Research Article

PPARα Enhances Cancer Cell Chemotherapy Sensitivity by Autophagy Induction

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PPARα (peroxisome-proliferator-activated receptor α) plays a critical role in regulation of inflammation and cancer, while the regulatory mechanism of PPARα on cancer cell autophagy is still unclear. Here we found that PPARα enhanced autophagy in HEK293T, SW480, and Hela cell lines, which was independent of PPARα transcription activity. PPARα induced antiapoptotic Bcl2 protein degradation resulting in release of the Beclin-1/VPS34 complex. Consistently, silenced PPARα reversed this event. PPARα-induced autophagy significantly inhibited tumor growth and enhanced SW480 cancer cell sensitivity to chemotherapy drugs. Moreover, PPARα agonist increased SW480 cancer cell chemotherapy sensitivity. These findings revealed a novel mechanism of PPARα/Bcl2/autophagy pathway suppressed tumor progression and enhanced chemotherapy sensitivity, which is a potential drug target for cancer treatment.

1. Introduction

As one of the nuclear hormone receptor family, peroxisome-proliferator-activated receptor α (PPARα) is a ligand-activated transcription factor. Ligand binding and activated PPARα heterodimerizes with RXRs (Retinoid X receptors) lead to binding peroxisome-proliferator response element (PPRE: AGGTCA N AGGTCA, N is any nucleic acid) that regulates the target gene expression, which is involved in atherosclerosis, diabetes, obesity, inflammation, and cancer [1–7]. Clinical observation shows that expression of PPARα contributes the survival of breast and ovarian cancer [8, 9]. The synthetic ligands of PPARα including fenofibrate, clofibrate, and wyeth14,643 suppress cancer cell proliferation [2, 5]. As a nuclear receptor, PPARγ induces NFκB/p65 and MUC1-C ubiquitination and degradation independent of its transcription activity [2, 3]. Similarly, PPARα serves as E3 ligase to induce Bcl2 ubiquitination and degradation leading to increased cancer cell apoptosis in response to chemotherapeutic agents [6]. As antiapoptotic protein, Bcl2 inhibits autophagy signaling by binding to Beclin-1 to inhibit Beclin-1/VPS34 complex [10]. Autophagy delivers cytoplasmic materials (proteins, lipids, etc.) or organelles (mitochondria, nucleus, etc.) into lysosomes for degradation, which is also a progress of nutrient recycling [11]. Autophagy contributes cancer cell survival during nutrient deprivation; however, cancer cells consume all of the cellar components resulting in cell death [11, 12]. Other reports show that ligand-activated PPARα increases autophagy of AML12 cells or livers via PPARα-mediated autophagy-associated gene expressions [13], while here we found that PPARα induced cancer cell autophagy independent of its transcription activity by release of Beclin-1/VPS34 complex.

