PPARγ Dependent and Independent Effects Contribute to the Anti-Proliferative Effect of Ciglitazone in ER Negative Breast Cancer Cells

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Abstract: Background: Ciglitazone is a synthetic ligand of PPARγ (Peroxisome Proliferator Activated Receptor gamma) and possess potent anti-proliferative effect in cancer cells. Given the complexity in the mechanism of action of synthetic ligands of PPARγ which is strictly context dependent, we analyzed the molecular alterations associated with the cytotoxic effect of pioglitazone in ER negative breast cancer cells. Methods: Effect of ciglitazone on cell proliferation and cell cycle progression was determined in MDA MB 231 cells. PPARγ activation studies were performed using luciferase reporter and transactivation assay. PPARγ independent effects of ciglitazone were studied in PPARγ knock-down cells. Changes in expression of downstream effectors were analyzed using western blot assay. Correlation of in vitro expression data was done in different grades of breast cancer tissues using immunohistochemical analysis. Results: Ciglitazone effectively induced activation of PPARγ in MDA MB 231 cells. The activation of PPARγ was inversely correlated with cyclin D and skp2 levels and directly correlated with p27 levels. However, the reduction in skp2 and increase in p27 were partially independent on PPARγ activation. Correlation of our in vitro findings with the expression of PPARγ, skp2, p27 and cyclin D in breast cancer tissue samples further substantiated the presence of PPARγ dependent and independent mechanisms for ciglitazone in ER negative breast cancer cells. Conclusion: Ciglitazone reduces skp2 levels in ER negative cancer cells through PPARγ dependent and independent mechanisms.

Key words: PPARγ, ciglitazone, breast cancer, estrogen receptor

1. Introduction:

Breast cancer is most common among women worldwide. Anti-estrogen therapy is highly appreciated currently for breast cancer prevention and treatment [1]. However, anti-estrogen therapy has little or no effect on estrogen receptor (ER) negative tumors [2]. This fuelled the quest for novel approaches for treating ER negative breast cancer.

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors. PPARγ is one of the well-studied isoforms of PPAR. PPARγ regulates lipid metabolism, cell growth, cell differentiation, and apoptosis. Roles of PPARγ in development and progression of cancer is controversial [3]. Most of the studies which attempted to elucidate the role of PPARγ in cancer cells heavily rely on data generated from its synthetic ligands. However, PPARγ non-specific actions of the synthetic ligands compounds hurdles in understanding the exact nature of contribution of PPARγ in cancer cells [4]. Complicating further, PPARγ ligands have multiple mechanisms depending on the cell type and context. For example troglitazone reduces skp2 levels in hepatoma cells whereas pioglitazone and ciglitazone do not show such effects [5-10].

Recent reports indicated that thiazolidinedine class of PPARγ ligands, especially troglitazone, pioglitazone and rosiglitazone, have little clinical value because of their severe side effects [11]. At the same time, preclinical studies suggest that ciglitazone may be an alternative approach for certain subsets of cancers due to its high efficacy and minimal side effects. Therefore in the current study, we tried to comprehend the possible

mechanisms involved with ciglitazone mediated cytotoxicity in ER negative MDA MB 231 cells. Our results indicate that the cytotoxic effect of ciglitazone is mediated through both PPARγ dependent and independent mechanisms.

2. Materials and Methods

2.1 Cell culture

MDA MB 231 cell line was purchased from the American Type Cell Culture Collection (ATCC). The cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1X antimycotic-antibiotic cocktail in a humidified 5% CO_2 atmosphere at 37°C. Media change was given every 2 days and the cells were passaged *via* trypsinization before reaching confluency.

2.2 Tissue Collection and Primary culture

Tissue specimens from clinically verified ER negative breast cancer patients were collected from Division of Surgical Oncology, Regional Cancer Centre, Thiruvananthapuram. Informed written consent was obtained from each patient for the use of individual biopsy material. The study has been approved by the Institutional Review Board and Human Ethics Board. Fresh tissue samples were washed extensively in PBS to remove blood cells and cell debris, mechanically minced, and digested for 3 h at 37°C in a shaking incubator with 0.2% collagenase type IV (Invitrogen, USA) in DMEM. The single cell suspension was washed twice with DMEM/F12 supplemented with 20% FBS and cultured in DMEM/F12 supplemented with 20% heat inactivated FBS, 0.5 μg/ml of hydrocortisone, 10 ng/ml of human recombinant epidermal growth factor and 5 μg/ml of human recombinant insulin in a humidified atmosphere at 37°C. The medium was replaced every second day. Under these conditions, outgrowths of primary cells were observed. The cells were then sub-cultured. Fibroblasts were removed by differential trypsinization at 70% confluency. After the third passage, the cells were considered for the experiments.

