were observed for individual cell lines. In general, non-adenocarcinoma (H520 and H1299) cells did not respond to PPAR γ ligands as well as the adenocarcinoma (H358 and A549) cell lines (Fig. 3A and B). Also, as observed in various other cancer cell lines, significant differences between the relative activation of the reporter were seen with individual ligands within single cell lines. In H1299 cells, treatment

with Cig, PGJ $_2$, or GW resulted in significantly higher activation of the PPRE reporter when compared to vehicle control while Ros and Cig treatment caused significant increases in activity in H520 cells (Fig. 3A). In H358 cells, Ros, Cig, and PGJ $_2$ all resulted in increased activation when compared to control (Fig. 3B). However, Ros treatment resulted in significantly greater reporter activity when compared to both Cig and PGJ $_2$ as well. Exposure of A549 cells to Ros, Cig, or PGJ $_2$ also caused a significant increase in activation of the PPRE reporter when compared to control (Fig. 3B). However, in these cells reporter activation was greatest in Cig treated cells and the fold change for this treatment was significantly greater than that in PGJ $_2$ treated cells. GW9662 treatment was not significantly different from control in H520, H358, or A549 cells.

3.4. Expression of PPAR γ and RXR α mRNA

mRNA levels of PPAR γ and RXR α were measured in all cell lines (Fig. 4). Total RNA was isolated from untreated cells. H1299 had the lowest expression of both PPAR γ and RXR α when compared to all other cells. H520 cells had the second lowest levels of PPAR γ and RXR α , while HMEC and A549 cells were next highest. H358 cells had similar expression of PPAR γ as HMEC and A549 cells, but had significantly more RXR α expression when compared to the same cell lines. T47-D cells had significantly lower levels of PPAR γ than all of the cell lines except the H520 and H1299 cells. However, these cells had the highest expression of RXR α among all cell lines. Caco-2 cells expressed the second largest amount of PPAR γ mRNA and had high RXR α levels with only MCF-7 and T47-D cells expressing more. HT-29 cells had higher mRNA levels of PPAR γ when compared to all other cell lines and RXR α expression similar to Caco-2 and H358 cells. MCF-7 cells express significantly higher levels of PPAR γ expression than all but four cell lines and higher RXR α than all of the cells tested except T47-Ds. MDA-MB-231 cells had PPAR γ mRNA levels similar to MCF-7 cells, but had lower RXR α expression with only two cell lines having significantly lower levels.

3.5. Effect of Ros and 9-cis-retinoic acid co-treatment on reporter activation in selected cell lines

Three cell lines were selected to determine if the relative expression of PPAR γ and RXR α are predictive of the effect that co-treatment with a PPAR γ agonist (Ros) and RXR α (9-cis-retinoic acid) agonist have on the PPRE reporter assay. HT-29, MCF-7, and MDA-MB-231 cells were selected to test this principal because Ros was shown to activate the reporter and the cell lines expressed varying levels of PPAR γ to RXR α . MCF-7 cells were found to express more RXR α than PPAR γ . Conversely, MDA-MB-231 and HT-29 cells expressed more PPAR γ than RXR α . These three cell lines were transfected with the PPRE reporter construct and treated with

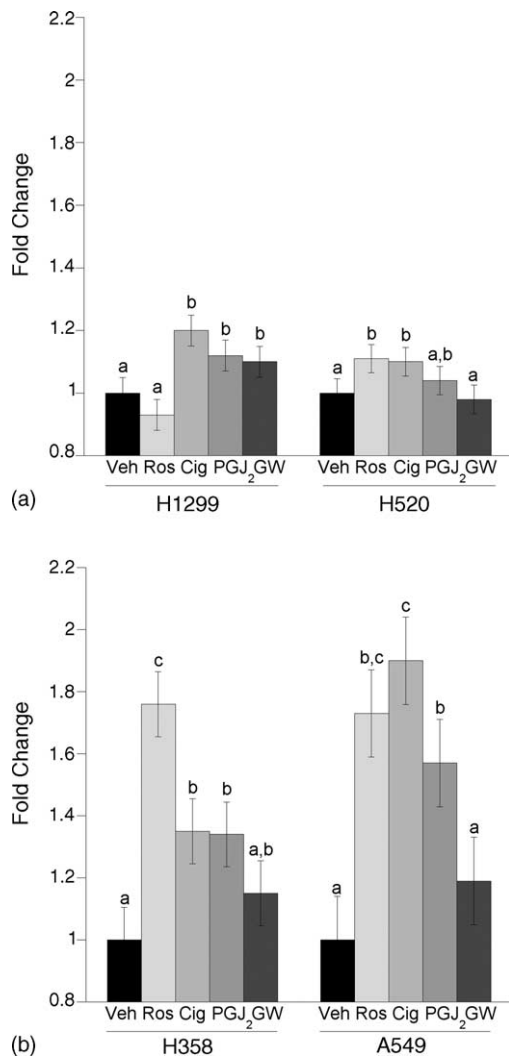


Fig. 3. (A and B) Effect of PPAR γ ligands on reporter activation in lung cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPAR γ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

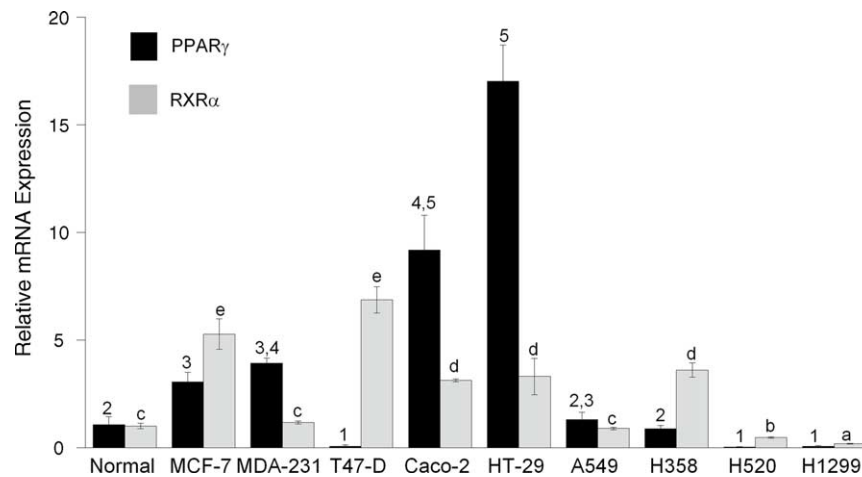


Fig. 4. Relative expression of PPAR γ and RXR α cancer cell lines of the breast, colon, and lung. PPAR γ and RXR α were detected by real-time PCR. Total mRNA was collected from untreated cells for each of the 10 cell lines. The relative expression levels of PPAR γ and RXR α as compared to the endogenous control, human GAPDH were normalized to the expression of the targets in the normal mammary epithelia. Error bars represent the standard error of the mean.

either vehicle, Ros alone, or co-treated with Ros and 9-*cis*-retinoic acid. Ros alone and the co-treatment resulted in significantly higher activation of the reporter in all three cell lines when compared to vehicle controls (Fig. 5). In HT-29 cells, activation of the reporter was higher in the cells treated with Ros alone compared to those receiving the co-treatment. Conversely, co-treatment resulted in significantly greater reporter activity in the MCF-7 and MDA-MB-231 cells when compared to Ros treatment alone.

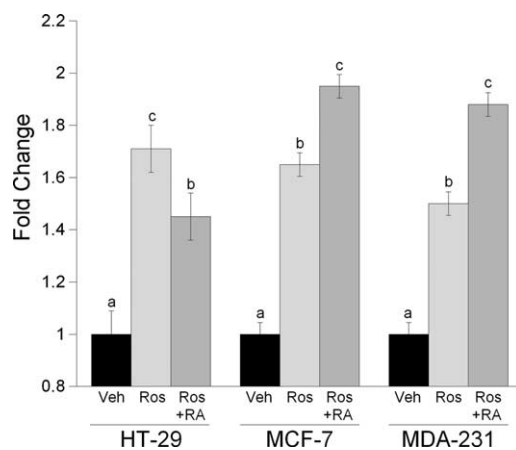


Fig. 5. Effect of cotreatment with Rosiglitazone and 9-*cis*-retinoic acid on reporter activation in various. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with either rosiglitazone (Ros) or rosiglitazone plus 9-*cis*-retinoic acid (Ros + RA) for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

4. Discussion

Data from the present study demonstrate that individual PPAR γ ligands have the ability to selectively activate a PPRE reporter in cancers of the breast, colon, and lung. Differences in PPRE reporter activation were observed between cells derived from different tissue types as well as between cell lines of the same cancer type. Also, within a single cell line, individual ligands selectively induced PPRE reporter activity. Expression of PPAR γ and RXR α mRNA were measured in all cell lines in the absence of treatment, but expression was not predictive of how individual cell lines responded to ligand and treatment. Finally, differences in how individual cell lines responded to co-treatment with PPAR γ and RXR α agonists were observed.

A growing body of evidence indicates PPAR γ is involved in both breast cancer development and progression. PPAR γ (+/-) mice had almost three-fold increased incidence of mammary adenocarcinomas and decreased survival rate when compared to PPAR γ (+/+) littermates (Nicol et al., 2004). Several reports have demonstrated that treating animals with PPAR γ ligands prior to chemical induction of mammary tumors is protective against tumor development (Mehta et al., 2000; Suh et al., 1999). It appears that expression and transactivation of PPAR γ is protective against breast tumor formation particularly when activated by PPAR γ ligands. Our data demonstrate that some ligands are more effective than others in transactivating PPAR γ in normal mammary epithelia.

Once a breast tumor has formed, PPAR γ appears to have multiple effects. In vitro, treatment of breast cancer cells with troglitazone results in lipid accumulation, changes in gene expression associated with cellular differentiation, reduction in growth rate and clonogenic capacity (Mueller et al., 1998). Others have observed that distinct PPAR γ ligands induce apoptosis (Eltner et al., 1998). Conversely, a recent

report by Saez et al. (2004) found that when mice expressing a constitutively active form of PPAR γ in the mammary gland were crossed with mice prone to mammary gland cancer, bigenic animals develop tumors that express higher levels of markers of malignancy. The authors conclude that once an initiating event takes place, increased PPAR γ signaling serves as a tumor promoter in the mammary gland of these experimental animals. Collectively, these data suggest that the physiological consequence of PPAR γ activation is dependent on many factors including the stage of development of the specific breast cancer cell. Our demonstration that individual PPAR γ ligands distinctively modulate PPRE reporter activity in breast cancer cell lines differently has implications for breast cancer treatment. Specifically, T47-D cells were fairly unresponsive to any of the three PPAR γ agonists tested, whereas, Ros, Cig, and PGJ₂ significantly increased reporter activity in MCF-7 and MDA-MB-231 cells. It can be concluded that individual breast cancer cell types are likely to respond to PPAR γ ligands in unique physiological ways and our data suggests that, in part, variant cellular responses are the result of selective PPAR γ transactivation.

PPAR γ also influences colon tumor development and growth. In mice predisposed to the development of intestinal polyps caused by a mutation in the adenomatous polyposis coli (APC) gene, treatment with troglitazone or Ros increases both the number and size of intestinal polyps (Lefebvre et al., 1998; Saez et al., 1998). These data are partially explained by studies demonstrating that PPAR γ loses its ability to influence colon tumorigenesis in mice with a mutated APC gene, whereas in wild-type APC mice, PPAR γ functions as a tumor suppressor (Girnun et al., 2002). Conversely, PPAR γ ligands reduce aberrant crypt foci (ACF) formation in mice following tumor induction by azoxymethane (Osawa et al., 2003). Differences in the effects of PPAR γ ligands in these two models demonstrates that like mammary cells, colon cancer cells respond to a single PPAR γ ligand differently dependent on the cell characteristics. When we examined the ability of PPAR γ ligands to activate the PPRE reporter construct in two colon cancer cell lines, differences in cellular responsiveness was revealed. Ros significantly increased reporter activity in both Caco-2 and HT-29 cells though the level of responsiveness was much greater in the HT-29 cells. Furthermore, Cig and PGJ₂ did not significantly enhance reporter signal in Caco-2 cells, but were strong agonists in the HT-29 cells. Selective modulation of PPAR γ transactivation can explain the variant physiological responses observed in different colon cancer animal models. Differences in ligand activity could have significant impact on colon cancer treatment strategy as a number of studies have shown that PPAR γ ligands affect colon tumor cell progression. Treatment of colon cancer cells with PPAR γ agonists inhibits their growth in vivo (Brockman et al., 1998; Kitamura et al., 1999; Sarraf et al., 1998; Shimada et al., 2002) and in vitro (Sarraf et al., 1998). Inhibition of growth is often attributed to PPAR induced apoptosis and DNA fragmentation (Chen et al., 2002; Shimada et al., 2002; Yang and Frucht, 2001).