2. Results

2.1. PPARα Induces Autophagy Independent of Its Transcription Activity. Western blot analysis showed that PPARα shRNA silence significantly decreased the LC3-II levels in SW480, Hela, and HEK293T cell lines (Figure 1(a)). Transfected GFP-LC3 plasmid in SW480 cells showed that PPARα silence decreased autophagosome formation and GFP-LC3 puncta (Figure 1(b)), which was consistent with the transmission electron microscopy analysis (Figure 1(c)).
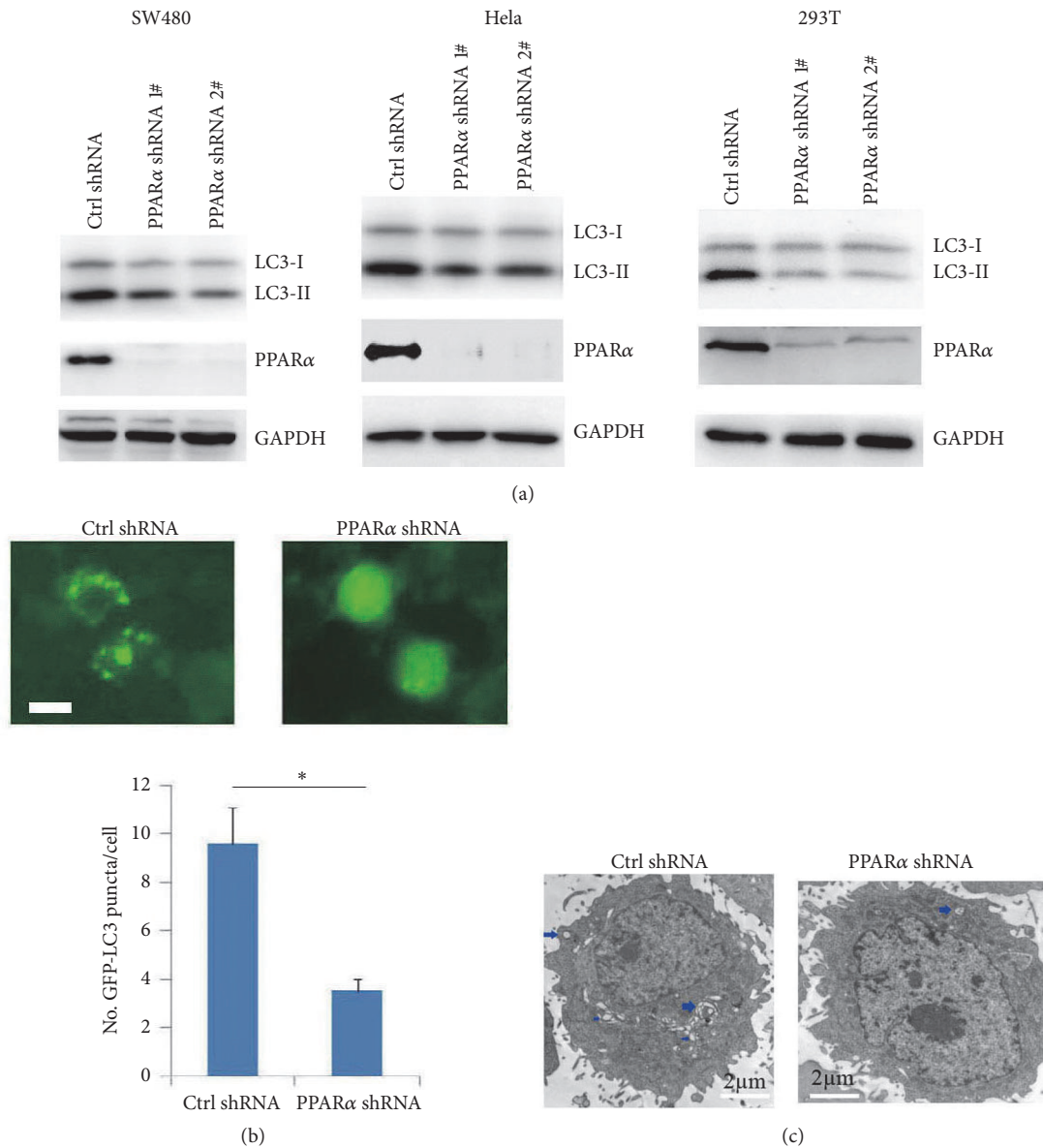
FIGURE 1: **Silenced PPARα inhibits autophagy.** (a) PPARα shRNA silenced SW480, Hela and 293T cell lysates were subjected to Western blot. (b) Representative images of GFP-LC3 puncta (autophagosomes) in PPARα silenced SW480 cells. Scar bar: 20 μm. The GFP-puncta was quantified. Results are expressed as means ± SEM (n=5). ∗$P<0.05$. (c) TEM analysis of PPARα shRNA silenced SW480 cells. Arrows show the autophagosomes.

