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Down-regulation of PPAR γ 1 suppresses cell growth and induces apoptosis in MCF-7 breast cancer cells

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Abstract

Background: Peroxisome proliferator-activated receptor gamma (PPAR γ) is a member of the nuclear hormone receptor superfamily and is highly expressed in many human tumors including breast cancer. PPAR γ has been identified as a potential target for breast cancer therapy based on the fact that its activation by synthetic ligands affects the differentiation, proliferation, and apoptosis of cancer cells. However, the controversial nature of current studies and disappointing results from clinical trials raise questions about the contribution of PPAR γ signaling in breast cancer development in the absence of stimulation by exogenous ligands. Recent reports from both *in vitro* and *in vivo* studies are inconsistent and suggest that endogenous activation of PPAR γ plays a much more complex role in initiation and progression of cancer than previously thought.

Results: We have previously demonstrated that an increase in expression of PPAR γ 1 in MCF-7 breast cancer cells is driven by a tumor-specific promoter. Myc-associated zinc finger protein (MAZ) was identified as a transcriptional mediator of PPAR γ 1 expression in these cells. In this study, using RNA interference (RNAi) to inhibit PPAR γ 1 expression directly or via down-regulation of MAZ, we report for the first time that a decrease in PPAR γ 1 expression results in reduced cellular proliferation in MCF-7 breast cancer cells. Furthermore, we demonstrate that these changes in proliferation are associated with a significant decrease in cell transition from G₁ to the S phase. Using a dominant-negative mutant of PPAR γ 1, Δ 462, we confirmed that PPAR γ 1 acts as a pro-survival factor and showed that this phenomenon is not limited to MCF-7 cells. Finally, we demonstrate that down-regulation of PPAR γ 1 expression leads to an induction of apoptosis in MCF-7 cells, confirmed by analyzing Bcl-2 expression and PARP-1 cleavage.

Conclusion: Thus, these findings suggest that an increase in PPAR γ 1 signaling observed in breast cancer contributes to an imbalance between proliferation and apoptosis, and may be an important hallmark of breast tumorigenesis. The results presented here also warrant further investigation regarding the use of PPAR γ ligands in patients who are predisposed or already diagnosed with breast cancer.

Background

Breast cancer is the most common malignancy and the second leading cause of cancer related death among American women [1]. Despite of the fact that recent research efforts have significantly improved the outcome of breast cancer, the complexity and heterogeneity of this disease still urges the necessity to explore new and more specific drug targets. Peroxisome proliferator-activated receptor gamma (PPAR γ), a member of the nuclear-hormone receptor family, has shown potential as a therapeutic target for prevention and treatment of breast cancer. PPAR γ is a ligand-activated transcription factor. There are two isoforms of PPAR γ protein, PPAR γ 1 and PPAR γ 2, the latter of which has the addition of 30 N'-terminal amino acids as a result of the usage of a different promoter and alternative splicing [2]. PPAR γ plays an important role in adipocyte differentiation, insulin sensitivity, energy metabolism, immune response, and the development of the nervous system [3-5]. It is predominantly expressed in adipose tissues; although, it is also detected in various tissues such as cardiac and skeletal muscle, intestine, vascular smooth muscle, lung, breast, colon, and prostate [6,7]. Some polyunsaturated fatty acids [8-10] and arachidonic acid metabolites [11] are considered to be the natural ligands of PPAR γ . Synthetic ligands of PPAR γ include the thiazolidinedione class of anti-diabetic drugs (TZDs) such as rosiglitazone, pioglitazone, troglitazone [12,13], some non-steroidal anti-inflammatory drugs (NSAID) [14], and non-thiazolidinedione tyrosine [15]. In addition, a ligand-independent mechanism of PPAR γ activation has also been observed due to altered phosphorylation status of the receptor [16].

Recently, PPAR γ has emerged as a promising target for cancer therapy based on the fact that its activation by synthetic ligands such as TZDs have been shown to induce cell cycle arrest, apoptosis and differentiation in many human malignancies [17,18]. Several studies have demonstrated that PPAR γ activation by agonists can promote growth inhibition and apoptosis in both primary and metastatic breast malignancies [19-22]. In addition to the anti-proliferative and pro-apoptotic effects, PPAR γ ligands have also been reported to inhibit invasion and metastasis of human breast cancer cells [23,24]. However, these results were questioned by several studies that demonstrated the ability of PPAR γ ligands to elicit anti-tumor effects via PPAR γ -independent pathways and in the absence of PPAR γ receptors [25,26]. Moreover, there is a debate that the concentrations of PPAR γ ligands used in many studies are above the saturation level of the receptor. In fact, Rosiglitazone, a widely studied PPAR γ agonist, has shown to induce opposing effects when used in low versus high doses [27]. Furthermore, PPAR γ antagonists have also shown anticancer effects in a wide range of epi-

thelial cancer cell lines, usually with greater potency than agonists [28].

Existing data from *in vivo* studies is also controversial. Recent animal studies have demonstrated that PPAR γ agonists can prevent mammary carcinogenesis and reduce the development of tumors in nude mice [29]. In contrast, another study has demonstrated an increase in the number of tumors when PPAR γ ligand was administered [30]. To clarify the controversy arising from the use of pharmacological approaches, several animal studies utilized techniques that allowed evaluation of the consequences of PPAR γ transactivation in breast cancer independent of exogenous stimulation. Studies which employed a genetic approach to explore the intrinsic role of PPAR γ signaling have demonstrated that an increase in PPAR γ signaling accelerates mammary gland tumor development and constitutive over-expression of PPAR γ increases incidence of breast cancer in mice already susceptible to the disease [31]. This group has also shown that mice heterozygous for a null PPAR γ mutation develop tumors with the same kinetics as those that carry two functional copies [31]. Furthermore, the ablation of PPAR γ expression in the mouse mammary gland using a Cre-Lox recombination system has demonstrated that no tumors developed in mammary glands lacking PPAR γ suggesting that PPAR γ is not a tumor suppressor [32]. In summary, these observations suggest that reduced PPAR γ expression does not contribute to the initiation of breast cancer; however, acceleration of PPAR γ signaling after tumor initiation markedly promotes breast cancer development.

In this study, we have begun to elucidate the functional significance of endogenous PPAR γ activation in breast cancer using an *in vitro* model. We have previously reported that PPAR γ 1, not PPAR γ 2, is expressed in normal mammary epithelial cells and breast cancer cell lines [33]. Our lab and others have also demonstrated that the level of PPAR γ 1 expression is significantly higher in breast cancer cell lines as compared to normal epithelial cells [33-36]. In addition, we have shown that a distinct promoter regulates PPAR γ 1 expression in MCF-7 cells and that *promoter switching* mediates differential PPAR γ 1 expression levels between normal and cancer cells [33]. The Myc-associated zinc finger protein (MAZ) has been identified as a transcriptional mediator of PPAR γ 1 in MCF-7 cells [37]. MAZ is a transcriptional factor that controls the expression of various genes through interactions between GC-rich DNA binding sites within the promoter sequence of target genes and the carboxyl-terminal zinc finger motifs of MAZ [38]. Here, we demonstrated that an increase in expression and endogenous transactivation of PPAR γ 1 in MCF-7 breast cancer cells enhances cell proliferation by accelerating cell transition from G₁ to the S

phase. This data was confirmed using a dominant-negative PPAR γ 1 mutant as an alternative approach to inhibit endogenous activity of PPAR γ 1 in two different cell lines, MCF-7 and T47D. We also found that in the absence of exogenous stimulation high expression of PPAR γ 1 significantly inhibits apoptosis in MCF-7 cells.