In the lung, epithelial cells possess 15-lipoxygenases which produce a variety of metabolic products including 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) (Profita et al., 2000). In A549 cells, 15(S)-HETE has been demonstrated to induce apoptosis by binding to PPAR γ (Shankaranarayanan and Nigam, 2003). Similarly, treatment of adenocarcinoma (A549) cells with Cig resulted in growth inhibition (Chang and Szabo, 2002); however, this inhibition was not observed in either squamous cell carcinoma (H520) or large cell carcinoma (H1299) cell types (Chang and Szabo, 2002). In the present study, two adenocarcinoma (A549 and H358) and two nonadenocarcinoma (H520 and H1299) cell lines were selected for evaluation. The nonadenocarcinoma cell lines were highly unresponsive to the PPAR γ ligands when compared to the adenocarcinoma cells. Ros and Cig significantly increased reporter activity in H1299 and H520 cells, but fold change compared to control was relatively small in these cells. Conversely, A549 and H358 cells were highly responsive to the PPAR γ agonists. These data suggest that the varying effects of Cig on adenocarcinoma versus nonadenocarcinoma cells observed in Chang et al., 2002 are likely the down stream result of selective PPRE transactivation.

One focus of these studies was to determine whether individual ligands of PPAR γ could act as SPARMs. We present evidence indicating that within each tissue type, individual ligands are capable of selectively activating the PPRE reporter construct dependent on the individual cell line tested. However, individual ligands had unique effects across tissue types as well. For instance, we report that GW, a known PPAR γ antagonist in adipocytes (Leesnitzer et al., 2002; Starkey et al., 2003), significantly increased reporter activity in HMECs. This effect was observed in no other cell line except H1299 cells and in those cells the magnitude of change was very small leaving in question its biological significance. These findings are significant because they suggest that an individual compound can function as a PPAR γ antagonist in one tissue and as an agonist in other tissues. It is possible that the agonist activity of GW is specific to normal epithelial cells and that changes occur during cancer cell formation that results in the loss of this responsiveness. It is also possible that the actions of GW are mammary specific. Further, investigation is necessary to explore these possibilities.

Another objective of these studies was to determine if PPAR γ mRNA expression is predictive of a cell line's responsiveness to PPAR γ ligands with regards to PPRE activation. For three of the cell lines (MCF-7, MDA-MB-231, and HT-29) increased relative expression of PPAR γ over HMECs correlated with enhanced reporter activity when exposed to the PPAR γ agonists. However, A549 and H358 had higher reporter activity in response to the individual PPAR γ agonist compared to Caco-2 cells despite the fact that A549 and H358 cells express much lower levels of PPAR γ than the Caco-2s. Therefore, PPAR γ mRNA levels alone are not predictive of PPAR γ mediated PPRE activation. These data led us to ex-

plore the possibility that variances of expression of RXR α , the heterodimeric partner of PPAR γ , and its relation to the levels of PPAR γ in the different cell lines may play a critical role in PPAR γ 's ability to activate the PPRE reporter construct. Here we demonstrate that the cell lines differed in their relative expression of PPAR γ to RXR α ; however, there was no unifying pattern of receptor expression that was predictive of ligand activity in the reporter assay.

Unique expression patterns of PPAR γ relative to RXR α in certain cell lines led us to hypothesize that the relative expression of these receptors may be used to identify cell lines in which co-treatment with an RXR α agonist (9-*cis*-retinoic acid) would enhance PPRE reporter activation. Three cell lines were chosen to test this hypothesis. HT-29 and MDA-MB-231 cells express higher levels of PPAR γ relative to RXR α and would therefore, not be expected to have increased reporter activity with co-treatment (Ros + 9-*cis*-retinoic acid) compared to Ros alone. Conversely, MCF-7 cells having higher expression of RXR α relative to PPAR γ would supposedly demonstrate enhanced reporter activity when treated with both ligands compared to Ros alone. HT-29 cells showed no additional reporter activity in cells co-treated with Ros and 9-*cis*-retinoic acid over those treated with Ros alone. MCF-7 cells had enhanced activation of the PPRE reporter with co-treatment over Ros treatment alone. These two cell lines support the possibility that relative expression levels may be predictive in identifying cells that will more readily respond to co-treatment with both ligands. However, MDA-MB-231 cells did not support this hypothesis. Co-treatment with both ligands had a significantly higher effect on reporter activity when compared to single treatment with Ros even though relative PPAR γ expression was higher than RXR α expression in these cells. These data suggest that while this approach may work for certain cancer cell types, receptor expression alone may not be predictive for how cells will respond to co-treatment. A better understanding of how PPAR γ and RXR α function in each of the cell lines will be necessary before the predictive value of receptor expression can be realized.

In conclusion, PPAR γ ligands, have distinct activities within a cell type, between tumor cells derived from the same tissue, and across distinct tissues. Although we used only one type of PPRE reporter construct in these experiments, the selective modulation of PPAR γ within individual cells is likely to be a gene/promoter specific event. Nonetheless, utilizing this reporter of PPAR transactivation, differences were observed in all three cancer types (breast, colon, and lung) where a single ligand enhanced reporter activation in certain cell types, but had minimal to no effect in other cell lines of the same malignancy type. Finally, our data demonstrates that individual PPAR γ ligands can selectively activate reporter activity within a single cancer cell type. These data suggest that the effectiveness of PPAR γ as a target for chemotherapeutic treatment will greatly depend on the cell that is treated which opens the possibility of utilizing PPAR γ for targeted gene therapy.

Acknowledgments

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Transactivation of ER α by Rosiglitazone induces proliferation in breast cancer cells

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Abstract In the present study, we demonstrate that Rosiglitazone (Rosi), a thiazolidinedione and PPAR γ agonist, induces ERE (Estrogen Receptor Response Element) reporter activity, pS2 (an endogenous ER gene target) expression, and proliferation of ER positive breast cancer (MCF-7) cells. By performing a dose–response assay, we determined that high concentrations of Rosi inhibit proliferation, while low concentrations of Rosi induce proliferation. Using the anti-estrogen ICI, ER negative breast cancer (MDA-MB-231) cells, and a prostate cancer cell line (22Rv1) deficient in both ER α and PPAR γ , we determined that Rosiglitazone-induced ERE reporter activation and proliferation is through an ER α dependent mechanism. Rosiglitazone-induced ERE activation is also dependent on activation of the Extracellular Signal-Regulated Kinase–Mitogen Activated Protein Kinase (ERK–MAPK) pathway, since it is inhibited by co-treatment with U0126, a specific inhibitor of this pathway. We also demonstrate that when ER α and PPAR γ are both present, they compete for Rosi, inhibiting each others transactivation. To begin to unravel the pharmacological mechanism of Rosi-induced ER activation, sub-maximally effective concentrations of E₂ were used in combination with increasing concentrations of Rosi in luciferase reporter assays. From these assays it appears that E₂ and Rosi both activate ER α via similar

pharmacological mechanisms. Furthermore sub-maximally effective concentrations of E₂ and Rosi additively increase both ERE reporter activity and MCF-7 cell proliferation. The results of this study may have clinical relevancy for Rosi's use both as an anti-diabetic in post-menopausal women and as an anti-cancer drug in women with ER positive breast cancer

Keywords PPAR γ · ER α · Crosstalk · Breast cancer · Rosiglitazone

Introduction

Thiazolidinediones (TZDs) are a class of drugs used in the treatment of type II diabetes that include Troglitazone (Rezulin), Rosiglitazone (Avandia), Pioglitazone (Actos), and Ciglitazone. TZDs, through a mechanism that is not thoroughly understood, cause insulin sensitization and a decrease in glucose levels in Type 2 diabetic patients. These effects are mediated, at least in part, by activating the peroxisome proliferator-activated receptor-gamma (PPAR γ) [1]. PPAR γ belongs to the nuclear receptor superfamily, which are ligand-mediated transcription factors [2]. Ligand binding allows PPAR to heterodimerize with RXR α [3] and interact with a specific DNA sequence termed a PPAR Response Element (PPRE) in the nucleus [4]. This in turn mediates the recruitment of co-activators resulting in transcriptional regulation of target genes [5, 6]. PPAR γ is expressed in numerous tissues including adipocytes, monocytes, the large intestine, and breast epithelia [7–10]. Although the endogenous ligand remains unknown, PPAR γ ligands include polyunsaturated fatty acids [11, 12], 15-deoxy-delta^{12,14}-prostaglandin J2 (PGJ₂) [13, 14] and some non-steroidal anti-inflammatory drugs [15].

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Several labs have shown that PPAR γ is expressed at higher levels in breast carcinoma cells than in normal human mammary epithelia cells [16–18]. The function of PPAR γ in breast cancer cells, however, is unclear. PPAR γ ligands have been shown to reduce malignancy by promoting differentiation [19] and apoptosis in both primary and metastatic breast cancer cells [18] suggesting that PPAR γ is a potential tumor suppressor. However, another study [20] demonstrated that PPAR γ may instead be a tumor promoter and suggested that inhibition of cellular growth seen in other studies may reflect PPAR γ -independent effects of the ligands used. TZDs have been found to elicit varying effects in clinical studies. Troglitazone was the first TZD that was shown to have anti-cancer effects in patients with liposarcomas [21] but had no effect when used as treatment for patients with refractory breast cancer [22]. Data from the PRO-Active Study, however, have shown that diabetic patients on Pioglitazone had a decreased occurrence of breast cancer [23]. One explanation for the variance of the effect of TZDs is their ability to elicit PPAR independent effects. The interaction of TZDs with other nuclear hormone receptors, however, has not been thoroughly examined.

Arguably one of the most important hormone receptors in breast cancer is ER α . Approximately 65% of all breast cancers are ER positive [24] and are dependent on estrogen for growth. Consequently, a great deal of research has focused on exploring novel methods to selectively inhibit the actions of estrogen in breast cancer. Furthermore, the identification of selective estrogen modulators (SERMs) has opened new approaches in breast cancer prevention and treatment. It is clear that PPAR γ itself can interact with ER signal transduction pathway [25, 26] and many studies have employed TZD's to demonstrate this interaction [27, 28]. Whether these results are from a direct interaction between PPAR γ and ER α or a ligand-mediated receptor independent effect of the TZDs on the ER signal transduction pathway remains unclear. In this study, we sought to determine the effect of the thiazolidinedione and PPAR γ agonist Rosiglitazone (Rosi) on the ER signal transduction pathway. Here we demonstrate that Rosi can also be an ER α agonist. The results of this study may explain Rosi's varying effects in breast cancer studies and contraindicate its use both as an anti-cancer drug in women with ER positive breast cancer and as an anti-diabetic in post-menopausal women.

Materials and methods

Reporter and expression vectors

The reporter construct, 3XPPRE-TK-pGL3 [8], contains three copies of a PPRE sequence (AGGACAAAGGTCA)

upstream of the minimal thymidine kinase promoter ligated into pGL3 basic vector (Promega, Madison, WI). The ERE-TK-pGL3 reporter construct contains one copy of an ERE sequence (AGGTCAATGACCT) upstream of the minimal thymidine kinase promoter ligated into pGL3 basic vector (Promega, Madison, WI) [29]. The CMV-promotor controlled β -galactosidase, a kind gift from Dr. Melinda Wilson (University of Kentucky, College of Medicine), was used as a control to analyze constitutive activation. Bluescript plasmid was purchased from Stratagene (La Jolla, CA).

Treatments

17 β -estradiol (E₂) was purchased from Sigma (St. Louis, MO). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI) and ICI 182,780 was purchased from Tocris (Ellisville, MO). All treatments were dissolved in dimethyl sulfoxide (DMSO).

Cell culture and conditions

MCF-7 and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and the human prostate carcinoma cells (22Rv1) were a kind gift from Dr. Natasha Kyprianou (University of Kentucky). All cells were maintained at 37°C in 5% CO₂ atmosphere. MCF-7 cells were maintained in DMEM, MDA-MB-231 cells in IMEM, and 22Rv1 in RPMI1640 medium and all contained 10% FBS and lacked phenol red. The cells were changed to medium containing charcoal stripped serum 72 h prior to all experiments.