2.3 Growth inhibition assay

MDA MB 231 cells were cultured in 96-well plates at a density of 5×10^3 cells/well. Following overnight incubation, the medium was replaced with fresh medium containing varying concentrations of ciglitazone (6.25–100 μM; Cayman Chemicals, St Louis, MO, U.S.A.) or DMSO. The final concentration of DMSO in all cases did not exceed 0.1% and was not found to be cytotoxic. Cell viability was assessed at 24 h, 48 h and 72 h periods. Briefly, MTT (3-[4, 5-dimethylthiazolyl]- 2,5-diphenyltetrazolium bromide) was added to the culture 4 h prior reading The formazan crystals formed was dissolved in isopropanol and colorimetrically read at 570 nM. Percentage growth inhibition, compared to control, was then calculated based on the cell viability data.

2.4 Cell cycle analysis

For cell cycle analysis, 5×10^5 cells/well were seeded in 6-well plates and treated with ciglitazone for 24 h. Cells were harvested by trypsinization, centrifuged at 5000 rpm for 5 min, washed with PBS, and fixed for 1 h in 70% ethanol at 4°C. The cells were then resuspended in PBS containing 5μg/ml RNase and incubated at 37 °C for 1 h. The cells were filtered through 75μM cell strainer to remove the debris and incubated with 50μg/ml propidium iodide (PI) for 10 min, protected from light. DNA content was then analyzed using Becton Dickinson FACS Aria II and Diva software.

2.5 Nuclear extract preparation

Briefly, cells were seeded at a density of 1×10^6 cells per 100 mm dish. After 24 h, medium was replaced with fresh medium containing varying concentrations of ciglitazone. Following 24 h of treatment, nuclear extracts were isolated using a nuclear extraction kit (Cayman Chemicals, St Louis, MO, U.S.A.) according to the manufacturer's instructions. The total protein content was determined using the Bradford's assay.

2.6 PPRE binding assay

The binding efficiency of PPARγ to PPRE was assayed in nuclear extracts using Transcription Factor Assay kit (Cayman Chemicals St Louis, MO, U.S.A.) and according to the manufacturer's instructions. Briefly, after blocking nonspecific binding in wells (Binding buffer supplied with the

kit), 50 μg of nuclear extract or positive control extract (supplied with the kit) was added to the appropriate wells and the plate incubated for 60 min at room temperature. Primary anti-PPARγ antibody (supplied with the kit) was added to each well and the plate incubated for 60 min. Secondary antibody (anti-rabbit IgG-HRP supplied with the kit) was then added to each well and incubated for another 60 min., washed five times with wash buffer followed by developing solution was added and incubated for 45 min with mild agitation. The reaction was stopped using stopping solution and the colorimetric detection of bound fraction of antibody was performed by measuring the absorbance at 450nm using a microplate reader.

2.7 Luciferase Reporter Assay

To test for transcriptional activity of PPARγ, cells were seeded at a density of 2.5×10^5 cells per well in six-well plates one day prior to transfection. Cells were transiently transfected with PPRE-x3-TK-LUC reporter (2 μg) using FuGENE6 Transfection Reagent (Roche Diagnostics) according to the manufacturer's instructions. After 12 h, the FuGENE6-DNA mix-containing media was replaced with fresh media and incubated for another 24 h. This was followed by treatment with vehicle or ciglitazone for the indicated time periods. Cells were then harvested and lysed with 1 × Reporter Lysis Buffer (Promega). Luciferase activity was measured using the Dual Luciferase Assay System (Promega). Luciferase activities were normalized against renilla expression levels to adjust for transfection efficiency.

2.8 PPARγ Knockdown

PPARγ knock down was done using PPARγ specific siRNA (Santa Cruz Technologies). Cells were transiently transfected with siRNA using silentfect reagent (BioRad) and according to the manufacturer's instructions. After 12h, the silentfect- siRNA mix containing media was replaced with fresh media and incubated for another 24 h. Some wells received follow up treatment with ciglitazone or vehicle. Cells were then harvested and cell lysates were collected and used for immunoblot analysis.