Ligand-activated PPARα promotes autophagy of AML12 cells or livers by inducing autophagy-associated gene expressions (LC3a, LC3b, etc.) [13]. To further detect whether PPARα-mediated autophagy was involved in autophagy-associated gene expressions, qPCR analysis was performed. The results showed that PPARα had no significant effect on autophagy-associated gene expressions (SFigure. 1). In contrast, overexpression of PPARα in SW480 cells increased the LC3-II levels (Figure 2(a)) and GFP-LC3 puncta (Figure 2(b)), which had no effect on autophagy-associated gene expressions (SFigure. 2), suggesting that PPARα promoted cancer cell autophagy independent of its transcription activity. Our previous results show that PPARα induces the antiapoptotic Bcl2 protein

ubiquitination and degradation [6]. Further analysis showed that PPARα induced Bcl2 degradation, while it had no effect on the activation of caspase-3 in SW480 cells (SFigure. 3), suggesting that PPARα-induced Bcl2 degradation had no effect on cancer cell apoptosis.

2.2. PPARα-Mediated Bcl2 Degradation Increases Beclin-1/VPS34 Complex.

Our results demonstrated that PPARα induced cancer cell autophagy without effect on autophagy-associated gene expressions. To further detect PPARα induced cancer cell autophagy independent of its transcription activity, the PPARα nuclear location signal (NLS) was deleted and overexpressed in SW480 cells. The results
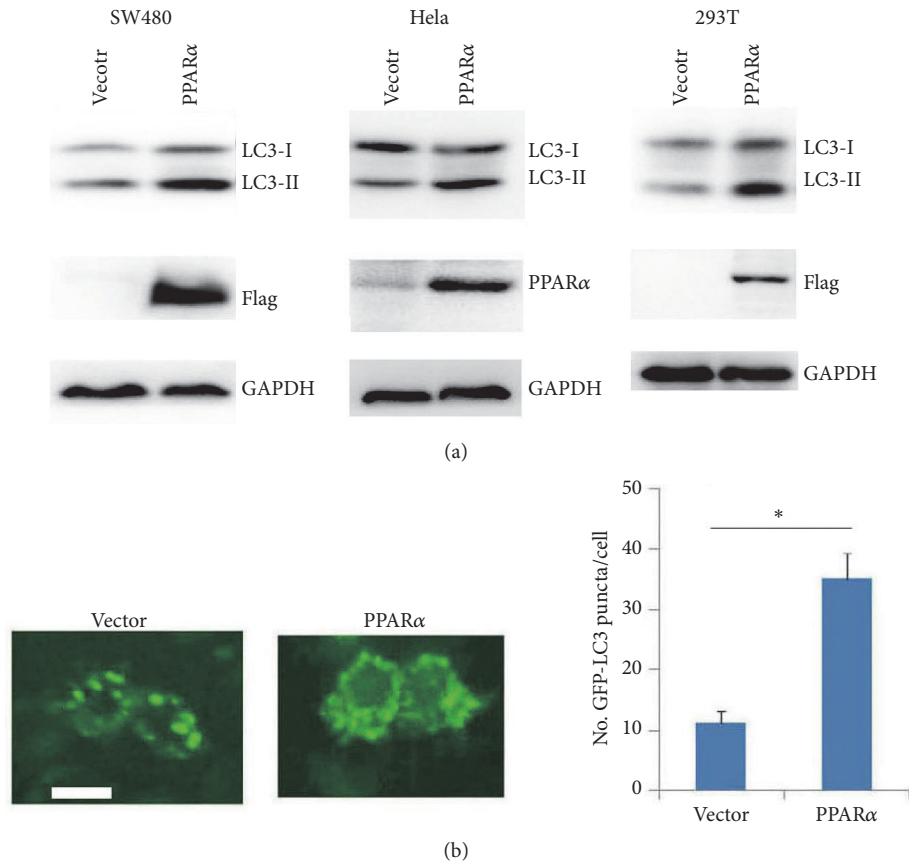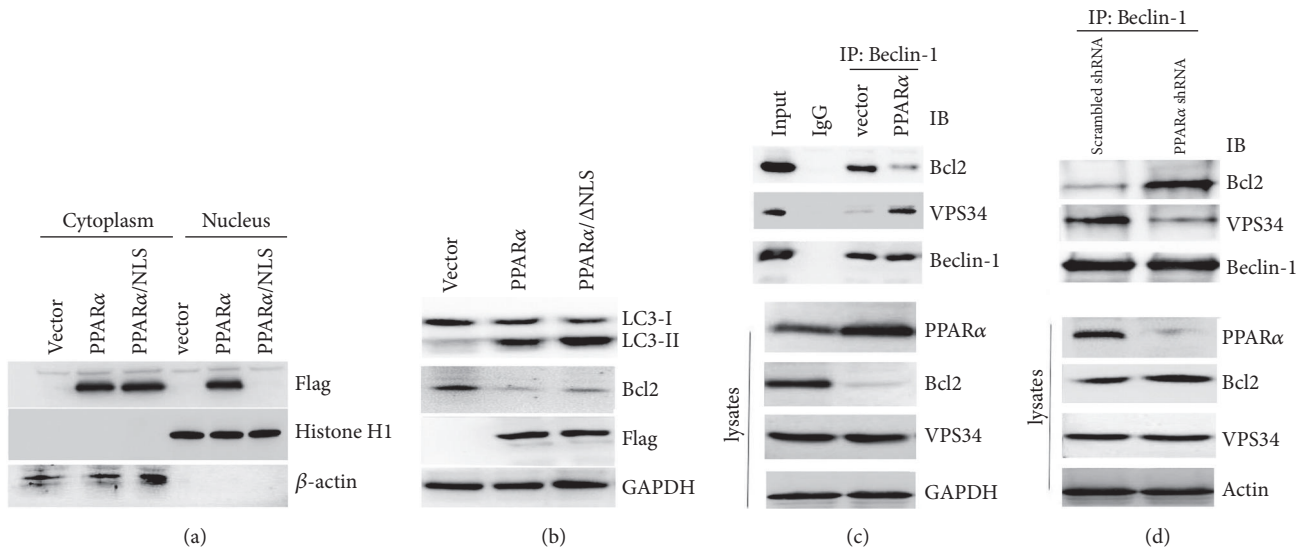
(a)