Transfection and luciferase assays

All cells were transiently transfected using ESCORT lipofectamide transfection reagent in 24-well plates. MCF-7 cells were transfected with 2 μ g ERE-luciferase and 0.5 μ g β -gal reporter plasmids. MDA-MB-231 cells were transfected with 2 μ g ERE or PPRE-luciferase reporter plasmids, 0.5 μ g β -gal reporter plasmid, and either 0.5 μ g ER α or bluescript expression plasmids. All 22Rv1 cells were transfected with 1 μ g ERE-luciferase and 250 ng β -gal reporter plasmids per plate. In addition to the luciferase and β -gal reporter plasmids, 22Rv1 cells were transfected with either 1 μ g bluescript expression plasmid (control cells), 500 ng each PPAR γ and bluescript expression plasmids (cells expressing PPAR γ), 500 ng each ER α and bluescript expression plasmids (cells expressing ER α), or 500 ng each ER α and PPAR γ expression plasmids (cells expressing ER α and PPAR γ). Four hours after transfection, cells were treated for 18 h

with the described treatments. The cells were then washed and lysed with 100 μ l passive lysis buffer (Promega). Quantification of induced firefly (*Phontius pyralis*) luciferase protein was performed according to manufacturer's instructions (cat #E1501). About 10 μ l cell lysate was analyzed using a Berthold Lumat 9507 to calculate luciferase quantities while another 10 μ l of cell lysate was used in combination with β -gal enzyme assay system and a plate reader (420 nm) to measure β -gal quantities. The luciferase numbers were divided by the β -gal numbers in order to normalize the data. Each treatment was conducted in triplicate and repeated 3 times for an $n = 9$ for each treatment.

MTS proliferation assay

MCF-7 cells were treated for 3 days with treatment media which was replaced every other day. The cells were then treated with 80 μ l of MTS/PMS solution (Promega—CellTiter® Aqueous Non-Radioactive Assay) for 3 h to measure proliferation. Absorbance was read using a plate reader at 492 nm. Mean fold changes were calculated by dividing each treatment absorbance by the average of the control absorbance values for that day. Each treatment was conducted in triplicate and repeated 3 times for an $n = 9$ for each treatment.

BrdU proliferation assay

MCF-7 cells were treated for 3 days with treatment media which was replaced every other day. The cell proliferation ELISA BrdU (colorimetric) kit (Roche Applied Science, Germany) was then used to calculate proliferation. This assay is based on the incorporation of the pyrimidine analog BrdU into the DNA of proliferating cells. The BrdU is then detected by immunoassay. The protocol, in brief, begins with the BrdU labeling solution being incubated with the treated cells, which are then fixed and the DNA denatured. A monoclonal Anti-BrdU antibody conjugated with peroxidase that binds the BrdU labeling reagent is added to each well. The immune complexes are detected by the subsequent substrate reaction. The reaction product is then quantified by measuring the absorbance using an ELISA-plate reader.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

MCF-7 cells were treated for 24 h with E₂ or Rosi alone or with ICI. The cells were harvested and the RNA was isolated using the Rneasy kit (Qiagen). About 1 μ g of total RNA was reverse-transcribed in a total volume of 20 μ l using 200 units of reverse transcriptase, 50 pM of random hexamers, and 1 mM dNTP (Stratagene Sybr Green Kit).

The Oligogreen Assay (Molecular Probes) was then used to calculate the concentration of the resulting DNA. Each real-time PCR reaction consisted of 0.5 μ g of DNA, 1 $\times$ SYBR Green PCR Master Mix, and 50 nM of forward and reverse primers. The primers used in the real-time PCR were for pS2: pS2 255f, 5'-ATACCATCGACGTCCCT CCA-3' and pS2 401r, 5'-AAGCGTGTCTGAGGTGTC CG-3' and for 36B4: 36B4 574f, 5'-GTGTTTCGACAATGG CAGCAT-3' and 36B4 657r, 5'-GACACCCTCCAGGAA AGCGA-3'. Reactions were carried out in a Stratagene Mx 3000P for 40 cycles (95°C for 15 s, 60°C for 1 min) following an initial 10-min incubation at 95°C. The fold change in gene expression was calculated using the $\Delta\Delta C_t$ method with the ribosomal protein 36B4 mRNA as an internal control.

Western blots

After treatment for 24 h, MCF-7 cells were washed with PBS, lysed with passive lysis buffer, and centrifuged to collect the whole cell lysate. A Bradford Assay (BioRad) was then used to quantify the concentration of the total cell lysate. About 10 μ g of total cell lysate per treatment was run on a 10% SDS electrophoresis gel. The protein was then transferred to nitrocellulose membrane, blocked in 5% milk/Tris Buffer Saline Tween 20 (TBST), and incubated with 1/1,000 dilution of primary ER α antibody (Santa Cruz Biotechnology) in 5% milk/TBST solution overnight at 4°C. The membrane was then washed 3 times with TBST and incubated for 2 h with an HRP-conjugated secondary antibody. The membrane is again washed 3 times in TBST, treated with ECL detection reagent (Pierce SuperSignal® West Pico Chemniluminescent Substrate), and visualized after exposure with film.

Statistics

Mean fold changes were calculated by dividing each treatment value by the mean of the control for that particular day. The mean and standard error of these fold changes over all the experiments were then calculated. Differences in luciferase to β -gal ratios and/or proliferation fold changes were subject to a two-way analysis of variance (ANOVA) hypothesis testing ($\alpha = 0.05$) using a custom designed program running on the StatServer 6.1 (Insightful, Seattle, WA) from the server housed and maintained by the University of Kentucky's Department of Statistics. In every case, the post-hoc test, Tukey's pair-wise comparison, was performed to identify significant differences between all possible combinations of treatments by mean estimations across the entire balanced set of pair-wise comparisons using the studentized range distribution, q . Mean fold changes in luciferase/ β -gal ratios of

treatments compared to vehicle controls were displayed by column graph with standard error of the mean as an estimation of error. Significant differences within those comparisons are designated by differences in letter designations.

Results

Rosi induces ERE-reporter activity in MCF-7 cells and is inhibited by ICI

To assess how Rosi affects the ER signal transduction pathway in breast cancer cells, MCF-7 cells were transiently transfected with a luciferase reporter under the control of an ER response element (ERE) and treated with E₂, Rosi, or E₂ together with Rosi for 18 h (Fig. 1A). E₂ treatment significantly increased ERE reporter activity and

interestingly, Rosi treatment also resulted in a significant increase in ERE reporter activity. E₂ and Rosi treatments combined did not significantly alter ERE-reporter activity as compared to E₂ treatment alone. The pure ER α antagonist, ICI 182,780 (ICI), was used to determine whether Rosi activation of an ERE reporter is dependent on the ER signal transduction pathway. MCF-7 cells transfected with the ERE luciferase reporter were treated with E₂, Rosi, or ICI alone or in combination for 18 h (Fig. 1B). Although ICI treatment alone did not alter ERE reporter activity, it completely blocked ERE reporter activation by both E₂ and Rosi.

Rosi-induced expression of pS2 is inhibited by ICI in MCF-7 cells

To investigate Rosi's ability to regulate an endogenous ER target gene, the expression of a well-characterized estrogen

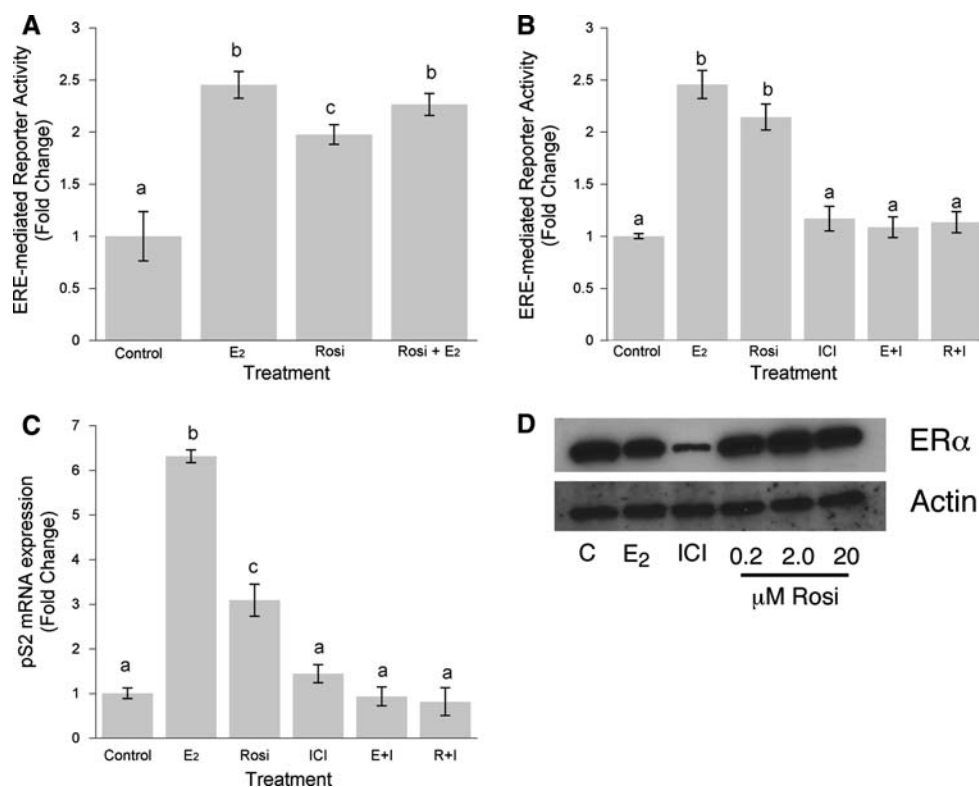


Fig. 1 Rosi, independent of ER α expression, induces ERE-reporter activation and pS2 expression and both are inhibited by ICI. ERE-mediated reporter activity was measured in MCF-7 cells transiently transfected with an ERE-TK-pGL3 reporter plasmid and treated with (A) 10 nM E₂ and 1 μ M Rosi both alone and together or (B) 10 nM E₂, 1 μ M Rosi, or 10 μ M ICI alone or E₂ or Rosi together with ICI. Luciferase activity was normalized to constitutively active β -gal. Data is expressed as mean fold changes in luciferase to β -gal ratios compared to vehicle control. (C) RT-PCR to determine expression of pS2 was run on RNA isolated from cells treated for 24 h with 10 nM E₂, 1 μ M Rosi, or 10 μ M ICI alone or E₂ or Rosi together with ICI.

The cycle threshold (Ct) of ps2 was detected by SYBR-GREEN RT-PCR method. The fold change in gene expression was calculated using the $\Delta\Delta$ Ct method (with the ribosomal protein 36B4 mRNA as an internal control). Error bars represent the standard error of the mean and bars that do not share a letter designation are significantly different. (D) Western blot analysis was performed on MCF-7 cells treated with 10 nM E₂, 10 μ M ICI, or increasing concentrations of Rosi (0.2–20 μ M) for 24 h; whole cell lysates were analyzed for immunoreactive ER α and actin (internal control) proteins by Western blot analysis as described in “Materials and methods”

responsive gene in breast cancer cells, pS2, was examined. To determine if Rosi could induce the expression of pS2, mRNA was isolated from MCF-7 cells treated for 24 h with E₂, Rosi, or ICI alone or in combination (Fig. 1C). Relative mRNA was assessed using a SYBR-green RT-PCR assay. While E₂ and Rosi both significantly increased pS2 expression, ICI alone did not alter pS2 expression. ICI did, however, completely inhibit E₂ and Rosi-induced pS2 expression.

Rosi does not increase ER α expression

One potential mechanism by which Rosi induces ER activation could be by simply increasing the expression of ER α . To test this, Western blots were performed on whole cell extracts from MCF-7 cells treated for 24 h with 10 pM E₂, 10 μ M ICI, or increasing concentrations of Rosi (0.2–20 μ M) (Fig. 1D). As reported previously [30], ER α expression was significantly decreased by treatment with ICI, but only slightly decreased by E₂. No significant difference in ER α expression was detected with increasing concentrations of Rosi.

