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# PPARγ inhibits ovarian cancer cells proliferation through upregulation of miR-125b

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ABSTRACT

miR-125b has essential roles in coordinating tumor proliferation, angiogenesis, invasiveness, metastasis and chemotherapy recurrence. In ovarian cancer miR-125b has been shown to be downregulated and acts as a tumor suppressor by targeting proto-oncogene BCL3. PPARγ, a multiple functional transcription factor, has been reported to have anti-tumor effects through inhibition of proliferation and induction of differentiation and apoptosis by targeting the tumor related genes. However, it is unclear whether miR-125b is regulated by PPARγ in ovarian cancer. In this study, we demonstrated that the miR-125b downregulated in ovarian cancer tissues and cell lines. Ligands-activated PPARγ suppressed proliferation of ovarian cancer cells and this PPARγ-induced growth inhibition is mediated by the upregulation of miR-125b. PPARγ promoted the expression of miR-125b by directly binding to the responsive element in miR-125b gene promoter region. Thus, our results suggest that PPARγ can induce growth suppression of ovarian cancer by upregulating miR-125b which inhibition of proto-oncogene BCL3. These findings will extend our understanding of the function of PPARγ in tumorigenesis and miR-125b may be a therapeutic intervention of ovarian cancer.



## 1. Introduction

MicroRNAs (miRNAs) are short (about 20–25 nucleotides) regulatory noncoding RNAs that can post-transcriptionally regulate the expression of specific target genes and that play important roles in the control of physiological and pathological cellular processes [1,2]. A growing amount of reports have proved that miRNAs have a crucial role in human cancers as it can either act as oncogenes or tumor suppressors that control the cancer onset, growth, and progression [3]. miR-125b is deregulated in many human cancers including lung, gastric, colorectal, ovarian, breast, bladder and hepatocellular cancers [4], and plays a key role in several signaling networks such as IGF-signaling pathway, PI3K/Akt/mTOR pathway, MAPK signaling pathway [5–7]. miR-125b has been shown to be downregulated in ovarian cancer tissues, and acts as a tumor suppressor by targeting BCL3(B-cell CLL/lymphoma 3) and ERBB2/3 (Her2/Her3), which associated with tumor growth and angiogenesis [8,9]. miR-125b regulates so many target genes which related to tumor growth, invasion and metastatic, progression survival and chemotherapy recurrence [10–12], but its own regulation is not well. It has been reported that transcriptional factors CDX2 and STAT3 can modulate miR-125b expression [13,14]. However, there is no concern with nuclear receptor (NR)-mediated regulation of miR-125b.

Peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear receptor superfamily, regulates lipid metabolism, inflammation and cancer progression [15]. Synthetic PPARγ ligands are thiazolidinediones (TZDs), which consist of ciglitazone (CGZ), troglitazone (TGZ), pioglitazone (PGZ) and Rosiglitazone (RGZ). Ligand-activated PPARγ binds to response elements of target genes behaves as transcriptional transrepressors or transactivators [16–18]. PPARγ expression has been shown to be upregulated in ovarian cancer [19,20], making it a potentially important regulator in the cancer progression. PPARγ displays anti-tumor effects through inhibition of proliferation and induction of differentiation and apoptosis by targeting the tumor related genes,

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such as p63, p73, p21, Bax, caspase-3, Bcl-2, c-myc [21–24]. However, it is unclear whether PPARγ can regulate the miR-125b in ovarian cancer.

In this study, we demonstrated that miR-125b downregulated in ovarian cancer tissues and cell lines. Ligands-activated PPARγ suppressed proliferation of ovarian cancer cells and this PPARγ-induced growth inhibition is mediated by the upregulation of miR-125b. PPARγ promoted the expression of miR-125b by directly binding to the responsive element in miR-125b gene promoter region. These results suggest that miR-125b can be regulated by an important transcriptional factor PPARγ and may be a therapeutic intervention of ovarian cancer.

## 2. Materials and methods

### 2.1. Reagents

miR-125b antagomir and negative control (NC) were synthesized by Shanghai GenePharma (Shanghai, China). Ciglitazone (CGZ), Troglitazone (TGZ), and Pioglitazone (PGZ), GW9662 were purchased from Cayman Chemical (Ann Arbor, MI). All-in-One miRNA quantitative reverse transcriptase PCR Detection Kit was purchased from GeneCopoeia (Guangzhou, China). Dual luciferase assay systems were from Promega (Madison, USA). ChIP kit was from Upstate (Lake Placid, USA).

### 2.2. Human tissue samples and cell lines

20 primary serous ovarian carcinomas and 10 normal ovarian tissues were collected by surgical resection. All tissue specimens were snap-frozen in liquid nitrogen. This study was approved by our hospital Ethics Committee in Suining central Hospital.