Results

PPAR γ 1 is highly expressed in breast cancer cell lines

Our previous studies revealed that MAZ is a critical transcriptional regulator of PPAR γ 1 in MCF-7 breast cancer cells [37]. We also showed that both PPAR γ 1 and MAZ are highly expressed in MCF-7 cells as compared to normal mammary epithelial cells (HMEC) and tumor-specific expression of PPAR γ 1 is MAZ dependent [37]. To evaluate expression of PPAR γ 1 within other breast cancer cell lines, whole cell lysates from a panel of eight different breast cancer cell lines originated from heterogeneous tumors (ranging from adenocarcinoma to metastatic ductal carcinoma) were examined by Western blot analysis. HMEC cells were used as a control. Figure 1A shows the representative immunoblot for PPAR γ 1. To demonstrate the reproducibility of these data, statistical analysis of three Western blots was performed (Fig. 1B). The results showed that PPAR γ 1 is expressed at significantly higher level in cancer cell lines as compared to HMEC.

RNAi effect on PPAR γ 1 expression and activity in MCF-7 breast cancer cell line

As discussed above, evaluation of PPAR γ 1 as a potential breast cancer therapy target revealed the complexity of PPAR γ 1 signaling in cancer. The mechanism of PPAR γ 1 activation during cancer development and the functional role of this event in tumorigenesis still remain unclear. To address these questions and elucidate the role of PPAR γ 1 activation in cancer, we utilized the advantages of shRNA techniques. A set of five shRNAs for each of PPAR γ or MAZ gene was purchased from The RNAi Consortium (TRC). Each shRNA was evaluated using Western blot analysis (data is not shown). The most efficient shRNA for each gene was chosen for further investigation. MCF-7 cells were transiently transfected with PPAR γ and MAZ shRNAs, as well as with a scrambled shRNA as control. MCF-7 cells treated only with the transfection reagent were also used as a control. To evaluate the specificity of shRNAs to their target genes and the extent of PPAR γ 1 down-regulation when either PPAR γ or MAZ shRNA were applied to MCF-7 cells, Real-time PCR, Western blot analysis and Luciferase assay were performed. Real-time PCR data revealed that both PPAR γ and MAZ shRNAs are highly specific to their targets and their application to the cells leads to a significant decrease in PPAR γ 1 or MAZ mRNA levels respectively (Fig. 2A). We also tested whether the observed changes in PPAR γ 1 or MAZ mRNA lead to changes in PPAR γ 1 protein expression. Figure 2B shows

the representative immunoblot for PPAR γ 1 and demonstrates the level of PPAR γ 1 down-regulation by both PPAR γ and MAZ shRNAs as compared to controls. The statistical evaluation of three different Western blots showed that the direct inhibition of PPAR γ 1 by PPAR γ shRNA resulted in an average 50 percent decrease in PPAR γ 1 expression. A lower level of inhibition was observed when PPAR γ 1 knocked-down was achieved via down-regulation of MAZ expression. This was anticipated since we believe transcription factors other than MAZ are involved in regulation of PPAR γ 1 in cancer cells.

It is known that PPAR γ activates gene transcription by interacting with a Peroxisome-Proliferator Response Element (PPRE) located within the promoter sequence of target genes [2]. To confirm that shRNAs-mediated down-regulation not only affects expression but also activity of PPAR γ 1, a PPRE functional response was measured. MCF-7 cells were transfected with a 3XPPRE-mTK-pGL3 reporter plasmid and then co-transfected with scrambled, PPAR γ , or MAZ shRNA expression plasmids. Following transfection, cells were treated with 10 μ M Rosiglitazone (Rosi), a well known PPAR γ agonist [39]. Cells were lysed 24 hours after the second transfection and a Luciferase assay was performed. Data demonstrated that direct down-regulation of PPAR γ 1 expression by PPAR γ shRNA led to a significant decrease in PPRE-mediated reporter activity in both Rosi treated and untreated MCF-7 cells (Fig. 2C). This confirms that PPAR γ shRNA is specific and efficient for the inhibition of PPAR γ 1 expression and activity. Moreover, the fact that PPRE activity falls below the control level when PPAR γ shRNA is applied to the cells is additional evidence for endogenous transactivation of PPAR γ 1 in breast cancer cells. Although we observed a decrease in reporter activation when MAZ shRNA was transfected to the cells, these changes were not statistically significant, suggesting the complexity of PPAR γ transcriptional regulation and that the knock-down of MAZ seen in transient transfection assays is not sufficient to block PPRE-mediated reporter activity.

Down-regulation of PPAR γ 1 expression by PPAR γ or MAZ shRNA decreases proliferation of MCF-7 breast cancer cells

Since one of the most important characteristics of tumor development is enhanced cell growth, we tested whether inhibition of PPAR γ 1 expression affects cellular proliferation in breast cancer cells. MCF-7 cells were transiently transfected with scrambled, PPAR γ , or MAZ shRNA and the rate of BrdU incorporation during DNA synthesis was assessed by using the BrdU proliferation assay (Roche). The results revealed that down-regulation of PPAR γ 1 by both PPAR γ and MAZ shRNAs significantly decreased cellular proliferation in MCF-7 breast cancer cells (Fig. 3A). To confirm these results, we used a different approach to