ER α is necessary for Rosi-induced ERE-reporter activation in MDA-MB-231 cells

Rosi's ability to induce ERE reporter activation was then examined in the ER negative MDA-MB-231 breast cancer cells. These were transiently transfected with or without an ER α expression plasmid and treated with 10 nM E₂ or 1 μ M Rosi (Fig. 2A). In cells that were not transfected with an ER α expression plasmid, neither E₂ nor Rosi were capable of inducing ERE reporter activity; however, in MDA-MB-231 cells expressing ER α , both Rosi and E₂ significantly induced ERE reporter activity.

ER α inhibits Rosi-induced PPRE-reporter activity in MDA-MB-231 cells

Having demonstrated that Rosi is a ligand for ER α , we hypothesized that the expression of ER α would compete for Rosi binding therefore inhibiting PPAR's activation. To test this, MDA-MB-231 cells transfected with a PPRE (PPAR Response Element) reporter construct and with or without an ER α expression plasmid, were treated with 10 nM E₂ or 1 μ M Rosi (Fig. 2B). The expression of ER α results in a slight decrease in PPRE reporter activity in untreated cells yet treatment with E₂ did not increase PPRE reporter activity in cells regardless of the presence of ER α . Rosi, however, significantly induced PPRE reporter activity and this transactivation was inhibited by ER α expression. This is in direct agreement with what was seen

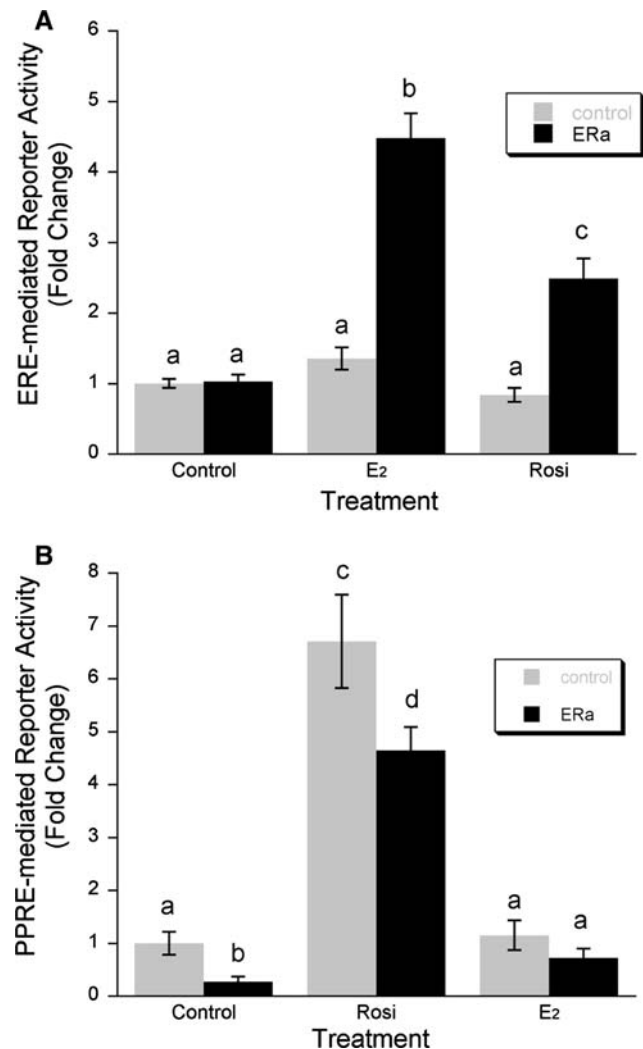


Fig. 2 ER α is necessary for Rosi-induced ERE reporter activation but inhibits Rosi-induced PPRE reporter activation in MDA-MB-231 cells. MDA-MB-231 cells were transiently transfected with (A) an ERE-TK-pGL3 reporter or a (B) PPRE-TK-pGL3 reporter plasmid in addition to either an ER α expression plasmid or a bluescript expression plasmid. Transfected cells were then treated with 10 nM E₂ or 1 μ M Rosi for 18 h. Luciferase activity was normalized to constitutively active β -gal and data is expressed as mean fold changes in luciferase to β -gal ratios compared to vehicle control. Error bars represent the standard error of the mean and bars that do not share a letter designation are significantly different

in our previous studies using another selective PPAR γ ligand, BRL 48,482 [29].

PPAR γ suppresses Rosi-induced ERE-reporter activation in 22Rv1 cells

To examine both ER and PPAR's individual role in Rosi-induced ERE activation, we used 22Rv1 cells, an androgen responsive prostate cancer cell line that are deficient in both ER α [31] and PPAR γ (data not shown).

22Rv1 cells were transfected with an ERE reporter construct as well as an ER α or PPAR γ expression plasmid alone or in combination. Transfected cells were treated for 18 h with E₂ or Rosi alone or together (Fig. 3). In the absence of ER α expression, no ERE-reporter activity was observed. The expression of ER α re-established E₂ responsiveness and yet E₂-induced ERE-reporter activity was not significantly altered by the co-expression of PPAR γ . Rosi also increased ERE-reporter activity in 22Rv1 cells expressing ER α alone but not PPAR γ alone. Furthermore, in the presence of Rosi, the expression of PPAR γ significantly inhibited ER α -mediated reporter activity. Combining Rosi and E₂ treatment did not significantly alter ERE-reporter activity as compared to E₂ treatment alone regardless of the expression of either receptor.

Rosi-induced proliferation is inhibited by ICI in MCF-7 cells

Since Rosi, like E₂, can induce ERE reporter activation and the expression of at least one target gene in MCF-7 cells, we examined the effect on cellular proliferation. MCF-7 cells were treated for 4 days with E₂ and Rosi alone or in combination (Fig. 4A). E₂ and Rosi treatments each significantly increased proliferation. Treatment with both ligands also significantly increased proliferation but there was no change as compared to either ligand alone. ICI was

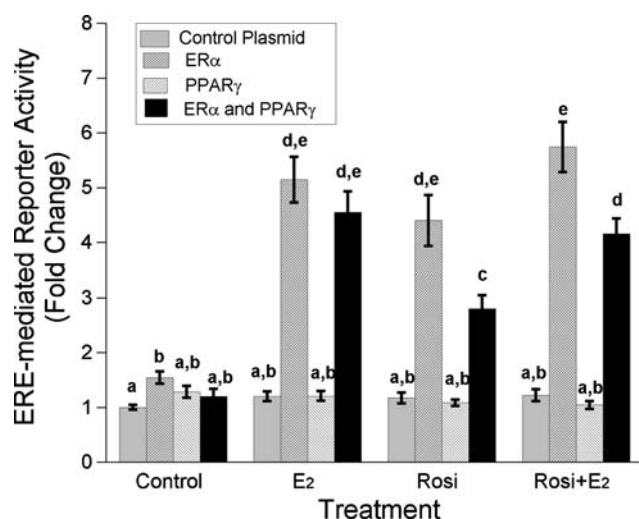


Fig. 3 E₂ and Rosi induce ERE-reporter activity in 22Rv1 cells only when expressing ER α . 22Rv1 cells were transiently transfected with an ERE-TK-pGL3 reporter plasmid in addition to an ER α or PPAR γ expression plasmid alone or in combination. Transfected cells were then treated with either 1 μ M Rosi, 10 nM E₂, or both for 18 h. Luciferase activity was normalized to constitutively active β -gal and data is expressed as mean fold changes in luciferase to β -gal ratios compared to vehicle control. Error bars represent the standard error of the mean and bars that do not share a letter designation are significantly different

used to determine whether Rosi-induced proliferation is mediated through ER dependent mechanisms. The ability of ICI to inhibit E₂-induced proliferation has been shown extensively and was verified by our lab (data not shown). Proliferation was assessed in MCF-7 cells treated for

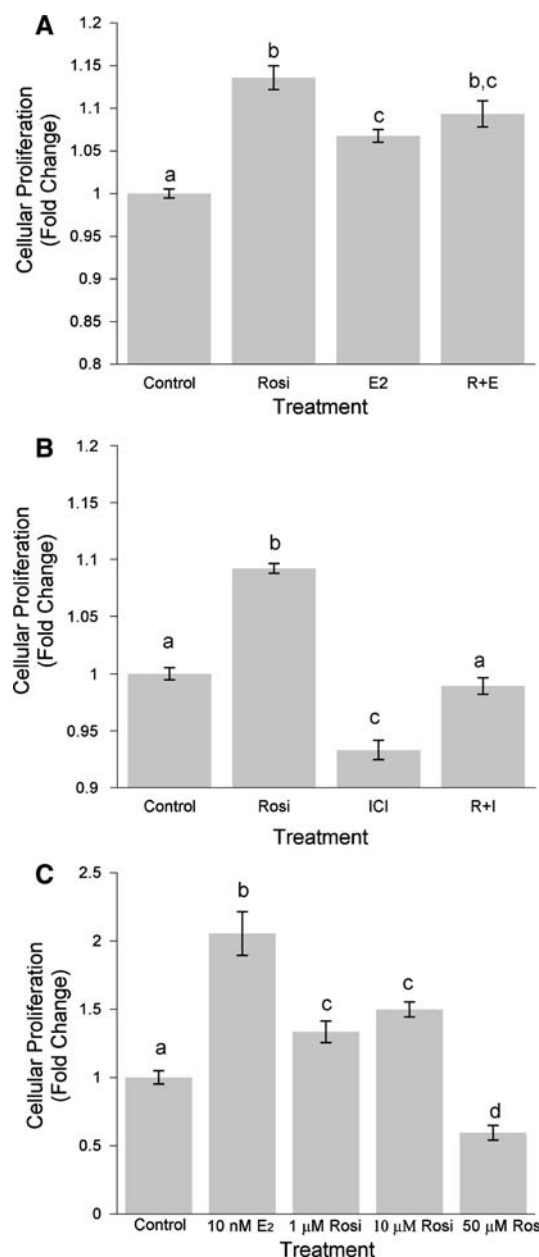


Fig. 4 Rosi-induced MCF-7 cell proliferation is concentration dependent and inhibited by ICI. MTS assays were used to measure proliferation in MCF-7 cells treated for 4 days with (A) 10 nM E₂ and 1 μ M Rosi both alone and together or (B) 1 μ M Rosi and 10 μ M ICI alone or together. (C) BrdU incorporation was measured in MCF-7 cells treated for 3 days with 10 nM E₂ or increasing concentrations of Rosi (1, 10 and 50 μ M). Data is expressed as mean fold changes in proliferation compared to vehicle control. Error bars represent the standard error of the mean and bars that do not share a letter designation are significantly different

4 days with Rosi and ICI alone or ICI together with Rosi (Fig. 4B). ICI treatment alone suppressed MCF-7 cell proliferation and completely inhibited Rosi-induced proliferation.

Rosi's effect on proliferation is concentration dependent

To validate the previous MTS assays and determine if there is a dose-dependent proliferative effect of Rosi, ELISA-based BrdU incorporation assays were performed. MCF-7 cells were treated for 3 days with increasing concentrations (1–50 μ M) of Rosi or 10 nM E_2 (Fig. 4C). The results of this assay were consistent with the MTS assays clearly demonstrating that E_2 and 1 μ M Rosi increased proliferation of MCF-7 cells. A dose-dependent effect of Rosi was also seen. At lower concentrations (≤ 10 μ M), Rosi increased proliferation, while at higher concentrations (50 μ M) Rosi inhibited proliferation.

Rosi-induced ER activation is dependent on MAP kinase activation

It has been previously shown that ER and PPAR are differentially regulated by the Extracellular Signal-Regulated Kinase–Mitogen Activated Protein Kinase pathway (ERK–MAPK). To determine the role of this pathway on Rosi-induced ER activation, MCF-7 cells were transiently transfected with an ERE reporter construct and treated with E_2 or Rosi alone or with increasing concentrations (1, 10, and 20 μ M) of U0126 (U) (a specific MEK inhibitor) (Fig. 5). Rosi- and E_2 -induced ER activation was completely abolished with the inhibition of the ERK–MAPK pathway by U0126.