(b)

FIGURE 2: **Overexpression of PPARα enhances autophagy**. (a) SW480 cells were transfected with vector or Flag-PPARα plasmids for 36 h. Cell lysates were subjected to Western blot. (b) representative images of GFP-LC3 puncta (autophagosomes) in overexpression of PPARα in SW480 cells. Scar bar: 20 μm. The GFP-puncta was quantified. Results are expressed as means ± SEM (n=5). ∗$P<0.05$.

showed that PPARα/ΔNLS did not locate into nucleus by Western blot analysis (Figure 3(a)), while PPARα/ΔNLS still induced autophagy (Figure 3(b)). These findings further demonstrated that PPARα-mediated autophagy was independent of transcription activity, as Bcl2 interacts with Beclin-1 leading to disruption of Beclin-1/VPS34 complex and autophagy suppression [14]. Our previous finding shows that PPARα acts as E3 ubiquitin ligase to induce Bcl2 ubiquitination and degradation [6]. Consistent with this, cytoplasmic PPARα reduced Bcl2 protein levels corresponding to the increase in LC3-II levels (Figure 3(b)). To further detect whether Bcl2 degradation by PPARα led to the increase in Beclin-1/VPS34 complex, immunoprecipitation analysis was performed. The results showed that overexpression of PPARα increased the Beclin-1/VPS34 complex associated with reduction of Bcl2 protein levels (Figure 3(c)). In contrast, PPARα shRNA silence reversed this event (Figure 3(d)), suggesting that PPARα-mediated Bcl2 degradation increased the Beclin-1/VPS34 complex resulting in autophagy induction.

2.3. PPARα/Autophagy Signaling Suppresses Tumor Progression. To detect the effect of PPARα-mediated autophagy on the tumor progression, xenograft tumor model was performed. The results showed that PPARα shRNA silence promoted tumor growth (Figure 4(a)) and increased tumor weight (Figure 4(b)). Western blot analysis by using tumor lysates showed that silenced PPARα reduced LC3-II levels and increased Bcl2 protein levels (Figure 4(c)). These findings showed that PPARα-mediated autophagy suppressed tumor progression, which was involved in reduced Bcl2 protein levels.