Ovcar3, CaOv3, Skov3 cells were obtained from American Type Culture Collection. Ovcar3 cells were grown in RPMI-1640 medium with 0.01 mg/ml bovine insulin and 20% fetal bovine serum. CaOV3 cells were cultured in Dulbecco's Modified Medium (DMEM) supplemented with 10% fetal bovine serum. Skov3 cells were maintained in McCoy's 5a medium with 10% fetal bovine serum. The human immortalized ovarian epithelial cells (HIOSE) were generated by transfecting normal ovarian surface epithelial cells with the immortalizing simian virus 40 early genes [25]. HIOSE cells were grown in 199/MDCB 105 (1:1) medium supplemented with 5% fetal bovine serum.

### 2.3. Luciferase reporter vectors construction, transfection and luciferase assays

The promoter of miR-125b was amplified by PCR using CaOV3 cell-derived genomic DNA as template. The PCR product was cloned into pGL3-basic luciferase reporter vector (Promega). The mutations in two PPARγ binding sites were synthesized and cloned into the pGL3-basic by SangonBiotech (Shanghai, China). CaOV3 cells were transfected with reporter luciferase vectors and internal control luciferase vector pRL-TK using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After transfection for 24 h, cells were treated with vehicle DMSO or TGZs (5 μM) for 24 h, then, cells were harvested for luciferase assays using the Dual-Luciferase Assay kit according to the manufacturer's instruction (Promega). All transfection experiments were performed in triplicate and repeated at least three times.

### 2.4. Real-time quantitative reverse transcription PCR

The total RNA was extracted from cells using TRIzol reagent (Invitrogen), and the first-strand cDNA was synthesized by PrimeScript RT Master Mix (TaKaRa). The BCL3 mRNA expression were detected by qRT-PCR using SYBR Green qPCR Master Mix (Promega). The BCL3 primers: forward, 5′-CCATGATGTGCCCCA TGGAA-3′, and reverse, 5′-CTGCTGGAAGAGGTTGACCA -3′. β-Actin was used as an internal control with the pimers5′- GTGA AGGTGACAGCAGTCGGTT-3′ (forward) and 5′-GAAGTGGGGTGG CTT TTAGGA -3′ (reverse). The miR-125b expression was determined using All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia) according to the manufacturer's protocol. The primers for miR-125b: forward: 5′- TCCCTGAGACCCTAACTTGTGA -3′, and the reverse universal primer was supplemented in the detection kit. U6 small nuclear RNA (U6 snRNA) was used as an internal control with the pimers 5′-CGCTTCGGCAGCACATATA CTAA-3′(forward) and 5′-TATGGAACGCTTCACGAATTTGC-3′(reverse).

### 2.5. Western blot analysis

The whole cell proteins were extracted and the protein concentrations were determined by BCA assay (Beyotime, China). Proteins(50 μg) were separated with 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). After incubated with 5% fat-free dry milk in Tris-buffered saline tween-20 (TBST) for 1 h, the membranes were incubated with anti-BCL3 antibody (1:250, Santa Cruz), or GAPDH (1:000, Santa Cruz) at 4°Covernight. And then the membranes were HRP-conjugated anti-goat or anti-rabbit secondary antibodies (1:10,000; Sigma–Aldrich). Signal detection was performed using enhanced chemiluminescence detection reagents (Pierce).

### 2.6. Cell proliferation assay

Cell proliferation was determined by CellTiter 96® AQueous One Solution Reagent (Promega) according to manufacture's instructions. Briefly, $2 \times 10^3$ cells per well were seeded in 96-well plates and cultured for 24 h. Then, the cells were transfected with miR-125b antagomir or NC. After 8 h, cells were treated with DMSO or TGZs (5 μM) for 24 h, then added 20 μl of CellTiter 96® AQueous One Solution Reagent into each well of the 96-well assay. Subsequently, the cells were incubated at 37 °C for 4 h, and recorded the absorbance at 490 nm using a 96-well plate reader.

### 2.7. ChIP assays

ChIP assay was performed using ChIP Assay kit (Upstate) according to the manufacturer's instructions. Briefly, CaOv3 cells were treated with 5 μM TGZ, CGZ, or DMSO for 48 h, then cells were treated with 1% formaldehyde for 10 min at 37 °C to cross-link proteins to DNA. The chromatin was sonicated to shear DNA to an average length between 200 and 1000bp. Subsequently, the chromatin was immunoprecipitated with anti-PPARγ (Santa Cruz) and normal goat IgG (negative control) antibodies. The ChIP DNA was extracted and the purified sample was subjected to PCR amplification with primer pairs spanning the PPRE sequence in the miR-125b promoter region (−344 to −224). Sequences of the primers for PCR were 5′-TGTTTTGTTCTTAACTGCAACG-3′(forward); 5′-CCTAATTGAAATTTTGCTTCCCA-3′(reverse).

### 2.8. Statistical analysis

Data are expressed as mean ± standard deviation (SD). The statistical differences between experimental and control groups were determined by Student's t test. $P < 0.05$ was considered statistically significant.