2.9 Western blotting

Briefly, cells were scraped out and lysed in cell lysis buffer (50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, 0.5% NP40, 0.2 mM Na_3VO_4, 1 mM dithiothreitol and protease inhibitor cocktail) and centrifuged. Cell lysates (60 μg protein per lane) were separated on a 10% SDS–poly acrylamide gel and transferred to nitrocellulose membrane (Hybond; Amersham, U.K.). Nonspecific protein binding was blocked using 5% skimmed milk in 1X TBST. Blots were incubated overnight with primary antibody, followed by horseradish-peroxidase-conjugated secondary antibody for 1 h. Immunoreactive bands were visualised using an enhanced chemiluminescence (ECL) detection kit (Amersham) according to the manufacturer's instructions and images captured in an X-ray film or using a BioRad Versadoc imaging system.

2.10 Immunohistochemistry

A total of 30 tissue specimens obtained from ER negative breast cancer patients were used for immunohistochemical analysis. 5μm thick sections were deparaffinized and rehydrated, followed by elimination of endogenous peroxidase activity using hydrogen peroxide method. The slides were treated with primary antibodies overnight at 4°C. The primary antibody dilution for PPARγ and skp2 were in the order of 1: 100. Reactions were visualized using a streptavidin-biotin-immunoperoxidase system (ABC Vectastain Kit) with DAB as chromogen (Sigma Fast DAB; Sigma, St. Louis,MO). All sections were then counterstained with hematoxylin. The staining intensity was graded as follows: No staining (0%), weak (1-20%), moderate (20-60%) and intense (>60%).

2.11 Statistical analysis

Data are expressed as the mean ± S.E.M and analyzed by ANOVA followed by postdoc Tukey analysis using GraphPad Prism 5.0. The P value, $p < 0.05$ is considered significant.

3. Results

3.1 Ciglitazone induces cytotoxicity in MDA MB 231 cells

Ciglitazone exposure has reduced the viability of MDA MB 231 cells in a dose and time dependent manner with IC_{50} values of 75µM, 50µM and 25µM obtained at 24h, 48h and 72h respectively (Fig. 1A). The cell death was preceded by cell cycle arrest as evidenced by the accumulation of cells at the G0/G1-S phase (Fig. 1B). Additionally, the growth inhibitory effect of ciglitazone was analyzed in primary cell cultures isolated from clinically verified ER negative breast tumor tissues. Upon morphological examination we observed a loss of anchorage dependent growth in these cells following ciglitazone treatment (Fig. 1C).

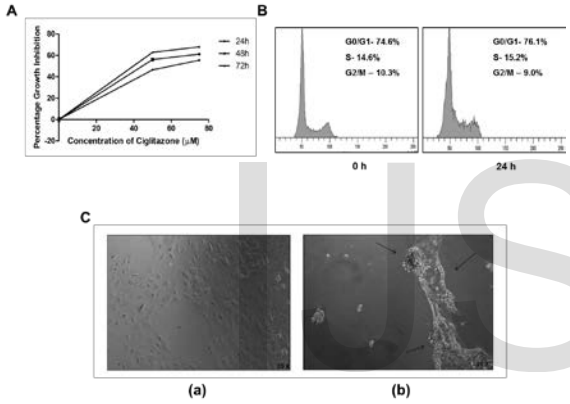

Figure 1: (A) Effect of Ciglitazone on cell proliferation. MDA MB 231 cells were treated with vehicle (DMSO), ciglitazone at the indicated doses and times (24, 48, and 72 h). Cell proliferation was determined by MTT assay. Data are expressed as the percentage control. Shown are the results of triplicate experiments and presented as mean ± SEM. (B) Histogram of cell cycle in MDA MB 231 cells treated with ciglitazone. MDA MB 231 breast cancer cells treated with 50µM ciglitazone for 24 h. The ciglitazone increased the G1-S phase proportion of MDA MB 231 cells, as analyzed by flow cytometry. (C) Effect of Ciglitazone on primary breast cancer cells (a) – before ciglitazone treatment and (b) – after ciglitazone treatment).