Rosi and E_2 activate ER via similar pharmacological mechanisms

To begin to define the pharmacological mechanism of Rosi's effect on ER transactivation, dose–response assays were performed. For this purpose, MCF-7 cells were transiently transfected with an ERE reporter construct and treated with increasing concentrations of Rosi (0.2–25 μ M) (Fig. 6A) or increasing concentrations of E_2 (1 pM–10 nM) (Fig. 6B). The maximally effective concentration was seen at 25 μ M (Fig. 6A) and 10 nM (Fig. 6B), for Rosi and E_2 , respectively. The concentration of Rosi and E_2 that were sub-maximally effective in activating ER was 2 μ M (Fig. 6A) and 10 pM (Fig. 6B), respectively. To determine if E_2 and Rosi transactivate ER α through a similar mechanism, ERE reporter activation was examined in MCF-7 cells treated with the sub-maximally effective

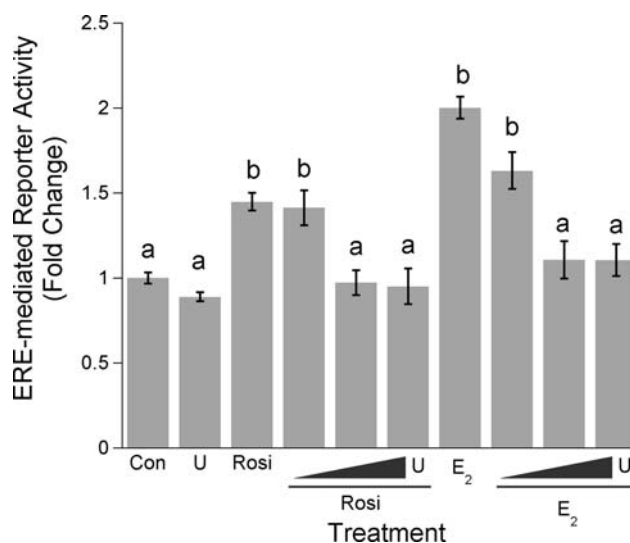


Fig. 5 Rosi-induced ER activation is blocked by inhibition of MEK. ERE-mediated reporter activity was measured in MCF-7 cells transiently transfected with an ERE-TK-pGL3 reporter plasmid and treated for 18 h with 10 μ M U0126 (U), 10 nM E_2 (E) or 1 μ M Rosi (R) alone or in combination with 1, 10, or 20 μ M U0126. Luciferase activity was normalized to constitutively active β -gal and data is expressed as mean fold changes in luciferase to β -gal ratios compared to vehicle control. Error bars represent the standard error of the mean and bars that do not share a letter designation are significantly different

concentration of E_2 (10 pM), in combination with increasing concentrations of Rosi (Fig. 6A). In the absence of Rosi, this concentration of E_2 resulted in a 2-fold induction of ERE reporter activity. When sub-maximally effective doses of E_2 and Rosi are combined, an additive effect on reporter activity was seen; however when 25 μ M Rosi is used, no additional increase in reporter activation was seen by adding a sub-maximally effective concentration of E_2 . There was a similar effect when a sub-maximally effective concentration of Rosi (2 μ M) was used in combination with increasing concentrations of E_2 (Fig. 6B). In the absence of E_2 , this concentration of Rosi resulted in a 2-fold induction of reporter activity. When sub-maximally effective doses of E_2 (10 pM) and Rosi (2 μ M) are combined, here too an additive effect was seen. When 10 nM E_2 is used, no additional increase in reporter activation was seen by adding sub-maximally effective concentrations of Rosi. These results suggest that E_2 and Rosi bind to and activate ER α via a similar pharmacological mechanism.

Sub-maximally effective concentrations of Rosi and E_2 are additive to MCF-7 cell proliferation

Since sub-maximally effective concentrations of Rosi and E_2 additively induced ERE reporter activity, we

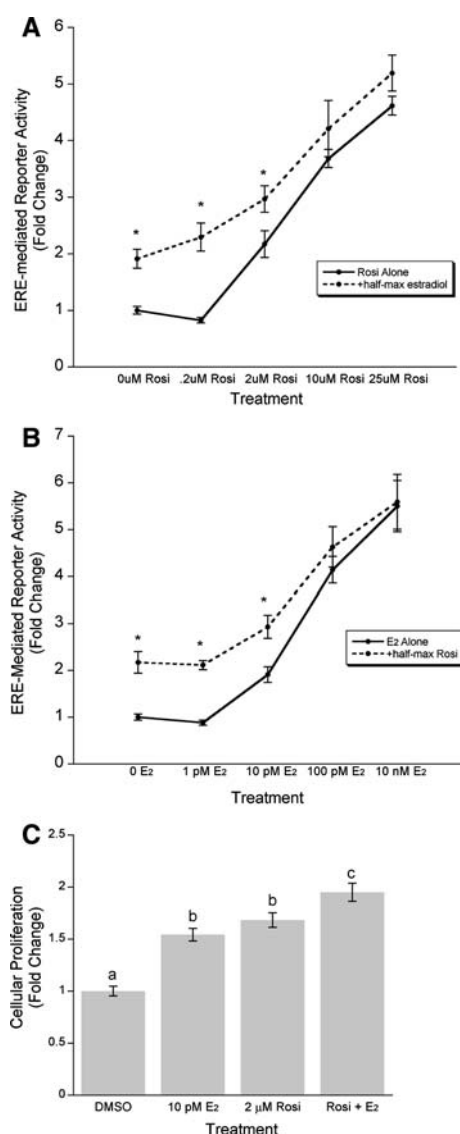


Fig. 6 Sub-maximally effective concentrations of Rosi and E₂ are additive in inducing ERE reporter activity and proliferation in MCF-7 cells. ERE-mediated reporter activity was measured in MCF-7 cells transiently transfected with an ERE-TK-pGL3 reporter plasmid and treated for 18 h with (A) increasing concentrations of E₂ alone (1 pM, 10 pM, 100 pM, or 10 nM) or in combination with a sub-maximally effective concentration of Rosi (2 μM) or with (B) increasing concentrations of Rosi alone (0.2, 2, 10, or 20 μM) or in combination with sub-maximally effective concentrations of E₂ (10 pM). Luciferase activity was normalized to constitutively active β-gal and data is expressed as mean fold changes in luciferase to β-gal ratios compared to vehicle control. Error bars represent the standard error of the mean and bars denoted by a* were determined to be significantly different. (C) BrdU incorporation was measured in MCF-7 cells treated with sub-maximally effective concentrations of E₂ (10 pM) and Rosi (2 μM Rosi) both alone and together for 3 days. Data is expressed as mean fold changes in proliferation compared to vehicle control. Error bars represent the standard error of the mean and bars that do not share a letter designation were determined to be significantly different

hypothesized that they would also be additive to cellular proliferation. To examine this, MCF-7 cells were treated

with sub-maximally effective concentrations of both ligands alone or together (10 pM E₂ and/or 2 μM Rosi) for 3 days. Proliferation was then assessed using the ELISA-based BrdU incorporation assay (Fig. 6C). Both 10 pM E₂ and 2 μM Rosi alone increased MCF-7 cells proliferation. When these concentrations of E₂ and Rosi were combined, there was an additive increase in MCF-7 cell proliferation.

Discussion

We have demonstrated for the first time that Rosi treatment results in a significant increase in ERE reporter activity in MCF-7 breast cancer cells. It is possible that Rosi binds ERα directly resulting in ERE activation. An alternate possibility is that Rosi induces a conformational change in PPARγ allowing it to directly mediate ERE reporter activation. However, further results showing that the ER antagonist ICI inhibited Rosi-induced ERE activation and that the expression of ERα was necessary for Rosi-induced ER activation in MDA-MB-231 cells strongly suggest that these effects are mediated directly through ERα.

These findings were further validated by the use of 22Rv1 cells, a prostate cancer cell line devoid of both ERα and PPARγ expression. Use of this cell line enabled us to control the expression of both ERα and PPARγ to determine their individual and combined effect on Rosi-induced ERE reporter activation. Rosi only induced ERE activation in 22Rv1 cells expressing ERα. When PPARγ is co-expressed with ERα, however, there was a significant decrease in ERE reporter activity following Rosi treatment. This could be potentially caused by the sequestration of Rosi by PPARγ which would leave less available to activate ERα, suggesting that the relative levels of each receptor impact Rosi's ultimate effect. However, a squelching mechanism is also possible such that the presence of PPARγ sequesters other factors important in the activation of ERα. None-the-less, this data supports our finding in MCF-7 cells, which shows that Rosi mediates ERα transactivation and strongly suggests that it is through an ERα dependent process. Furthermore, it is apparent that ERα and PPARγ compete for Rosi when both receptors are present in the cell. This is further illustrated by the fact that the expression of ERα in MDA-MB-231 cells inhibited Rosi-induced PPRE activation. Notably Rosi-induced ER activation is not due to Rosi's ability to simply increase ERα expression which was shown by no significant change with treatment by Western blot analysis.

To further support the finding that Rosi mediates ERα transactivation, we also examined the regulation of pS2, an endogenous ER target gene, following Rosi treatment. Although not as prominently as E₂, RT-PCR clearly demonstrated that Rosi significantly up-regulated the

expression of pS2 indicating that Rosi cannot only activate ERE reporter constructs but also the endogenous transcriptional regulatory mechanism of ER α . Furthermore, E₂ and Rosi-induced upregulation of pS2 was inhibited by ICI treatment, again suggesting that the mechanism of action is through ER.

Low concentrations of Rosi (1 μ M) also increased the proliferation of MCF-7 cells. The ability of Rosi to induce proliferation was also shown in the mouse uterus where it enhanced the morphogenic and proliferative actions of estradiol [32]. Like ERE-mediated reporter activity and the regulation of pS2, ICI also inhibited Rosi-induced MCF-7 cell proliferation again suggesting that this effect is mediated by the ER signal transduction pathway. Furthermore, while low concentrations (1–10 μ M) of Rosi increased MCF-7 cell proliferation, high concentration (50 μ M) inhibited proliferation. This is in direct agreement with reports that in breast cancer cells, high concentrations (>10 μ M) of PPAR γ ligands decrease proliferation while low concentrations (<10 μ M) increase proliferation [33]. Additional data, however, suggests that Rosi (even at low concentrations) inhibits MCF-7 cell proliferation [26, 34]. The differences in these results could be due to experimental variability and will require further analysis by our lab and others.

It has been shown that transcriptional activity of ER and PPAR are differentially regulated by the ERK–MAPK pathway. Phosphorylation of ER by this pathway leads to its activation [35], while phosphorylation of PPAR has been reported to inhibit its activity [36]. Furthermore, the ERK pathway has been shown to play an important role in the anti-proliferative actions of TZDs [37]. Thus we sought to determine whether this pathway was important in Rosi-induced activation of ER α . Our studies demonstrated that increasing concentrations of U0126, a specific MEK inhibitor, effectively inhibited Rosi- and E₂-induced ERE reporter activation suggesting that the phosphorylation of ER by the ERK pathway is instrumental to the transactivation of ER α by both ligands.

To begin to examine the molecular mechanism of Rosi-induced ER α transactivation, dose–response experiments for both Rosi and E₂ were performed in MCF-7 cells transfected with an ERE reporter. The concentration of each ligand needed to yield close to half-maximal responsiveness was then used in co-treatment studies. We reasoned that if both Rosi and E₂ were sharing a common ligand binding pocket, treating cells with sub-maximal concentrations of both ligands should additively transactivate ER α . Furthermore, when maximally effective concentrations of both ligands are combined, no further ER α transactivation is observed. Although these data, do not demonstrate direct binding, they do lend strong support that E₂ and Rosi transactivate ER α through a similar

mechanism. The ability of MEK inhibition to block transactivation by both ligands further supports this common mechanism. Finally we found that combining sub-maximally effective concentrations of Rosi and E₂ was additive not only to induction of ERE reporter activity but also to MCF-7 cell proliferation. This is in contrast to the effect of combining maximally effective concentrations of both ligands which was not additive to either ERE reporter activation or proliferation.

In conclusion, we have demonstrated through the use of reporter assays, endogenous gene expression, and the ability to control ER α expression, that Rosi directly transactivates ER α . Similar to E₂, this transactivation induces proliferation and requires an endogenous ER α and ERK–MAPK pathway. We also show that the concentration of Rosi is critical in determining its response in MCF-7 cells since lower concentrations of Rosi induced proliferation while higher concentrations inhibited proliferation. Furthermore, Rosi seems to be using the same pharmacological mechanism as E₂ to induce ER activation. Notably, Rosi induces ER activation even in cells expressing PPAR though at a reduced level (compared to cells without PPAR) suggesting the competition of ER and PPAR for available ligand. Thus it is evident that Rosi responsiveness depends not only on the availability of PPAR γ but also on the expression of ER α which could explain its varying effects in different breast cancer studies. Lastly, and perhaps more importantly we found that combining sub-maximally effective concentrations of E₂ and Rosi is additive to both ER activation and cellular proliferation. These findings have potential implications for the use of Rosiglitazone clinically.