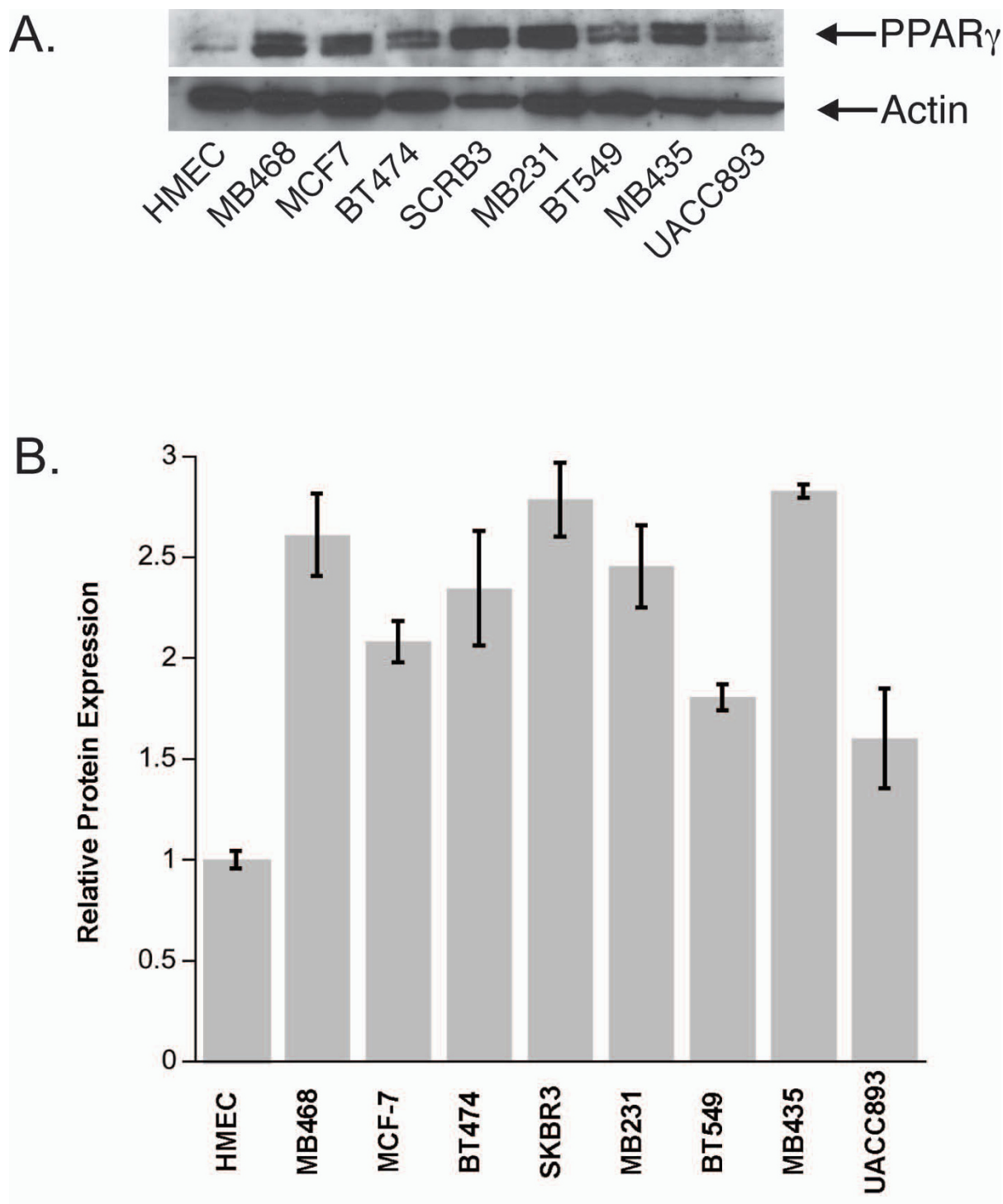


Figure 1
PPAR γ 1 is over-expressed in various breast cancer cell lines as compared to HMEC. **A.** The representative Western blot of PPAR γ 1 expression. Whole cell lysates (40 μ g of total protein) from nine different cell lines were analyzed by Western blot analysis as described in the methods section. **B.** Densitometry was used to quantify PPAR γ 1 expression. The chart represents data from the three different immunoblots (n = 3). Intensity of each band was normalized to actin. PPAR γ 1 expression is shown as a fold change in band intensity relative to HMEC. Statistical analysis was performed and demonstrated a significant difference in PPAR γ 1 expression in all tested cancer cell lines as compared to HMEC (p < 0.05).

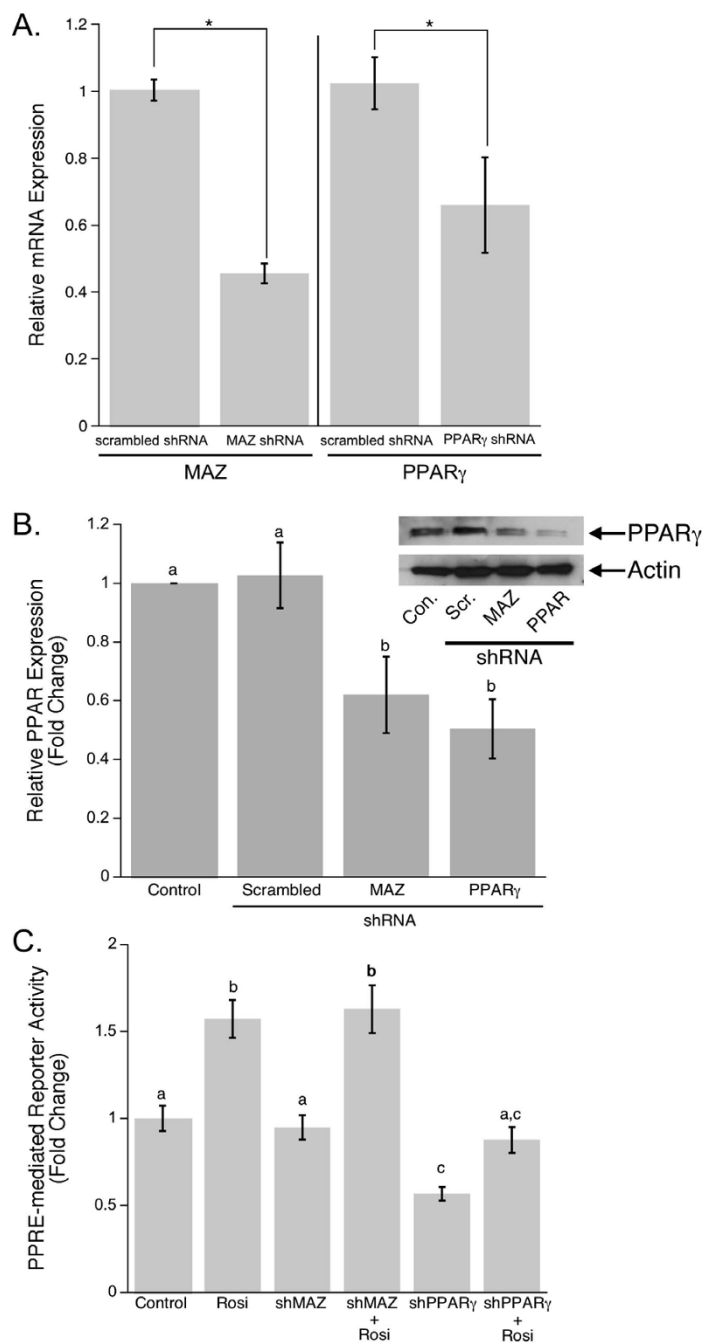


Figure 2

Effect of PPAR γ and MAZ shRNAs applications on down-regulation of PPAR γ I expression and activity in the MCF-7 breast cancer cell line. **A.** To test the specificity of MAZ and PPAR γ shRNAs for their target genes and estimate the efficiency of MAZ and PPAR γ knock-down, Real-time PCR analysis of MCF-7 cells transfected with scrambled, MAZ, or PPAR γ shRNA was performed. The fold change in gene expression was calculated using the $\Delta\Delta C_t$ method. 18S was included as an internal control. **B.** Representative Western blot analysis of PPAR γ I expression in MCF-7 cells transiently transfected with scrambled, MAZ, or PPAR γ shRNA. Densitometry was used to quantify PPAR γ I expression (n = 3). PPAR γ I expression is shown as a fold change in band intensity relative to control MCF-7 cells. Intensity of each band was normalized to actin. **C.** PPRE-mediated reporter activity was measured in MCF-7 cells transiently transfected with a 3XPPRE-mTK-pGL3 reporter plasmid and then co-transfected with MAZ or PPAR γ shRNA expression plasmids. Cells were also subsequently treated with 10 μ M Rosi for 20 hours. Data is expressed as mean fold change in luciferase to renilla ratios compared to control.