3.2 Ciglitazone activates PPARγ in MDA MB 231 cells

We analyzed the active fraction of PPARγ in ciglitazone treated cells with respect to its binding to PPRE. Since we got an IC_{50} value of 75µM for ciglitazone induced cytotoxicity at 24h, we used 50µM and 75µM doses for this study. Our assay showed increased binding of PPARγ to PPRE which almost reached to a saturation level at 50µM dose of ciglitazone (Fig. 2A**)**.To confirm the results, we performed luciferase assay in MDA MB 231 cells that were initially transfected with a reporter construct (PPRE-3-TK –Luc) containing three copies of PPRE upstream of the luciferase gene. Similar to PPRE binding assay, 50µM ciglitazone raised the luciferase activity to a saturation level (Fig. 2B**)**.

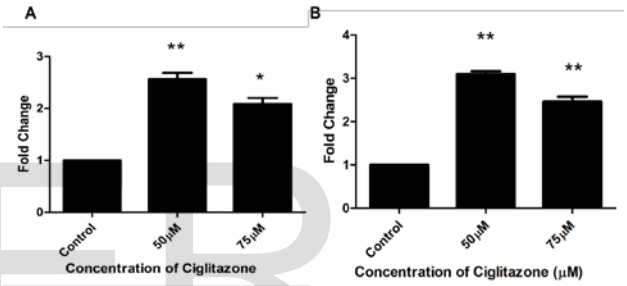

Figure 2: (A) Binding efficacy of PPARγ on PPRE, after activation with ciglitazone at concentrations 50 and 75µM for 24 h. (B) MDA MB 231 cells were transfected with either empty vector or pRL-TK and PPRE-3X-TK –Luc reporter construct, following treatment with vehicle or ciglitazone at indicated concentrations for 24 h. The firefly luciferase activity was measured in the cell lysates and was normalized by renilla luciferase activity. Shown is a representative figure for three experiments, each performed in triplicate, and presented as mean ± SEM. * $p<0.05$, ** $p<0.005$ compared with control by Tukey test after one way ANOVA.

3.3 Ciglitazone mediated growth arrest is associated with alterations of multiple cell cycle regulatory proteins

Cell cycle is tightly regulated with a complex network of positive and negative regulatory molecules including cyclin and cdk inhibitors [12]. Therefore, we analyzed the expression of cyclins A1, E1 and D1, p21, p27, β-TrCp, and survivin in

ciglitazone treated cells. As shown in figure 3 (A and B) ciglitazone up regulated the expression of PPARγ, p27, and β-TrCp while the expression of cyclin D1, survivin and skp2 were down regulated. There was not much change in cyclin A1 and cyclin E1 levels in the tested dose range, however, p21 was slightly increased at 75μM. Ciglitazone mediated up regulation of PPARγ peaked at 50μM and 75μM doses. Interestingly, the upregulation of PPARγ was positively correlated with p27 and β-TrCp but showed a negative correlation with cyclin D1, survivin and skp2 levels at the peak doses.

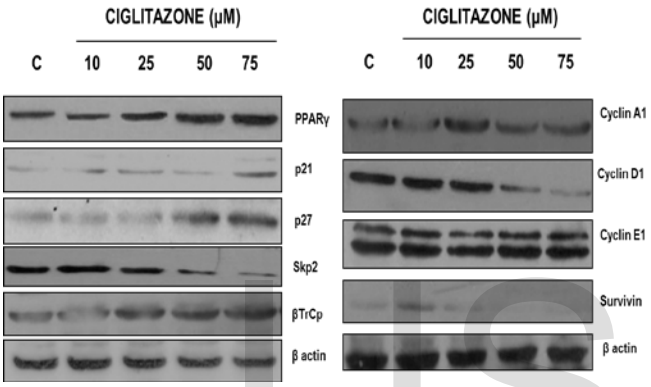

Figure 3: Dose dependent effect of ciglitazone on expression of PPARγ and cell regulatory proteins such as cyclin A, Cyclin B, Cyclin D1, p21, p27, p53, skp2, β-TrCp, and survivin. Cells were treated with the indicated concentrations of ciglitazone for 24 hr. Cell extracts were separated by SDS- PAGE, followed by western blotting. β-actin was used as a loading control.

3.4 Ciglitazone mediated skp2 down regulation is partially independent of PPARγ

Previous reports indicated a negative regulatory role for PPARγ on skp2 expression [13]. Therefore we initially believed that the reduction in skp2 levels in ciglitazone treated cells is dependent on PPARγ. Inclined to this, we observed a rise in skp2 levels in PPARγ knocked-down cells (Fig. 4A). However, ciglitazone treatment reversed the rise in skp2 in these cells indicating a PPARγ independent mechanism contributing to the ciglitazone effect on reducing the levels of skp2.