Most breast cancer cases (~75%) occur in post-menopausal women, and most are estrogen-dependent [38]. During the aging process, circulating E₂ levels dramatically decrease upon the onset of menopause due to loss of ovarian function [39, 40]. Under these conditions, Rosi treatment may induce ER activation—since at low concentrations of estrogen, Rosi may be capable of inducing further ER activation. This effect may be clinically irrelevant in pre-menopausal women (who have higher circulating concentrations of estrogen) since there was no additive effect of Rosi on ER activation at high concentrations of E₂. Thus the results of this study raise clinical concerns when considering the use of Rosi as anti-cancer therapy in post-menopausal women with estrogen-dependent breast cancer. Although Rosi may act either dependently or independently through PPAR γ to inhibit the growth of breast cancer cells at higher concentrations, at low concentrations it may contribute to the growth of ER+ breast cancer by activating ER. The use of Rosi (Avandia) in diabetic patients should also be closely monitored for estrogenic effects that could increase the risk for ER+

breast cancer. Recently a short pilot study showed that low concentrations of Rosiglitazone did not significantly alter proliferation in breast cancer patients [41]. However, both ER positive and negative cancers as well as pre- and post-menopausal women were included in this study which may obscure the proliferative effects of Rosi. A longer study may be necessary to accurately measure whether these drugs are truly proliferative in breast cancer.

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MAZ drives tumor-specific expression of PPAR gamma 1 in breast cancer cells

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Abstract The peroxisome proliferator-activated receptor gamma 1 (PPAR γ 1) is a nuclear receptor that plays a pivotal role in breast cancer and is highly over-expressed relative to normal epithelia. We have previously reported that the expression of PPAR γ 1 is mediated by at least six distinct promoters and expression in breast cancer is driven by a tumor-specific promoter (pA1). Deletional analysis of this promoter fragment revealed that the GC-rich, 263 bp sequence proximal to the start of exon A1, is sufficient to drive expression in breast cancer cells but not in normal, human mammary epithelial cells (HMEC). By combining the disparate technologies of microarray and computer-based transcription factor binding site analyses on this promoter sequence the myc-associated zinc finger protein (MAZ) was identified as a candidate transcription factor mediating tumor-specific expression. Western blot analysis and chromatin immunoprecipitation assays verify that MAZ is overexpressed in MCF-7 cells and is capable of binding to the 263 bp promoter fragment, respectively. Furthermore, the over-expression of MAZ in HMEC is sufficient to drive the expression of PPAR γ 1 and does so by recruiting the tumor-specific promoter. This results in an

increase in the amount of PPAR γ 1 capable of binding to its DNA response element. These findings help to define the molecular mechanism driving the high expression of PPAR γ 1 in breast cancer and raise new questions regarding the role of MAZ in cancer progression.

Keywords PPAR γ 1 · peroxisome proliferator activated receptor gamma · Breast cancer · HMEC · MAZ · Myc-associated Zinc finger protein · MCF-1

Introduction

Breast cancer is the second leading cause of malignancy related deaths among American women [1]. The majority of these breast tumors arise from the ductile epithelia and infiltrating ductal carcinomas account for over 70% of all cases of breast cancer [2]. Current chemotherapies entail significant toxicity and benefit only a limited number of patients. Due to limitations in current therapeutic options, and the high degree of prevalence, a great deal of research has focused on the search for new and more selective molecular targets in the treatment of breast cancer. A number of nuclear hormone receptors have been identified as potential candidates for use as drug targets including peroxisome proliferator-activated receptor gamma 1 (PPAR γ 1). Our identification of PPAR γ 1 in breast cancer [3] and the subsequent elucidation of its role in mediating differentiation [4, 5] has lead to the intensive examination of its role in mediating similar programs in breast adenocarcinomas [6–8].

Peroxisome proliferator-activated receptor gamma 1 is a member of the nuclear hormone receptor superfamily and plays a critical role in adipogenesis [4, 5, 9], insulin mediated glucose homeostasis [10], and development [11].

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Ligands for PPAR γ 1 include 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), dietary fatty acids and the thiazolidinedione class (TZDs) of hypoglycemic drugs [12–15]. At the protein level, two forms of PPAR γ (γ 1 and γ 2) are expressed from the same gene, γ 2 containing an additional 30 N' terminal amino acids not present on γ 1 [9, 16, 17]. PPAR γ 1 is expressed in normal, human mammary epithelial cells (HMEC) [7] and established breast cancer cell lines where it is functionally responsive to ligand-mediated transactivation [3, 18]; however, expression of PPAR γ 1 is higher in several different tumor types when compared to noncancerous tissue [19–23]. In the case of mammary tissue, normal epithelial cells also express much lower level of PPAR γ 1 compared to breast carcinoma cell lines [6, 7, 15, 18, 24, 25]. Furthermore, Mueller et al. demonstrated that PPAR γ 1 expression was higher in metastatic lesions in the lung compared to the primary breast tumor from the same patient indicating that increasing PPAR γ 1 expression correlates with the progression of breast tumors from formation through metastases [26].

Although PPAR γ 1 is thought to mediate differentiation in most tissues, its role in tumor progression or suppression is poorly understood. Indeed, in some tissues it has been shown that a reduction in the expression of PPAR γ 1 can increase the risk of carcinogenesis. In these studies, PPAR γ 1 heterozygous (+/–) knockout mice had a much greater rate of colon tumor formation following exposure to azoxymethane, an inducer of colorectal cancer [27]. These animals also develop more mammary tumors following exposure to 7,12-dimethylbenz(a)anthracene [28]. By contrast, constitutive over-expression of PPAR γ 1 in animal studies increases the risk of spontaneous breast cancer in mice already susceptible to the disease [29]. It has been suggested that this paradox might be resolved by careful dose–response studies, where both the level of PPAR γ 1 gene expression and transactivation are carefully controlled [30]. This would suggest that the level of expression is a critical factor in determining the physiological outcome of PPAR γ 1 transactivation in a cell-specific context. Since benign breast ducts express lower levels of PPAR γ 1 protein compared to infiltrating carcinoma cells [6] and the expression of PPAR γ 1 is positively correlated with breast cancer metastasis [26], it is critical that we understand the mechanisms that drive these changes in PPAR γ 1 expression that accompany tumor progression. Therefore, we sought to identify the molecular mechanisms that mediate the increase in PPAR γ 1 as cells progress from normal mammary epithelial to breast carcinoma cells.

We have previously reported that the expression of PPAR γ 1 is under complex regulatory mechanisms [17]. Although we identified distinct promoters associated with

untranslated first exons that mediate the changes in expression seen during cellular transformation, we were unable to identify the factor(s) mediating this event. Here we report that through the novel combination of standard promoter analysis, computer-based *cis*-element prediction and microarray analysis we have identified the myc-associated zinc finger protein (MAZ) as the tumor-specific regulator of PPAR γ 1 in human breast cancer cell lines.

Methods

Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM lacking phenol red (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Hyclone) at 37°C in a 5% CO₂ atmosphere. Cells were grown in T-75 flasks prior to transfer to 12-well plates (Corning) used in transfection assays. Normal, HMEC were obtained from Cambrex/Clonetics. HMEC cells were cultured in the Cambrex/Clonetics MEGM[®] media containing SingleQuot[®], tissue culture media supplements optimized for the growth of these cells. HMEC were grown in phenol red free medium at 37°C in a 5% CO₂ atmosphere. HEMC were grown in T-75 flasks prior to transfer to 12-well plates used in transfection and assays.

Plasmid construction

The pRL-TK vector (Promega, Madison, WI, USA) was used as an internal control in all transient transfection assays. The PPAR γ 1 3 kb promoter section and the series of 5'-end deletion fragments from the 5'-flanking region of exon A1 were cloned into the multiple cloning site of the pGL3-basic plasmid (Promega). The MAZ cDNA from human B-cells was obtained from ATCC. Using a combination of Xho I and EcoR I restriction enzymes, the MAZ gene was released from the pOTB7 plasmid and cloned into the pCI/IRES-hgfp mammalian expression vector where the CMV promoter drives MAZ gene expression. PCR was used to introduce the mutated MAZ response element, which substituted one of the guanine triplet sequences to a non-functional TCC sequence. As shown, the native MAZ response element found in the 263 bp sequence of the PPAR γ pA1 promoter was –217 GGGAGGGGA –209, and was changed to the mutant form –217 GGGATCCA –209 while maintaining the context of the 263 bp pA1 promoter in the pGL3-basic luciferase reporter plasmid.

Transient transfection analysis

Cells were transiently transfected with either 2 µg of a 3XPPRE-TK-pGL3 reporter vector [17], 2 µg of MAZ expression vector, or 0.5 µg pRL-TK (Promega) per plate using ESCORT (Sigma, St Louis, MO, USA). After 18–24 h, cells were lysed in 100 µl passive lysis buffer (Promega) and treated according to manufacturer's instructions for use with the Daul-Luciferase Assay Kit. Luminometry was performed on a Berthold Technologies Lumat (LB9507, Bad Wildbad, Germany) and data calculated as raw luciferase units divided by raw *Renilla* units (RLU's). Data are presented as the mean fold induction. These values were obtained by dividing the RLU data from each treatment well by the mean of the control values $\pm$ the standard error of the mean (sem) as shown.

Sequence analysis for putative *cis*-elements

Transcription Element Search Software (TESS), a string-based search tool similar to local alignment software [31] was used to search the 263 bp promoter sequence of PPAR γ pA1. Using sequence position weight matrices from TRANSFAC, IMD, and the CBIL-GibbsMat databases, possible transcription factor binding sites within the 263 bp region of the PPAR γ promoter A1 were identified.

Formaldehyde cross-linking and chromatin immunoprecipitation

Cell growth and chromatin preparation were performed according to the manufacturer's instructions included with the ChIP-ITTM kit (Active Motif North America, Carlsbad, CA, USA). Chromatin from MCF-7 cells was formaldehyde cross-linked in a 1.6% solution for 10 min followed by enzymatic shearing for 11 min. Soluble chromatin from MCF-7 cells was immunoprecipitated with a MAZ polyclonal antibody (Santa Cruz Biotechnology, sc-28745, Santa Cruz, CA, USA). Specific primer pairs (Integrated DNA Technologies, Coralville, IA, USA) were designed to amplify the PPAR gamma promoter regions from –210 to –1, forward primer: 5' GCCGCTCCCTCCCAGTCGTCGCG 3'; reverse primer: 5' CTCGAGGCCCGACCCAAGC 3'. PCR fragments were analyzed by 5% polyacrylamide gel (37.5:1, acrylamide–bisacrylamide) in TBE buffer. The 210 bp fragment was purified and subjected to DNA sequence analysis. Elim Biopharmaceuticals, Inc. (Hayward, CA, USA) performed all DNA sequencing.

Nuclear protein extraction

Nuclear proteins were prepared with the nuclear extract kit (Active Motif North America). In brief, cells were scraped into PBS containing phosphatase and protease inhibitors, centrifuged, resuspended in a 1 $\times$ hypotonic buffer and then kept on ice for 15 min. After the addition of a detergent, the lysates were centrifuged at 14,000 $\times$ g for 30 s at 4°C. The pellets were resuspended in complete lysis buffer and vortexed for 10 s at the highest setting. After a 30-min incubation on ice and centrifugation at 14,000 $\times$ g for 10 min at 4°C, the supernatants were collected, and the protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

PPAR gamma transactivation analysis

Peroxisome proliferator-activated receptor gamma 1 activation was determined with the TransAM ELISA kit (Active Motif North America). Nuclear extract was added to each well of a 96-well plate into which an oligonucleotide with a PPAR consensus binding site had been immobilized. After 1 h of incubation with smooth agitation, wells were washed three times with washing buffer and then incubated with PPAR γ 1 antibody (1:250 dilution in 1 $\times$ antibody binding buffer) for 1 h at room temperature. The wells were washed three times and incubated for 1 h with diluted anti-rabbit HRP-conjugated antibody (1:1,000 dilution in 1 $\times$ antibody binding buffer). After four wash cycles, 100 µl of developing buffer was added to each well and incubated for 5–8 min. The reaction was stopped by the addition of 100 µl stop buffer. The final A₄₅₀ was read on a Kinetic microplate reader with a reference wavelength of 650 nm.