inhibit endogenous activity of PPAR γ 1. MCF-7 cells were transiently transfected with a vector driving the expression of a dominant-negative mutant of PPAR γ , Δ 462, or an empty vector as control, and then a BrdU proliferation assay was performed. Inhibition of endogenous PPAR γ 1 activity using the Δ 462 mutant caused a decrease in cellular proliferation in MCF-7 cancer cells (Fig. 3B). To test whether endogenous activation of PPAR γ 1 plays a similar role in other types of breast cancer cells, the same experiment was performed using the breast cancer cell line, T47D. The level of PPAR γ 1 expression in this cell line was evaluated using Western blot analysis (Fig. 3D). T47D cancer cells were transiently transfected with either a control vector or a Δ 462 expression vector and then a BrdU proliferation assay was performed. Inhibition of PPAR γ 1 activity in these cells also led to a significant decrease in cellular proliferation (Fig. 3C). To confirm that the observed changes in cellular proliferation in both MCF-7 and T47D cell lines are indeed in response to the inhibition of PPAR γ 1 activity in a dominant-negative manner by Δ 462, a Luciferase assay was performed. Cells were transfected with 3XPPRE-mTK-pGL3 reporter plasmid and then co-transfected with control or Δ 462 expression plasmids. Following transfection, cells were treated with 10 μ M Rosi. In Rosi treated and untreated cells, application of a Δ 462 mutant resulted in a significantly lower level of PPRE-mediated reporter activity (Fig. 3E), thus, demonstrating that Δ 462 efficiently inhibits endogenous activity of PPAR γ 1 in MCF-7 and T47D cancer cells.

Down-regulation of PPAR γ 1 gene expression affects cell cycle distribution by decreasing the number of cells entering S-phase in MCF-7 cells

To elucidate the mechanism by which down-regulation of PPAR γ 1 expression leads to inhibition of cellular proliferation in MCF-7 cells, fluorescence-activated cell sorting (FACS) was performed. Analysis of cell cycle distribution (Fig. 4) revealed that PPAR γ 1 down-regulation by PPAR γ or MAZ shRNA primarily affects cell transition from G₁ to S-phase in MCF-7 cells. Approximately 25 percent fewer cells entered the S-phase when PPAR γ 1 expression was suppressed directly or indirectly. This is consistent with data from the BrdU proliferation assay. Therefore, these results confirm our hypothesis and demonstrate that an increase in PPAR γ 1 expression and its endogenous transactivation play an important functional role in promoting cellular proliferation in breast cancer cells.

FACS analysis allowed us to assess changes in apoptosis as well. Interestingly, the level of apoptosis (Fig. 4D) and the percentage of debris and aggregates (data not shown) in cells transfected with either PPAR γ or MAZ shRNA was significantly higher than in control cells or scrambled shRNA transfected cells. This observation suggests that in addition to its involvement in regulation of proliferation,

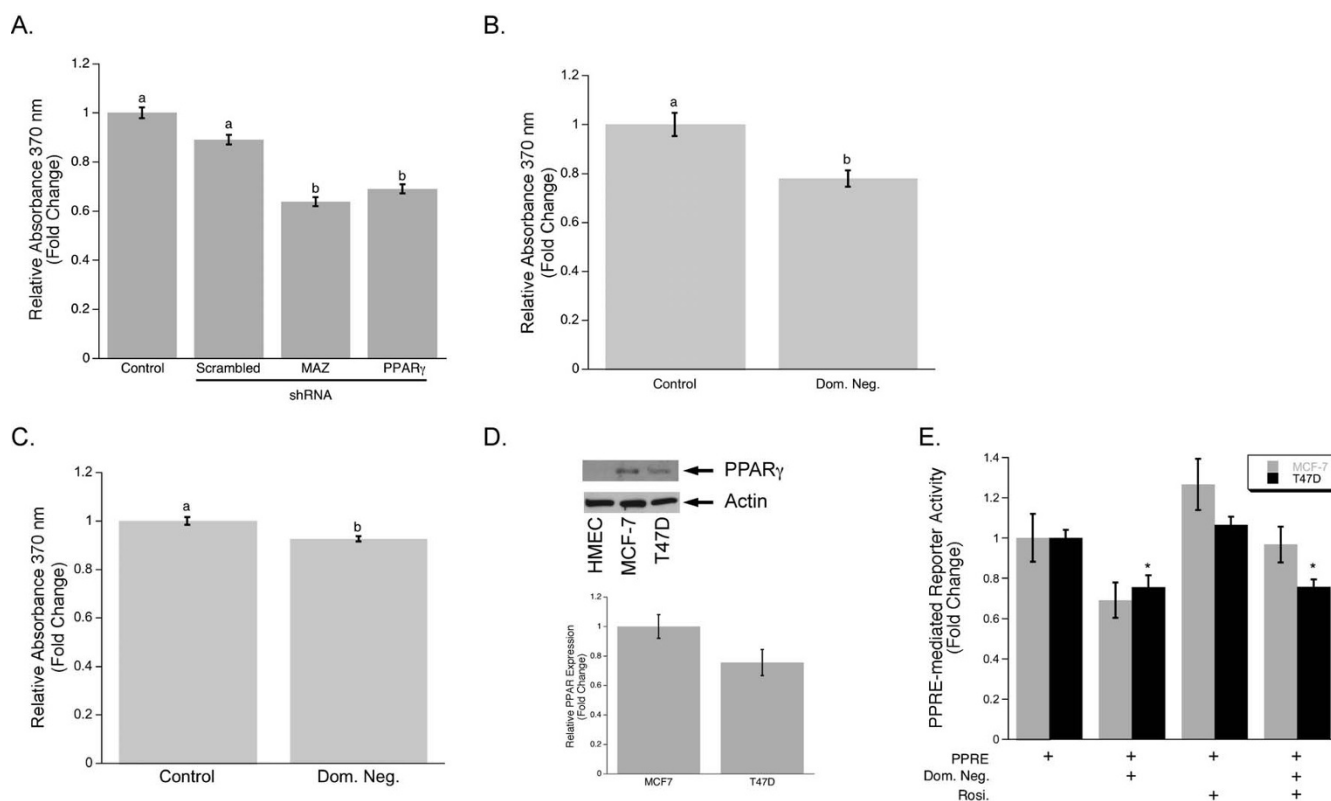
PPAR γ 1 may also be involved in regulation of apoptosis in MCF-7 cells.