2.4. PPARα Agonist Enhances Autophagy-Mediated Tumor Suppression. SW480 cells were treated with PPARα agonist (clofibrate); the results showed that clofibrate significantly increased autophagosome accumulation (Figure 5(a)). To further detect whether agonist-induced autophagy was PPARα dependent, the PPARα shRNA silenced SW480 cells were treated with clofibrate. The results showed that silenced PPARα had no significant effect on LC3-II levels in response to clofibrate (Figure 5(b)), suggesting that clofibrate induced autophagy in a PPARα-dependent manner. Further analysis showed that clofibrate reduced Bcl2 protein levels (Figure 5(c)). Moreover, agonist of PPARα did not affect the autophagy-associated gene expressions (SFigure.

FIGURE 3: **PPARα enhances Beclin-1/VPS34 complex formation.** (a) Extracts of cytoplasm and nucleus were subjected to Western blot by using overexpression of Flag-PPARα or mutant plasmids in SW480 cells. (b) SW480 cells were transfected plasmids as indicated for 36 h. Cell lysates were subjected to Western blot. (c) SW480 cells were transfected with vector or Flag-PPARα plasmids for 36 h. Cell lysates were subjected to immunoprecipitation and Western blot. (d) PPARα shRNA silenced SW480 cell lysates were subjected to immunoprecipitation and Western blot.
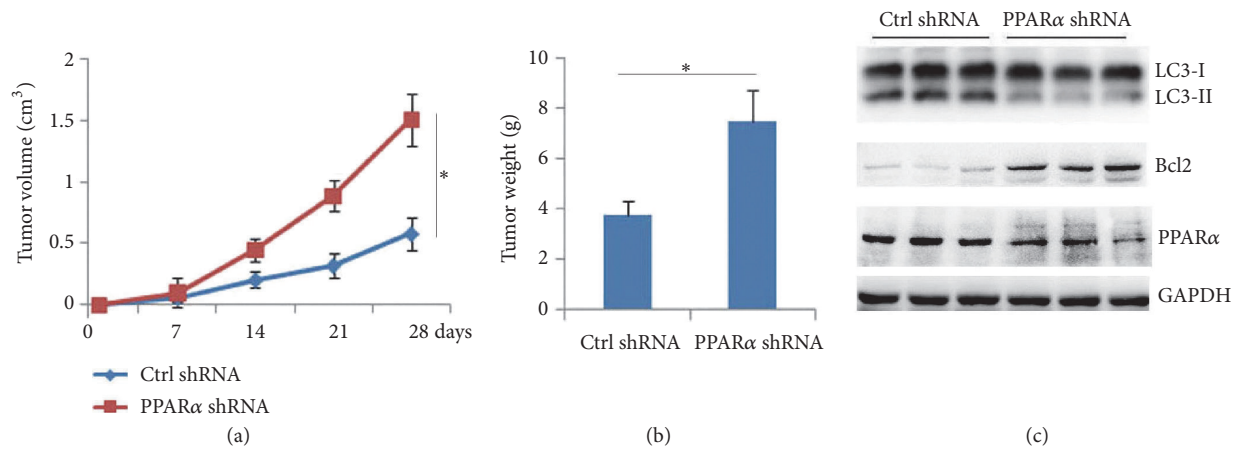