Western blot analysis

Western analysis was performed as described previously [17] using the nuclear fractions from MCF-7 and HMEC. The Anti-MAZ polyclonal antibody was purchased from Santa Cruz (1:200 dilution, sc-28745; Santa Cruz Biotechnology). To assess sample loading, α -Tubulin (1:1,000 dilution, sc-8035, Santa Cruz Biotechnology) was used as a loading control. An estimation of the relative quantity of MAZ was determined by densitometry using a Kodak Imaging System EL Logic 2200. The observed MAZ protein level was recorded as a ratio of MAZ to tubulin.

Affymetrix microarray analysis

Total RNA was isolated from cells following a 3-h treatment using the Qiagen RNeasy Mini Kit. The University of Kentucky Microarray Core Facility verified the total yield by both gel electrophoresis and Agilent Bioanalyzer. The core facility processed the total RNA samples to produce biotinylated, fragmented cRNA. The cRNA samples then were hybridized to the Affymetrix (Santa Clara, CA, USA) HG-U133A GeneChip and washed with a streptavidin phycoerythrin solution via an Affymetrix Fluidics Station 400. Finally, the resultant florescent intensities were collected and analyzed using an Affymetrix GeneArray Scanner coupled to a computer workstation running Affymetrix Microarray Suite (MAS 5.0). The MAS 5.0 data from each Gene Chip was saved and exported into Microsoft Excel spreadsheets (Redmond, WA, USA). MAS 5.0 signal intensity data collected were aggregated into a single Excel spreadsheet using the probe set IDs, signal intensity values, and signal detection flag for each sample and probe set description. Mean signal intensity values ($n = 3$ per cell type) were reported along with SE as an appropriate estimation of error. One factor analysis of variance (ANOVA, $\alpha = 0.05$) followed by t -test was used to determine significant differences in the observed treatment effect within a cell type.

Quantitative real-time PCR

A one-step quantitative real-time PCR technique was used to determine relative expression levels of PPAR γ 1 mRNA using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The RNeasy Mini Kit (Qiagen) was used to isolate the total RNA. The pre-optimized primers and probe with FAM reporter fluorescent dye for PPAR γ were purchased from Applied Biosystems, assay-on-demand number Hs00234592_m1. For the internal control, *18S*, a pre-optimized primer and probe also was used, assay-on-demand number Hs99999901_s1. A one-step reaction reagent mixture provided in the TaqMan One-step RT-PCR Master Mix kit (Applied Biosystems) was used for all of the amplifications. Cycle parameters for the one-step reverse transcription-PCR included a reverse transcription step at 48°C for 30 min, followed by 40 cycles of 95°C denaturation and 60°C annealing/extension. The house keeping gene *18S* was used for internal normalization.

Analysis methods as outlined in the ABI Prism 7700 Sequence Detection System User Bulletin 2 (October 2001) were performed using the relative C_t method. Briefly, this method uses the mathematical expression 2^{-C_t} to estimate the relative gene expression based on a calibrated sample,

$DC_t = C_{t,x} - C_{t,calibrator}$, and the gene target of interest normalized to the expression of an endogenous house-keeping gene like *18S*, $DDC_t = C_{t,PPAR} - C_{t,18S}$. The mean ($n = 4$ per cell line) values were reported along with the SE of the C_t because an appropriate estimation of error was calculated from the SDs of the C_t values for PPAR and *18S* through the formula $SE = s/n$ where the SD, $s = \sqrt{(C_{t,PPAR})^2 + (C_{t,18S})^2}$.

Results

Identifying transcription factors mediating tumor-specific expression of PPAR γ 1

We have previously reported that the regulation of PPAR γ 1 expression is under the control of at least six different promoters associated with distinct, first exons [17]. We have also demonstrated that the higher levels of PPAR γ 1 expression reported in breast cancer cells results from the recruitment of a distinct tumor-specific promoter termed pA1, which is not used in HMECs [17]. To define the factor driving expression from pA1, we initially used 5'-end deletion analysis of the 3,000 bp pA1 promoter fragment. These analyses allowed us to more narrowly define the promoter element(s) using luciferase reporters. In addition to the 3 kb pA1 promoter fragment, a 2-kb fragment from -1 to -2000, a 1-kb fragment from -1 to -1000, a 263-bp fragment from -1 to -263 and a 5' end 2 kb fragment from -1000 to -3000 were generated (Fig. 1a). These data indicate that the proximal 263 bp of the pA1 promoter fragment contain the element(s) necessary to drive expression of PPAR γ 1 in breast cancer cells.

The 263 bp pA1 promoter fragment is greater than 90% GC-rich and resisted efforts to more narrowly define the response element by standard deletional analysis (data not shown). To circumvent these limitations and define the element(s) within the 263 bp fragment driving expression, we integrated two distinct technological approaches to identify potential transcription factor binding sites. Using the Transcription Element Search System (TESS, (<http://www.cbil.upenn.edu/tess/>), developed at the University of Pennsylvania Computational Biology and Informatics Laboratory, we identified the potential transcription factor binding sites within this 263 bp tumor-specific promoter fragment [32]. This approach identified 29 different transcription factors that had the potential to bind at a total of 209 different DNA binding sites. We then used microarray analysis to determine which of the factors identified by TESS analysis were expressed at significantly higher levels in breast cancer cells relative to HMECs. These analyses revealed that of the transcription factors on

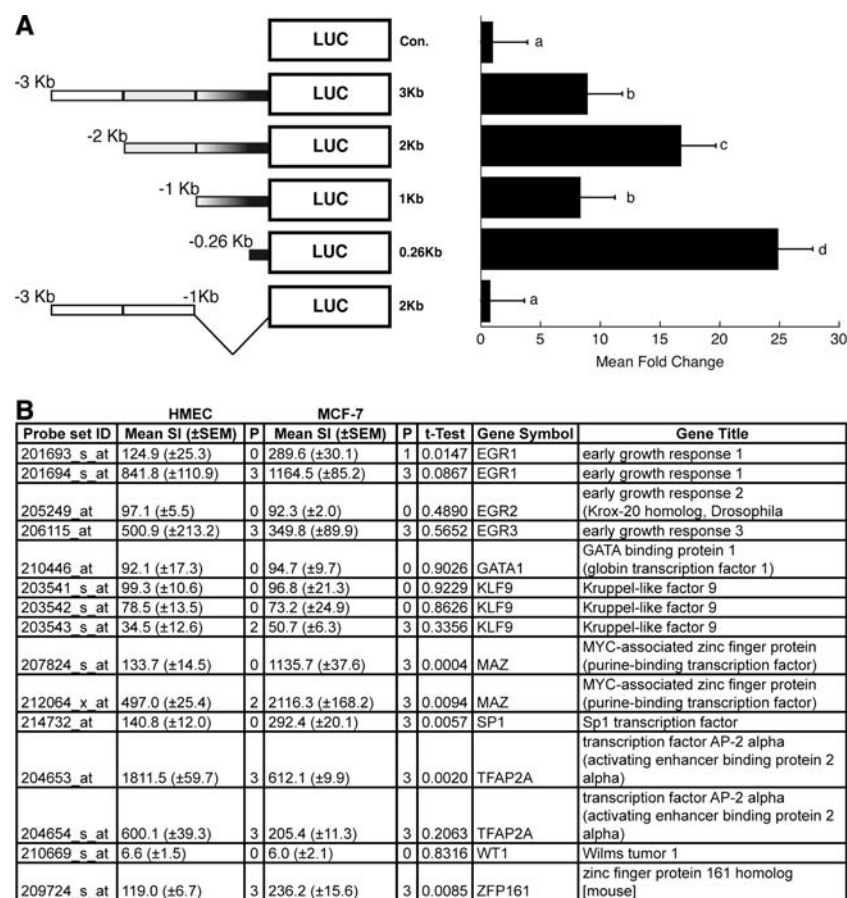


Fig. 1 Promoter analysis of the proximal 3 kb of exon A1 (pA1) from PPAR γ 1 in MCF-7 cells. **(A)** Promoter fragments were generated containing -2000 to +1 (2 kb), -1000 to +1 (1 kb), -263 to +1 (0.26 kb) and -3000 to -1000 (3 kb) controlling luciferase expression. The proximal 263 bp flanking the start site of transcription are sufficient to mediate expression. Constructs were transfected into MCF-7 cells and data were normalized to *Renilla* as a control for transfection efficiency. Error bars represent half of the critical value calculated from the Tukey's pair-wise comparison test and those that do not share a letter designation were determined to be significantly different ($p < 0.001$). The proximal 263 bp flanking the start site of

transcription are sufficient to mediate expression. The data shown represent three independent experiments composed of three biological replicates for each treatment for a total of nine observations per treatment. **(B)** The expression of transcription factors identified by TESS present on the HG-U133A chips was compared between HMEC and MCF-7 cells. Data was analyzed using a two-tailed heteroscedastic *t*-test. MAZ expression is significantly higher in MCF-7 cells compared to HMECs ($p < 0.01$). The data shown represent a single experiment composed of three Affymetrix GeneChips per cell line

the HG-U133A array, MAZ was significantly over expressed in estrogen-dependent breast cancer (MCF-7) cells compared to HMEC (Fig. 1b). This message was represented by two different probes sets, and both were significantly higher in the MCF-7 cells relative to HMEC. Densitometric analysis of the western blot confirms that is expressed at significantly higher levels in the nuclear extracts of MCF-7 cells (Fig. 2a, arrow marked bands).

To resolve whether the binding site identified by TESS analysis is recognized and bound by MAZ, Chromatin immunoprecipitation (ChIP) assay was preformed. Using the polyclonal antibody to MAZ and the gene-specific primers, the expected band size of 210 bp was amplified (Fig. 2b). This fragment was purified and sequenced confirming this to be the pA1 promoter. Electrophoretic

mobility shift assays (data not shown) confirmed the ChIP assay that MAZ binds to the MAZ response element (MAZ-RE) identified by TESS. Together these demonstrate that MAZ is overexpressed in tumors and physically binds to the endogeneous response element within the -263 bp pA1 promoter in MCF-7.

The expression of MAZ drives PPAR γ 1 expression in MCF-7 cells

Initially, we examined the ability of MAZ to mediate reporter activity of the 263 bp promoter shown to control tumor-specific expression of PPAR γ 1. In MCF-7 cells, this promoter is sufficient to confer transcriptional regulation

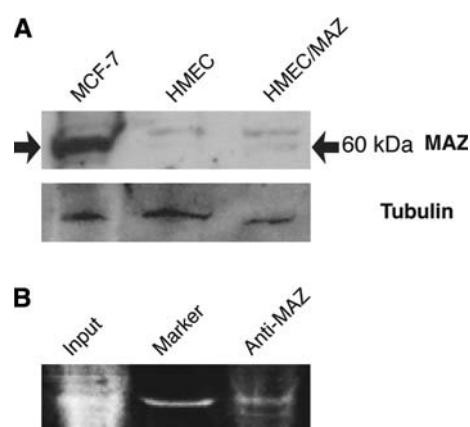


Fig. 2 The expression of MAZ in HMEC and MCF-7 cells. **(A)** Western blot analysis confirms that MAZ expression is much higher in nuclear extraction from MCF-7 compared to control. A small but significant increase in MAZ expression (as indicated by the bands marked with an arrow) is seen in HMEC following transfection with the MAZ expression plasmid. Alpha-tubulin was used as nuclear protein loading control. The data shown is representative of a single experiment with only one observation. **(B)** Formaldehyde cross-linking and chromatin immunoprecipitation assays were performed to confirm MAZ binding to the tumor-specific binding in MCF-7. The 210 bp amplified by tumor-specific promoter primers is clearly seen on 5% polyacrylamide gel from anti-MAZ sample and this 210 bp fragment was confirmed by DNA sequencing. The data shown is representative of a single experiment with only one observation

compared to the basic (pGL3-basic) luciferase reporter (Fig. 3a). Furthermore, when a MAZ expression plasmid is introduced into these cells reporter activity is significantly higher than MCF-7 cells not overexpressing MAZ (Fig. 3a). To confirm the need of the MAZ response element, this element was scrambled by site directed mutagenesis where the second G triplet sequence was replaced with TCC. When this mutated MAZ-RE was placed within the context of the 263 bp promoter, reporter activity was significantly reduced (Fig. 3b).