Down-regulation of PPAR γ 1 gene expression increases apoptosis in MCF-7 breast cancer cells

To evaluate data from FACS analysis (Fig. 4D) and determine whether changes in PPAR γ 1 expression can affect apoptosis, a Cell Death Detection ELISA assay was performed which distinguishes between necrotic and apoptotic cell death. MCF-7 cells were transfected with scrambled, PPAR γ , or MAZ shRNA using the same transfection protocol and time points as for the proliferation assay, FACS, and protein analysis. The results showed no significant difference in necrotic cell death between PPAR γ knock-down and control cells (data not shown). However, inhibition of PPAR γ 1 expression in MCF-7 cells using PPAR γ or MAZ shRNA resulted in a significant increase in apoptosis as compared to control (Fig. 5A). Interestingly, changes caused by down-regulation of PPAR γ 1 directly by using PPAR γ shRNA were more dramatic than through knock-down of MAZ. This suggests that MAZ probably contributes to regulation of apoptosis mostly through its mediation of PPAR γ 1 signaling in cancer cells. To confirm that down-regulation of PPAR γ 1 expression induces apoptosis, the expression of Bcl2, an anti-apoptotic protein, which has been shown to promote the survival of cancer cells [40] was evaluated. Statistical analysis of densitometry for four Western blots demonstrated that Bcl2 expression was significantly higher in control and MCF-7 cells transfected with scrambled shRNA versus cells transfected with PPAR γ or MAZ shRNA (Fig. 5B). We also assessed changes in poly (ADP-ribose) polymerase-1, PARP-1, cleavage in cells transfected with PPAR γ or MAZ shRNA as compared to controls. PARP-1 cleavage by caspases is a well-known marker for apoptosis [41]. Statistical evaluation of densitometry for three Western blots (Fig. 5C) showed a significant increase in an 89 kDa C-terminal fragment, the product of PARP-1 proteolysis, when PPAR γ shRNA was applied to MCF-7 cells. This data suggests that an increase in PPAR γ 1 expression followed by transactivation during cancer development may be an important factor that contributes not only to acceleration of cellular proliferation but also to cell evasion from apoptosis.

Discussion

In this report, we confirmed that PPAR γ 1 is highly expressed in cultured breast cancer cell lines as compared to HMEC [37,42]. High expression of PPAR γ 1 has also been reported in human breast cancer tissues [43]. However, questions about the mechanism and role of endogenous transactivation of PPAR γ 1 during development of breast cancer still remain unanswered. We have previously shown that the increase in expression of PPAR γ 1 from normal human mammary epithelia to breast cancer is due