FIGURE 4: **PPARα/autophagy signaling inhibits tumor progression.** Stabling expression of control or Flag-PPARα shRNA SW480 cells were injected subcutaneously in nude mice for four weeks, and tumor volume (a) and tumor weight (b) were measured. Results are expressed as means ± SEM (n=5). *$P<0.05$. (c) Tumor lysates were subjected to Western blot.
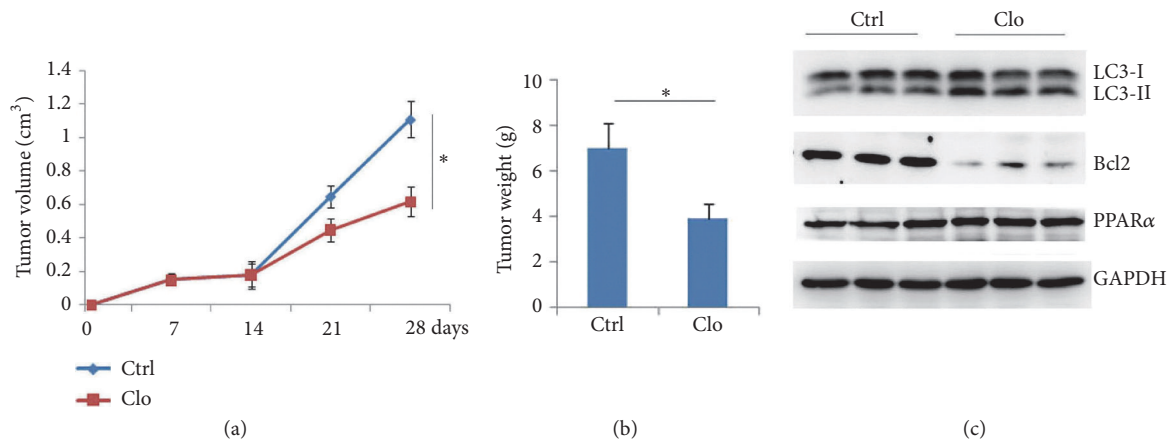
4). Xenograft mice model assay showed that PPARα agonist clofibrate significantly inhibited tumor growth (Figure 6(a)) and tumor weight (Figure 6(b)). The *in vivo* tumor tissues further demonstrated that agonist clofibrate reduced Bcl2 protein levels and increased LC3-II levels (Figure 6(c)). These findings suggest that agonist enhanced autophagy-mediated tumor suppression in a PPARα-dependent manner, which was not involved in autophagy-associated gene expressions.

2.5. PPARα/Autophagy Signaling Increased Chemotherapy Sensitivity to Cancer Cells. To further detect the interaction of autophagy with chemotherapy drugs, SW480 cells were treated with chemotherapy drugs (camptothecin, taxol,