Ciglitazone increased the knock-down efficiency of skp2 siRNA in MDA MB 231 cells which further confirms the skp2 lowering effect of ciglitazone at varying context (Fig. 4B). Skp2 targets both p27 and p21 for their degradation [13-16]. Therefore we reasoned that the augmentation of p27 and p21 in ciglitazone treated cells might be resulting from the loss of skp2.

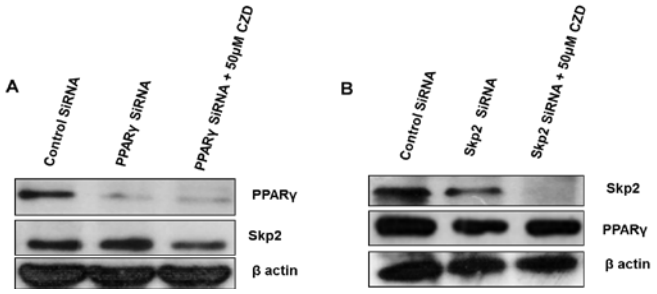

Figure 4: (A) PPARγ independent Skp2 ablation by ciglitazone after PPARγ knockdown. MDA MB 231 cells were transfected with control or PPARγ siRNA. After 12 h of transfection, the media were changed and cells were treated with vehicle or ciglitazone for 24 h. The knockdown of PPARγ was confirmed by western blotting. (B) MDA MB 231 cells were transfected with control or Skp2 siRNA. After 12 h of transfection, the media were changed and cells were treated with vehicle or ciglitazone for 24 h. The knockdown of Skp2 was confirmed by western blotting.

3.5 Expression of cell cycle regulatory proteins in breast cancer tissues

A total of 30 histopathologically confirmed ER negative breast cancer tissues were used for the immunohistochemical analysis (Table I). The staining intensity were graded 0 (no expression), 1(weak), 2 (moderate) and 3 (intense). Correlation analysis indicated an inverse correlation of PPARγ with cyclin D and skp2 in 86% of the total samples analyzed. Among them 53% samples showed a strong inverse correlation (weak to intense) between PPARγ and skp2 whereas 46% samples showed similar inverse correlation between PPARγ and cyclin D (Fig. 5). Surprisingly, 73% and 53% of the total samples showed rough inverse correlation (weak to moderate and weak to intense) between PPARγ and p27 and PPARγ and β-TrCP respectively. This observation was a deviation from

our *in vitro* results in MDA MB 231 cells where we observed positive correlation between PPARγ, β-TrCp and p27 expression at peak doses of ciglitazone. This reassures the involvement of PPARγ independent mechanisms involved with ciglitazone mediated cytotoxicity in ER negative breast cancer cells.

Table I : Expression level of PPARγ and various cell regulatory protein in Estrogen receptor negative breast cancer tissues.

	PPARγ	CyclinD1	CyclinA	Cyclin E	p53	p27	p21	Skp2	β-TrCp
No	2(6.7%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Weak	21(70%)	2(6.7%)	9(30%)	5(16.7%)	12(40%)	7(23.3%)	10(33.3%)	4(13.3%)	11(36.6%)
Moderate	4(13.3%)	18(60%)	10(33.3%)	12(40%)	8(26.7%)	17(56.7%)	15(50%)	14(46.7%)	12(40%)
Intense	3(10%)	10(33.3%)	11(36.7%)	13(43.3%)	10(33.3%)	6(20%)	5(16.7%)	12(40%)	7(23.3%)

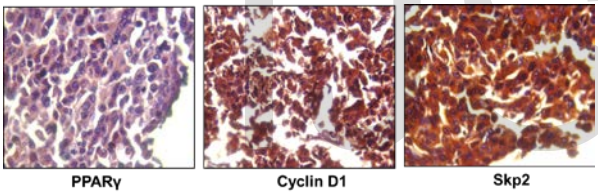

Figure 5: Representative immunostained tissue sections of estrogen receptor negative breast cancer showing PPARγ, cyclin D1 and Skp2 expressions.