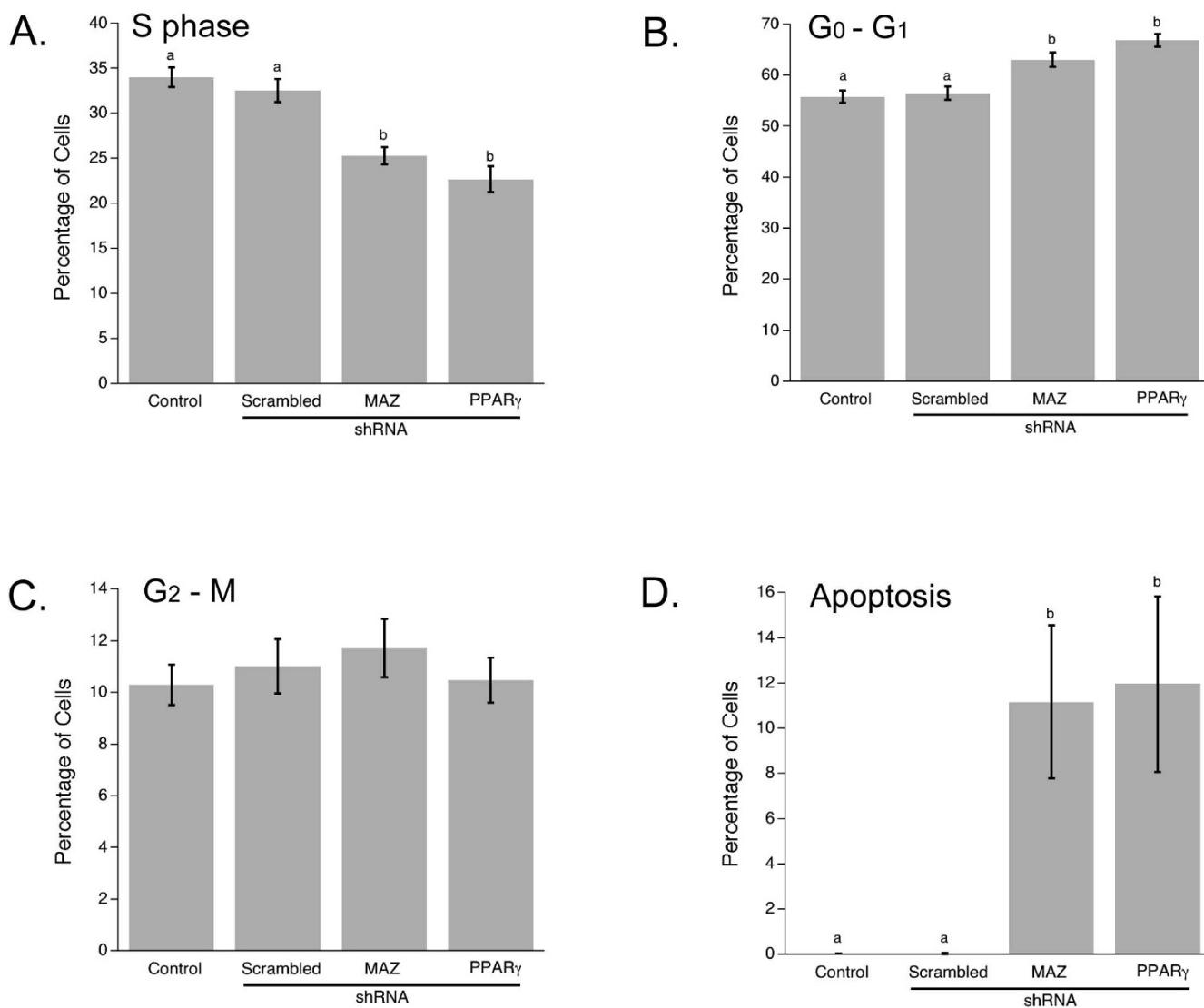
**Figure 3**

Decreased PPAR γ I expression is associated with a less proliferative phenotype for MCF-7 cells. **A.** Cell proliferation of MCF-7 cells transfected with or without scrambled, MAZ, or PPAR γ I shRNA was measured based on incorporation of the pyrimidine analog BrdU into DNA (BrdU proliferation ELISA). Data is shown as mean fold changes in cell proliferation compared to control cells. Error bars represent the standard error of the mean (s.e.m.) and the bars that do not share a letter designation were determined to be significantly different by Tukey's pairwise comparison ($p < 0.05$). **B.** MCF-7 cells were transiently transfected with a $\Delta 462$ expression plasmid or control plasmid. Cell proliferation was measured by BrdU proliferation ELISA. **C.** T47D cells were transiently transfected with a $\Delta 462$ expression plasmid or control plasmid. Cell proliferation was measured by BrdU proliferation ELISA. In Fig. B and Fig. C Student's t-test showed a significant difference ($p < 0.01$). **D.** The level of PPAR γ I expression in HMEC, MCF-7, and T47D cells was determined using Western blot analysis. Densitometry was used to quantify PPAR γ I expression in MCF-7 and T47D ($n = 3$). PPAR γ I expression in T47D is shown as a fold change in band intensity relative to MCF-7 cells. Intensity of each band was normalized to actin. **E.** PPRE-mediated reporter activity was measured by Luciferase assay in MCF-7 and T47D cells transfected with a dominant-negative mutant, $\Delta 462$, or control plasmid. Cells were also subsequently treated with 10 μ M Rosi for 20 hours. Data is shown as mean fold change in cell proliferation compared to control cells. Error bars represent the standard error of the mean (s.e.m.). * Significantly different from appropriate control at $p < 0.01$.

to the recruitment of a distal, tumor-specific promoter [34]. We identified MAZ as a transcription factor that directly binds to this promoter and drives expression of PPAR γ 1 in MCF-7 breast cancer cells [37]. Our results also indicated that MAZ is highly expressed in MCF-7 cells as compared to HMEC [36]. In this study, statistical analysis of three different Western Blots demonstrated an increase in PPAR γ 1 expression in a panel of different breast cancer lines and confirmed that it is a feature attributed not only to MCF-7 cells but also to other tested breast cancer cell lines. This observation suggests that the proposed model of endogenous PPAR γ 1 transactivation may apply not

only to a particular cell line, but also to breast cancer in general. Currently this hypothesis is being tested in the lab using pathological sections from normal and breast cancer specimens.

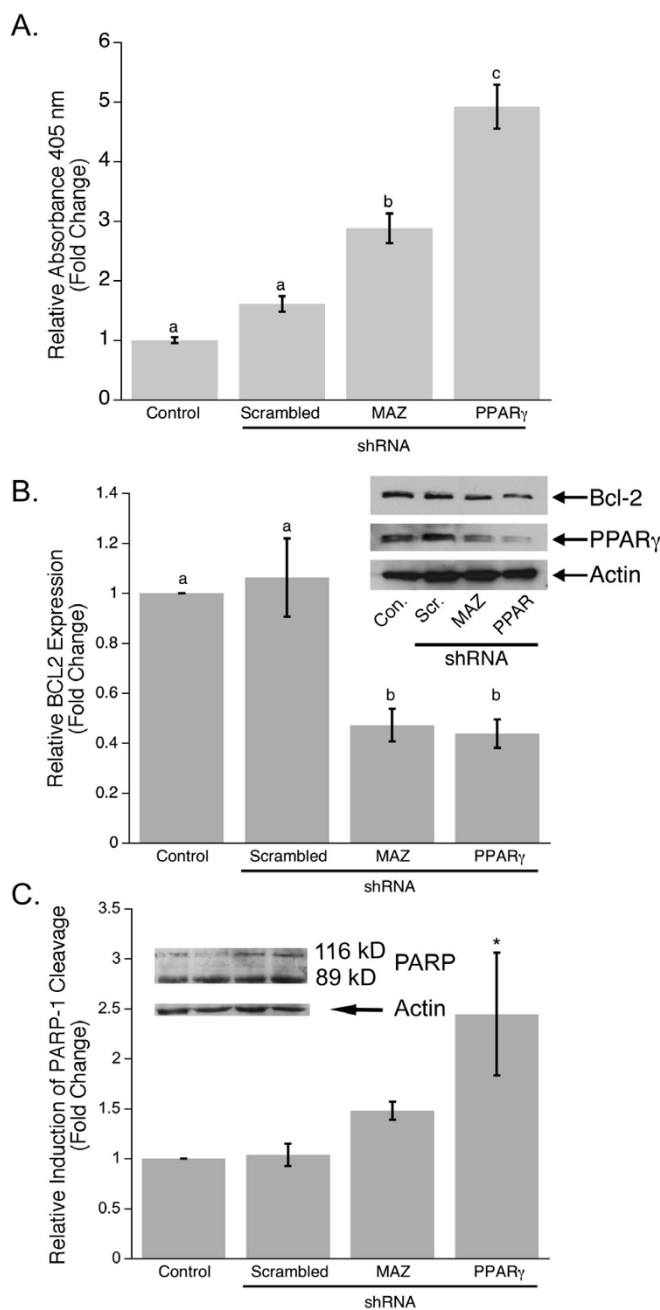
In efforts to explore the role of PPAR γ activation in cancer, most of the recent studies employed pharmacological approaches. The anti-cancer activity of PPAR γ ligands, such as TZDs, demonstrated in multiple *in vitro* studies, has raised discussion about the possibility of using PPAR γ receptors as a target for breast cancer therapy. However, the "off target" effects of PPAR γ agonists [44,45], the dual

**Figure 4****Down-regulation of PPAR γ 1 or expression of MAZ prevents S-phase entry in MCF-7 breast cancer cells.**

Changes in cell cycle distribution of MCF-7 cells transfected with or without scrambled, MAZ, or PPAR γ 1 shRNA were analyzed by FACS. Cells were stained with propidium iodide (PI). Mean and s.e.m. from five independent experiments are shown. Error bars that do not share a letter designation were determined to be significantly different by Fisher's LSD pairwise comparison ($p < 0.05$). **A.** Inhibition of PPAR γ 1 or MAZ expression leads to a decrease in a number of cells entering S-phase. **B.** A decrease in the number of proliferating cells in PPAR γ 1 or MAZ shRNA transfected cells is associated with an increase in the number of G₀-G₁ arrested cells. **C.** There was no significant difference in M-G₂ phase cell number observed among all groups. **D.** Down-regulation of PPAR γ 1 as well as MAZ leads to an increase in the number of apoptotic cells.

role that some ligands play when they are applied to the cells at different concentrations [46], and the paradoxical anti-cancer effect of PPAR γ antagonists [47] necessitated the use of other approaches to evaluate the consequences of PPAR γ transactivation in cancer. For the first time, using an *in vitro* model, we have addressed questions about the role that endogenous transactivation of PPAR γ 1 plays in the pathogenesis of breast cancer. Using RNAi techniques

to inhibit PPAR γ 1 expression we demonstrated that an increase in PPAR γ 1 signaling can significantly affect proliferation and apoptosis in breast cancer cells. It is widely accepted that the dysfunctional balance between cellular proliferation and apoptosis can contribute to the initiation and progression of cancer. Here, we demonstrated that down-regulation of PPAR γ 1, directly or indirectly via knock-down of its transcriptional regulator MAZ, leads to