etoposide, and cisplatin), the results showed that although these drugs increased LC3-II levels, overexpression of PPARα significantly enhanced this event (Figure 7(a)). In contrast, PPARα silenced cells inhibited chemotherapy drugs-induced autophagy (Figure 7(b)), suggesting that chemotherapy drugs induced autophagy in a PPARα-dependent manner. The above data demonstrated that the ligand induced autophagy in a PPARα-dependent manner. Consistent with this, clofibrate enhanced cisplatin-induced autophagy, which was terminated in PPARα silenced SW480 cells (Figure 7(c)). These findings suggest that the agonist enhanced chemotherapy drugs-induced autophagy in a PPARα-dependent manner. *In vivo* xenograft mice model assay showed that clofibrate
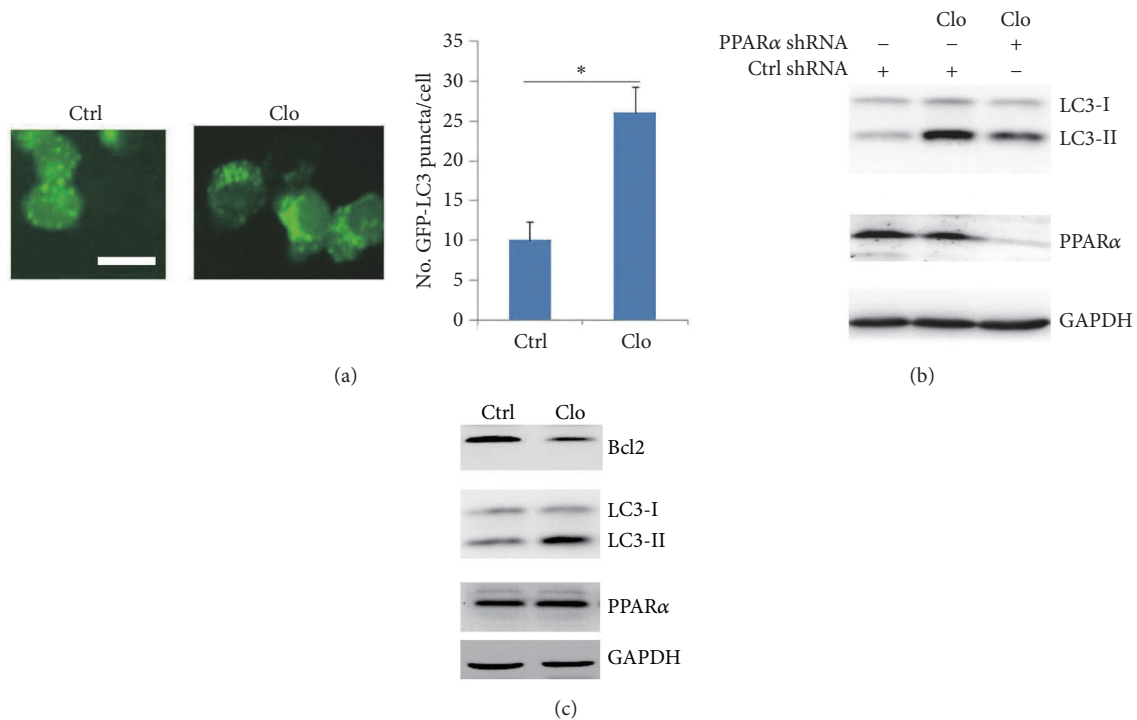
FIGURE 5: **Agonist enhances autophagy in a PPARα-dependent manner.** (a) Representative images of GFP-LC3 puncta (autophagosomes) in 10μM Clofibrate treated SW480 cells for 12 hours. Scar bar: 20 μm. The GFP-puncta was quantified. Results are expressed as means ± SEM (n=5). ∗$P<0.05$. (b) SW480 cells were transfected with ctrl shRNA or PPARα shRNA. After 36 h, cells were treated with 10μM clofibrate for 12 hours. Cell lysates were subjected to Western blot. (c) SW480 cells were treated with 10μM clofibrate for 12 hours. Cell lysates were subjected to Western blot. Data are triplicates from three independent experiments.


FIGURE 6: **Agonist of PPARα inhibits tumor growth.** SW480 cells were injected subcutaneously in nude mice. After two weeks, mice were treated without or with Clofibrate (20mg/kg/day) for another two weeks by intraperitoneal injection. Tumor volume (a) and weight (b) were measured. Results are expressed as means ± SEM (n=5). ∗$P<0.05$. (c) Tumor lysates were subjected to Western blot.

together with cisplatin significantly inhibited tumor growth (Figure 7(d)) and reduced tumor weight (Figure 7(e)). The *in vivo* tumor tissues further demonstrated that agonist clofibrate/cisplatin significantly increased LC3-II levels (Figure 7(f)). These findings suggest that PPARα/Bcl2/autophagy signaling increased chemotherapy sensitivity to cancer cells (Figure 7(g)).