4. Discussion

PPARγ gained wider attention to the cancer biologists by virtue of its projected anti-cancer properties [17-20]. However, majority of studies that assign PPARγ as a cancer therapeutic target heavily rely on the properties of its synthetic ligands [18-20]. Thiazolidinediones class of compounds is the synthetic ligands extensively studied in this direction. In-depth, molecular studies revealed that these ligands possess PPARγ independent actions as well [7-9, 20-23]. This raised the relevance of analyzing molecular effects of individual ligands in a cell specific manner. This approach could potentially reveal the biological characters of the pharmacophore associated with the specific ligands and may aid in developing novel anti-cancer compounds with more specificity and less off targeted side effects. ER activation modulates expression of hormone-responsive genes responsible for proliferation of mammary epithelial cells [24]. ER receptor antagonist such as tamoxifen thus works well as an effective anti-cancer agent [25]. During breast cancer progression, tumor cells acquire growth autonomy and may no longer require estrogen for survival. This leads to these cells becoming resistant to anti-estrogen therapy [26]. The anti-estrogen therapy resistant tumors are usually highly invasive and metastatic and respond poorly to chemotherapy and radiotherapy. Additional factors like mutation in ER, down-regulation of ER expression, dysregulation of ER negative responsive genes, and clonal selection of ER negative cells also contribute to anti-estrogen therapy resistant proliferation of breast cancer cells [27]. Since ER negative breast cancer types are comparatively less viable to drug treatment [2] we analyzed the effect of ciglitazone in ER negative breast cancer cell line MDA MB 231. We observed potent growth inhibitory effect of ciglitazone in these cells which is consistent with its effect reported in multiple other cell types [28]. Ciglitazone was also effective in primary breast cancer cells derived from ER negative breast cancer tissue indicating its potential to induce growth inhibition in clinically relevant ER negative tumors.

Ciglitazone down regulates cyclin D in breast cancer cells [6]. Hence, it was not surprising for us to see a reduction in cyclin D levels after ciglitazone

treatment. Recent studies indicate that the cyclin D lowering effect of thiazolidinediones class of compounds is strictly PPARγ independent [7-10,20,22,23,29]. However our results showed a correlation between PPARγ activation and cyclin D reduction in *in vitro* settings. Moreover, our *in vivo* tissue analysis showed an inverse correlation between PPARγ activation and cyclin D. This reinforces that PPARγ activation at least partially contributes to the cyclin D reduction in ciglitazone treated ER negative cancer cells. A previous observation indicated differential efficacy of troglitazone and ciglitazone in reducing the levels of cyclin D in ER positive MCF-7 cells [29]. This clearly indicates the diverse characteristic of synthetic ligands of PPARγ in eliciting similar response.

Upregulation of p27 and p21 by ciglitazone in MDA MB 231 cells can be partially explained by the concurrent upregulation of PPARγ. Breast cancer tissues showed positive correlation between PPARγ and p27 or p21 expression [30]. However our tissue analysis revealed inverse correlation between PPARγ and p27 in 73% of breast cancer tissues analyzed. This deviated observation might have resulted from the specific nature of ER negative tumors used in our study. Hence, the comparatively deficient correlation observed between our *in vitro* ciglitazone experiments and *in vivo* tissue analysis substantiate the involvement of PPARγ independent mechanisms also contributing to ciglitazone mediated molecular effects in MDA MB 231 cells.

Skp2 is an E3 ubiquitin ligase known to facilitate the degradation of p27 and p21 [13-16]. Thus skp2 down regulation may be the mechanism associated with ciglitazone induced p27/p21 upregulation in MDA MB 231 cells. Breast cancer tissues showed inverse correlation between PPARγ and skp2 [13]. The similar observation in our study using breast cancer specimens collected from Indian population indicates no demographic variation in this behavior. Interestingly, the efficacy of ciglitazone in reducing skp2 levels even in PPARγ knocked-down cells clearly demonstrate that the skp2 lowering effect of ciglitazone is at least partially independent of PPARγ. FoxM1 is a transcription factor known to promote transcription of skp2. Troglitazone has been shown to inhibit FoxM1 in hepatic cancer cells. This raise the possibility that FoxM1 may be involved in ciglitazone mediated skp2 down regulation in ER negative breast cancer cells and is worth further analyzing [31].

5. Conclusion

Our results add scientific evidence to the proposed potential application of ciglitazone in cancer treatment. Pooling the scattered data of ciglitazone in multiple cell lines and tissues may help dissect out further insights on its basic mechanism of action and expose additional targets and pharmacophores with potential anticancer properties.

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Conflict of Interest

The authors declare no conflict of interest.

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