**Figure 5**

Down-regulation of PPAR γ 1 increases apoptosis in MCF-7 breast cancer cells. **A.** Apoptosis was measured by specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates from control and shRNA transfected MCF-7 cells. Data is shown as mean fold changes in cell apoptosis compared to control. Error bars represent s.e.m. and the bars that do not share a letter designation were determined to be significantly different by Tukey's pairwise comparison ($p < 0.05$). **B.** Representative Western blot analysis of PPAR γ 1 and Bcl2 expression in control and shRNA transfected MCF-7 cells showed that a decrease in PPAR γ 1 expression leads to a decrease in Bcl2 expression. Densitometry was used to quantify Bcl2 expression ($n = 4$). Bcl2 expression is shown as a fold change in band intensity relative to control MCF-7 cells. Intensity of each band was normalized to actin. **C.** Western blot analysis of PARP-1 demonstrated an increase in induction of PARP-1 cleavage in MCF-7 cells transfected with PPAR γ 1 shRNA. Densitometry was used to quantify an 89 kDa fragment of PARP-1 cleavage ($n = 3$). It is shown as a fold change in the 89 kDa band intensity relative to control MCF-7 cells. Intensity of each band was normalized to actin. * Significantly different from control at $p < 0.05$.

a decrease in cellular proliferation in MCF-7 breast cancer cells. Interestingly, changes in cellular proliferation caused by direct PPAR γ 1 inhibition by PPAR γ shRNA were analogous to changes in PPAR γ 1 expression when inhibited via down-regulation of MAZ. This suggests that in addition to its role as a mediator of tumor-specific expression of PPAR γ 1, MAZ may also be involved in the regulation of other growth control genes in MCF-7 breast cancer cells. The ongoing project in our lab is to further investigate the role of MAZ in breast cancer development.

The observed pro-survival effect of PPAR γ 1 signaling in MCF-7 cancer cells was also confirmed by using a different approach to inhibit endogenous activity of PPAR γ 1. We took advantage of a PPAR γ 1 mutant, Δ 462, which lacks helix12, critical for ligand binding and co-activators recruitment. Thus, Δ 462 functions in a dominant-negative manner. Data from BrdU proliferation assays demonstrated that inhibition of PPAR γ 1 activity using Δ 462 decreases cell proliferation not only in MCF-7 cells but in another widely studied breast cancer cell line, T47D. In our previous study, we have shown that the T47D cell line also has a functional peroxisomal response [42]. Here, using Western blot analysis, we demonstrated that T47D cancer cells as well as MCF-7 cells have high level of PPAR γ 1 expression as compared to HMEC. However, the direct comparison of PPAR γ 1 expression in MCF-7 and T47D cells showed the lower level of PPAR γ 1 in a latter cell line. The differential expression of PPAR γ 1 in these cell lines can explain the more prominent changes in cellular proliferation in MCF-7 cells compare to T47D cells when Δ 462 are applied to the cells. The specificity of Δ 462 in the inhibition of endogenous PPAR γ 1 activity was confirmed using Luciferase assay. We measured PPARE-mediated reporter activity when either MCF-7 or T47D cells were transfected with Δ 462 or control plasmids and then treated with 10 μ M Rosi. Data revealed that PPARE reporter activity is significantly lower in cells transfected with the Δ 462 expression plasmid compared to control, thus, providing the evidence that this mutant acts in dominant-negative manner, decreasing activity of PPAR γ 1 in MCF-7 and T47D cancer cells. Moreover, a similar effect was observed with Rosi, confirming the specificity of Δ 462 action in these cell lines. In summary, these results suggest that PPAR γ 1 transactivation enhances cell growth in breast cancer cells and that this phenomenon is not specific to MCF-7 cells.

To further investigate the mechanism by which PPAR γ 1 regulates cell growth, we performed fluorescence-activated cell sorting (FACS). Cell cycle distribution analysis confirmed results from the BrdU proliferation assay and demonstrated that an increase in PPAR γ 1 signaling accelerates the transition of cells from G₁-phase to S-phase and, thus, increases cellular proliferation.

Blockage of apoptosis is a likely requirement for cancer maintenance [48]. In fact, FACS analysis revealed that the number of cells which undergo apoptosis is much higher in MCF-7 cells with decreased PPAR γ 1 expression. This observation was tested and confirmed by measuring DNA fragmentation in control and PPAR γ or MAZ shRNA transfected cells. The data demonstrated that down-regulation of PPAR γ 1 expression in MCF-7 cells leads to a significant increase in apoptosis. The induction of apoptosis in cells with PPAR γ 1 or MAZ knockdown was also confirmed by analyzing the expression of Bcl2, a protein that is known to block cell death [40], and by evaluation of PARP-1 cleavage, a widely accepted marker for apoptosis. The results showed that inhibition of PPAR γ 1 leads to down-regulation of Bcl2 which may in turn favor re-activation of signaling pathways to induce apoptosis. The increase in PARP-1 cleavage in MCF-7 cells, which have a decreased level of PPAR γ 1 expression, verified the induction of apoptosis as well. Together these results suggest that accelerated PPAR γ 1 signaling can interfere with apoptotic pathways and promote cancer cell survival during breast tumor development. However, the molecular mechanisms that drive these events are not known and will be the subject of future investigation in the lab.

In summary, this study demonstrates that the increase in PPAR γ 1 expression observed in breast cancer results in an increase in PPAR γ 1 signaling that in turn promotes proliferation and inhibits apoptosis and thus, may significantly contribute to the progression of disease to a more malignant stage. Our findings are consistent with results from a study that evaluated the consequences of intrinsic PPAR γ 1 activation using transgenic mice. This study demonstrated that constitutive over-expression of PPAR γ 1 in mice, which were predisposed to breast cancer, leads to a greater number of tumors and higher mortality in both male and female animals, thus suggesting that increased PPAR γ 1 signaling serves as a tumor promoter in the mammary gland [31]. Since constitutive PPAR γ 1 signaling did not affect mammary gland differentiation or function when introduced in wild-type mice, the authors emphasized that consequences of PPAR γ 1 transactivation are different in normal and transformed cells. This observation is consistent with our previous data, which demonstrated different mechanisms of transcriptional regulation of PPAR γ 1 in breast cancer cells as compared to HMEC [34,37].

Conclusion

This study provides insight into the functional significance of increased PPAR γ 1 expression and endogenous transactivation in breast cancer in an *in vitro* model. The results suggest that increased PPAR γ signaling can act as a pro-survival factor by enhancing cancer cell proliferation and blocking the ability of the cell to undergo apoptosis. Furthermore, modulation of the PPAR γ 1 signaling path-

way remains a promising tool for breast cancer therapy. The findings presented in this paper warrant further investigation regarding the use of PPAR γ 1 ligands, such as TZDs, in patients who are predisposed or already diagnosed with breast cancer. However, more broad and detailed studies are required to evaluate the impact of PPAR γ 1 signaling in breast cancer progression.