Expression of MAZ in HMEC cells drive PPAR γ expression from the tumor-specific promoter

Transient transfection of HMEC with the MAZ expression vector dramatically increased mRNA levels of PPAR γ 1 as seen by real-time PCR (Fig. 4a). This also resulted in a small but significant increase in protein levels (Fig. 2a, band marked by arrow). Since breast cancer cells drive expression of PPAR γ 1 from a tumor-specific promoter not used by HMEC, probes were designed to determine whether MAZ drives PPAR γ 1 expression from the tumor-specific promoter in HMECs as well. Exon-specific probes to the first exon (A1) present on PPAR γ mRNA from MCF-7 cells and the first exon present on PPAR γ 1 from HMEC (A3) [17] were used to amplify the mRNA from HMEC

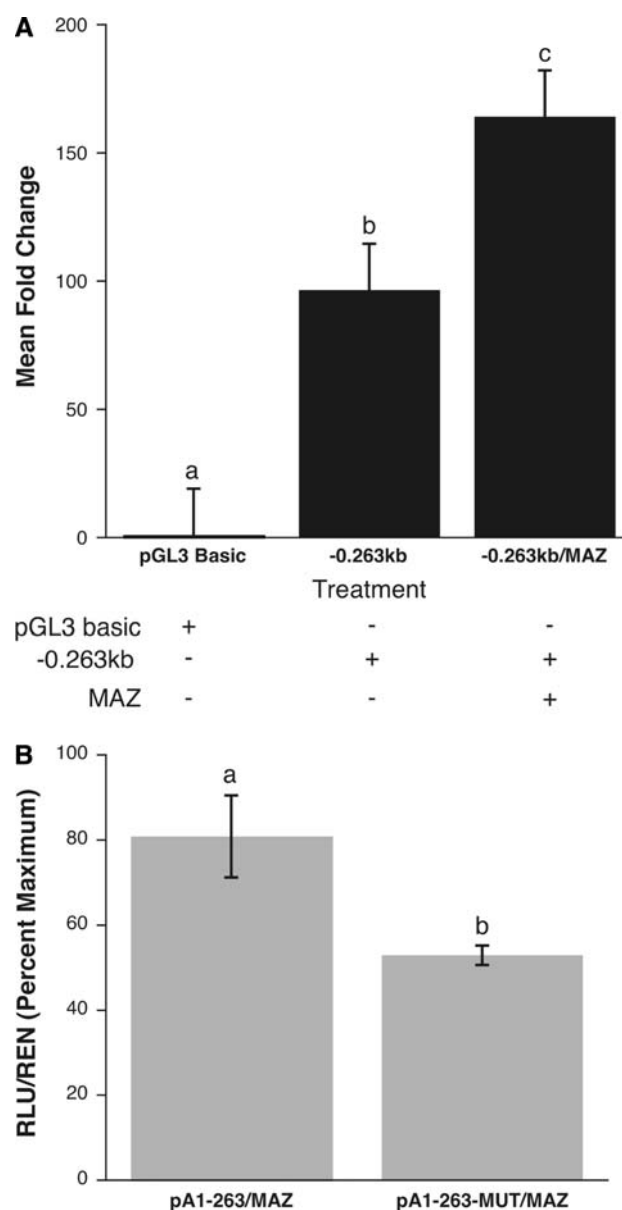


Fig. 3 Overexpression of MAZ in MCF-7 increases the expression of PPAR γ 1 from the MAZ response element. **(A)** MCF-7 cells were co-transfected with either pGL3 basic or pA1-263 alone or with the MAZ expression plasmid. Overexpression of MAZ increased the expression of the reporter from the -263 bp promoter. **(B)** Mutations (MUT) in the MAZ response element introduced into the -263 bp promoter significantly suppressed reporter activity. The reporter activity was measured by luciferase assay with *Renilla* used as a transfection efficiency control. Error bars represent half of the critical value calculated from the Tukey's pairwise comparison test and those that do not share a letter designation were determined to be significantly different. Data shown in both panel (A and B) each represent three independent experiments composed of three biological replicates for each treatment for a total of nine observations per treatment

expressing MAZ. These data demonstrate that MAZ drives expression of PPAR γ 1 in HMEC (Fig. 4a, insert) from pA1, the tumor-specific promoter [17]. Finally, to determine whether the MAZ-driven increase in PPAR γ 1

increases the amount of protein capable of binding DNA, an ELISA-based assay to quantitate the binding of PPAR γ to its response element was used. These data demonstrate that MAZ expression increases not only the level of PPAR γ 1 protein in HMEC but also the amount of PPAR γ 1 capable of binding to DNA.

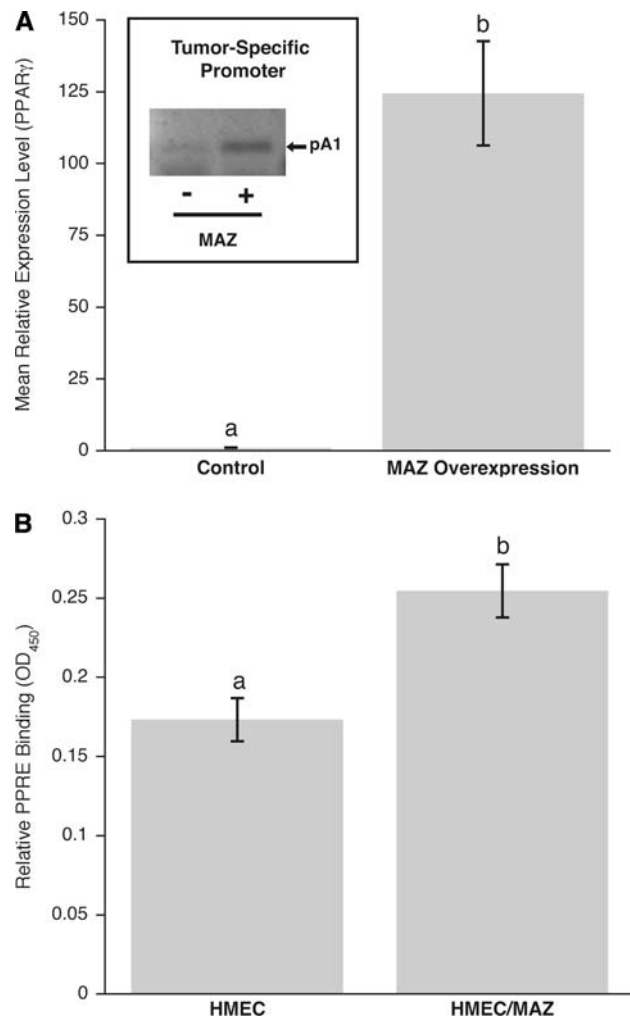


Fig. 4 MAZ recruits the tumor-specific promoter to drive the expression of PPAR γ 1 in HMEC. **(A)** HMEC were transfected with a control or a MAZ expression plasmid and PPAR γ expression measured by real-time PCR. Both real-time PCR and Western blot analysis (Fig. 2a) confirmed the expression of MAZ. PPAR γ 1 mRNA is dramatically increased in the presence of MAZ expression. PCR, using pA1 specific primers, verify that only in the presence of MAZ is the exon associated with the tumor-specific promoter used (*insert*). For real-time PCR, the data shown represent two independent experiments with three technical replicates for each treatment. **(B)** The ability of MAZ expression to increase the amount of PPAR γ able to bind to a PPRE was measured by ELISA using DNA as the immobilized target. Following the expression of MAZ the amount of PPAR γ bound to its response element is significantly increased. For transactivation analysis, the data shown represent three independent experiments each with three biological replicates for a total of nine observations per treatment

Discussion

A rapidly growing body of work has demonstrated that the transactivation of PPAR γ 1 by various exogenous ligands mediates a wide range of responses including growth arrest, differentiation and apoptosis making this nuclear receptor a possible target for cancer therapy [7, 8]. We have shown that PPAR γ 1 is highly over expressed in many tumors including breast, colon and lung [18]. In order to examine the transcriptional regulation of PPAR γ 1, we have determined its genomic structure and shown that the rise in expression from normal, human mammary epithelia to breast cancer is due to the recruitment of a distal, tumor-specific promoter element, termed pA1 [17]. The studies outlined in this report were designed to identify the mechanism driving this increase in PPAR γ 1 expression. Through standard 5'-end deletion analysis, we sought to determine the transcription factor that mediates the recruitment of the pA1 promoter. Although we were able to narrow the response element to a 263-bp fragment immediately flanking the start site of transcription, the GC-rich nature of this region made further promoter analysis by standard methods intractable. Therefore, we chose to take a novel approach that combined two disparate technologies to identify the factor mediating the tumor-specific expression of PPAR γ 1 in breast cancer. In addition to having identified a novel transcription factor whose expression could have an important role in cancer biology, this approach could find broader use in locating response elements and identifying the transcription factors that drive the expression of genes of interest.

By using the transcription element search system developed at the University of Pennsylvania, we were able to locate potential transcription factor binding sites within the proximal 263 bp PPAR γ 1 promoter fragment. Since PPAR γ 1 is highly expressed in MCF-7 cells relative to HMEC, we sought to determine which of the transcription factors identified by TESS were also over expressed in MCF-7 cells. For this, we employed data from a microarray analysis currently underway in the lab. We examined the expression levels of all the transcription factors identified by TESS that were present on the HG-U133A chip for factors overexpressed in MCF-7 cells compared to HMEC. Microarray analysis indicated that MAZ is expressed at significantly higher levels in MCF-7 cells. Indeed, MAZ is represented by two different probe sets on the HG-U133A chip and both were significantly higher in MCF-7 cells. Western blot analysis confirms that this is true at the protein level as well. Furthermore, ChIP assay proved that *in vivo* MAZ binds to the MAZ response elements in the 263 bp pA1 promoter fragment. The forced overexpression of MAZ in HMEC also drives an increase in the expression of PPAR γ 1. Furthermore, not only does MAZ drive

expression of PPAR γ 1 in HMEC, it does so from the tumor-specific promoter. Finally, mutating the MAZ-RE in the context of the tumor-specific promoter inhibits reporter activity and provides further support of its role in mediating the expression of PPAR γ 1 in breast cancer. These data have led us to hypothesize that MAZ plays a critical role in tumorigenesis and this is currently being tested in the lab. The prevalence of MAZ expression in breast cancer is unknown and is also currently under investigation in the lab.

Through development, differentiation and tumorigenesis, genes can be silenced and activated by several mechanisms. Clearly acetylation and deactivation of histones in the region of targeted genes has been shown to regulate expression, as has the methylation of CpG islands. Changes in the expression of transcription factors during these events can also play a critical role. These data demonstrate that the upregulation of MAZ is responsible, at least in part, for driving the increase in PPAR γ 1 expression and may also play a role in tumor progression. Although the tumor-specific promoter is very GC-rich, and therefore a potential target for methylation, this does not appear to be preventing use of this element. This is evident by the fact that when normal epithelial cells are forced to express MAZ, not only does this increase the expression of PPAR γ 1, but it does so using the tumor specific, GC-rich, promoter to drive expression. Although MAZ clearly drives an increase in the expression of PPAR γ 1 during tumor formation, it is not known what other genes are regulated by MAZ and what is the underlying mechanism that regulates MAZ expression. Therefore, it will be critical to understand the range of genes under the direct and indirect control of MAZ and define the consequences of MAZ expression to the mammary epithelia function. These questions are critical to our understanding of the consequences of regulation of both PPAR γ 1 and MAZ and are currently under investigation in the laboratory. These studies also highlight the usefulness of integrating the disparate technologies of computer based genomic analysis with expression patterns gleaned from microarray analysis to identify transcription factors involved in gene regulation. This is especially true of complex promoters such as that described here. This approach might also be useful in identifying the combination of factors that coordinate the expression of target genes including those that directly bind DNA as well as co-activators and co-repressors that coordinate the actions of the transcriptosome.

Experiments performed in vitro clearly demonstrate the potential of targeting PPAR γ 1 in the treatment of breast cancer [26, 28]. In addition, animal studies support the role for using PPAR γ 1 ligands for both the treatment and prevention of breast cancer [28], and clinical data is now emerging supporting the in vitro and animal studies that

demonstrate a protective role of PPAR γ 1 ligands in the treatment and prevention of breast cancer in women (<http://www.proactive-results.com>). In these studies, a significant reduction in the occurrence of breast cancer was seen in type 2 diabetic patients taking the thiazolidinedione Pioglitazone. It is critical, however, that we understand what role the level of PPAR γ 1 expression plays in mediating the responses of these ligands on growth suppression and apoptosis. The work outlined in these studies provide a much needed basis for assessing these changes and determining whether the expression of other factors will alter a patients response to drugs targeting PPAR γ 1.

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