Methods

Cell culture

MCF-7 and T47D breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in modified DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone). Normal human mammary epithelial cells (HMEC) (Cambrex) were cultured in MEGM[®] with SingleQuot[®] supplements. All cell lines were grown in media lacking phenol red at 37°C in a 5% CO₂ atmosphere.

Western blot analysis

The whole cell lysates were prepared using passive lysis buffer. Concentrations were determined using a Bradford Assay (BioRad). 30 μ g of total cell lysate per sample was run on a 10% or 12% SDS polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, blocked in 5% TBST, and incubated at 4°C overnight with primary antibody. The membrane was then washed and incubated for 4 hours with secondary IgG-HRP antibody. After incubation the membrane was washed, incubated with Chemiluminescence substrate (Pierce) for 5 min, and expose to film. The following primary antibodies were used: PPAR γ mouse monoclonal IgG antibody 1:200 dilution (Santa Cruz Biotechnology, sc-7273), Bcl2 mouse monoclonal antibody 1:1000 dilution (Santa Cruz Biotechnology, sc-509), PARP-1 rabbit polyclonal antibody 1:1000 dilution (Santa Cruz Biotechnology, sc-2578). Appropriate secondary goat anti-mouse (Santa Cruz Biotechnology, sc-2055), or bovine anti-goat (Santa Cruz Biotechnology, sc-2378), or goat anti-rabbit secondary antibody 1:1000 dilution (Santa Cruz Biotechnology, sc-2004) antibodies 1:1000 dilution were applied. Anti-actin raised in rabbit 1:2000 dilution (Sigma, A 5060) and goat anti-rabbit secondary antibody 1:1000 dilution (Santa Cruz Biotechnology, sc-2004) were used to visualize actin. Western Blot Stripping Buffer (Pierce, # 21059) was used to restore membranes.

shRNAs constructs

The set of five shRNAs for PPAR γ 1 (TRCN 000001670-74) and MAZ (TRCN 0000015343-47) genes as well as scrambled shRNA and non-hairpin TRC controls were purchased from The RNAi consortium (TRC) Human shRNA Library (Open Biosystems). The shRNA construct includes a hairpin of 21 base pairs, a sense and antisense

stem, and a 6 base-pair loop. Each hairpin sequence is cloned into a lentoviral vector (pLKO1). Based on structural evaluation and Western blot analysis the most efficient shRNAs for PPAR γ 1 (TRCN 000001672) and MAZ (TRCN 0000015345) were chosen for transient transfections.

Dominant-negative PPAR γ 1 construct

The dominant-negative PPAR γ 1 mutant was a kind gift of Dr. Stephen O'Rahilly and Dr. V. Krishna K Chatterjee, Department of Medicine, Addenbrook's Hospital, Cambridge, U.K. Sequence analysis revealed a single base deletion introducing a premature stop codon (5'-₁₃₈₀GACAGACTGA₁₃₉₀-3') leading to translation of protein truncated just before the AF-2 domain.

Transfection assays

Cells were transiently transfected with 3.6 μ g of pGL3 plasmid containing 3XPPRE-mTK-Luc and Renilla (Allred, 2005) per 24-well plate and then co-transfected with scrambled, PPAR γ or MAZ shRNAs, Δ 462, or control plasmids using FuGENE transfection reagent (Roche). 4 hours after transfection cells were subsequently treated with 10 μ M Rosi for 20 hours. Cells were lysed in 80 μ l of passive lysis buffer and treated according to manufacturer's instructions (Promega dual luciferase assay kit). Luminescence was performed on a Berthold Technologies Lumat 9507 (Wildbad, Germany). Results were calculated as raw Luciferase units divided by raw Renilla units (RLU's). Data is presented as mean fold changes in treated cells as compared to control cells.

Real-time PCR

Total mRNA was isolated using an RNeasy Mini Kit (Qiagen, CA) according to manufacturer's instructions. Real-time PCR was performed on total RNA using the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems). The pre-optimized primers and probes for MAZ, PPAR γ 1, and 18S were purchased from Applied Biosystems.

BrdU proliferation assay

MCF-7 cells were seeded at 0.1 \times 10⁴ cells/well in 96-well tissue culture plates. Cells were transiently transfected on the second and third day using 0.05 μ g of plasmid and 0.3 μ l of FuGENE 6 transfection reagent (Roche) per well. Control MCF-7 cells were treated with FuGENE6 only. 16 wells per each shRNA and control were used. The same experimental set-up was used when cells were transfected with a dominant-negative form of PPAR γ 1, Δ 462. The media was changed before the second transfection. Cell Proliferation ELISA, BrdU (colometric) (Roche) was performed on the fifth day according to the manufacturer instructions.

Apoptosis assay

The same protocol as for the proliferation assay was used to plate and transfect MCF-7 cells. A Cell Death Detection ELISA (Roche) was performed on the fifth day. The assay is based on quantitative sandwich-enzyme-immunoassay-principle and uses mouse monoclonal antibodies directed against DNA and histones. Cells were lysed in a 96-well plate, centrifuged, and 20 μ l of supernatant was transferred into streptavidin-coated wells. A mixture of antibodies was added and the plate was then incubated for 2 hours. The unbound components were removed by washing. ABTS substrate was added and the amount of mono- and oligonucleosomes were measured photometrically using the ELISA-plate reader according manufacture instructions (Roche).

Cell cycle analysis

The DNA content of control and shRNAs transfected MCF-7 cells was analyzed using a detergent-trypsin method (Vindelov, 1983). MCF-7 cells were seeded at 1×10^6 cells in 100 mm culture plates. Cells were transiently transfected on the second and third day with 6 μ g of plasmid using 18 μ l of FuGENE6 transfection reagent (Roche). On the fifth day the propidium iodide labeling procedure and fluorescence-activated cell sorting (FACS) using Mod FitLT V.3.1 software was performed (University of Kentucky Flow Cytometry Facility).

Statistics

Data was analyzed by a two-way analysis of variance (ANOVA) using the StatServer 6.1 (Insightful, Seattle, WA) from the server maintained by the University of Kentucky's Department of Statistics. In every 2-way ANOVA, Tukey's pair-wise comparison test was used post-hoc. P-values of less than 0.05 were considered to be significant. One-way ANOVA with Fisher's LSD or Tukey's pair-wise comparison post-hoc test were also used where appropriate. When appropriate, Student's t-test was also used for data analysis on Microsoft Excel.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors significantly contributed in the design of the study, data interpretation, and manuscript drafts. YYZ carried out all experiments. RCS performed all statistical analyses and figure design. NKW and XW assisted with Δ 462 and PPRE-Luciferase experiments. MWK coordinated this study.

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