3. Discussion

Autophagy is a conserved biochemical catabolic process that delivers cytoplasmic materials or organelles into lysosomes for degradation, which is also a progress of nutrient recycling [11]. Autophagy plays an important role in metabolic adaptation in cancer cell survival by digesting intracellular
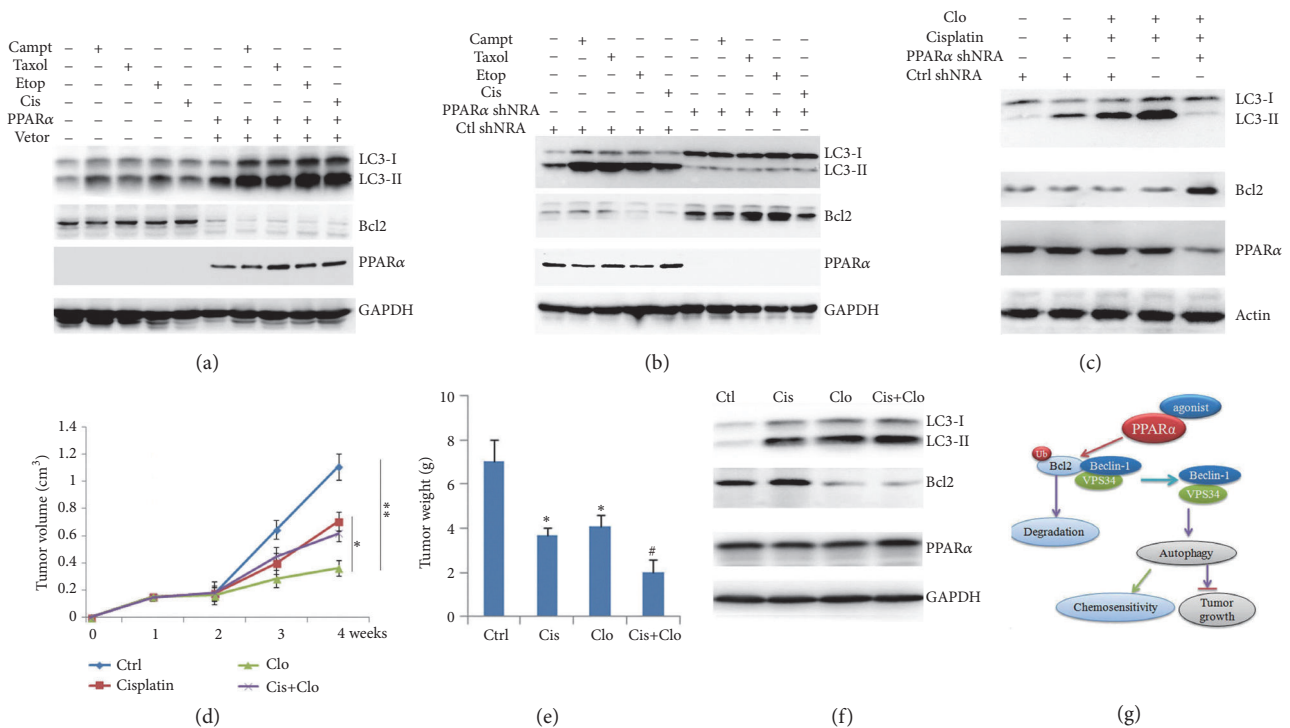
FIGURE 7: **PPARα/autophagy enhances chemotherapy sensitivity to cancer cells.** (a) SW480 cells were transfected with vector or PPARα plasmids for 36 h. After that, cells were treated with camptothecin (60μM), taxol (300μM), etoposide (600μM), and cisplatinum (30μM) for 6 h. Cell lysates were subjected to Western blot. (b) SW480 cells were transfected with ctrl shRNA or PPARα shRNA plasmids for 36 h. After that, cells were treated with camptothecin (60μM), taxol (300μM), etoposide (600μM), and cisplatinum (30μM) for 6 h. Cell lysates were subjected to Western blot. (c) SW480 cells were transfected with ctrl shRNA or PPARα shRNA plasmids for 36 h. After that, cells were treated 100μM cisplatin or 100μM cisplatin +10μm clofibrate for 12 h. Cell lysates were subjected to Western blot. SW480 cells were injected subcutaneously in nude mice. After two weeks, mice were treated without or with cisplatin (3mg/kg), Clo (20mg/kg/day), or Clo (20mg/kg/day) +cisplatin (3mg/kg) for another two weeks by intraperitoneal injection. Tumor volume (d) or weight (e) was measured. Results are expressed as means ± SEM (n=5). ∗P<0.05, ∗∗P<0.01. (f) Tumor lysates were subjected to Western blot. (g) The model of PPARα/Bcl2/autophagy signaling inhibits tumor growth and chemotherapy sensitivity. Data are triplicates from three independent experiments.

proteins and organelles in response to nutrient deprivation [11, 12]. Although autophagy increases cell survival under starvation stress, long-term autophagy without new nutrients replenishment leading to consumption of all available substrates and die (autophagy-associated cell death) [11, 12]. Therefore, autophagy is type II programmed cell death [12]. As a nuclear transcription factor, PPARα plays an important role in regulating gene transcription. Other report shows that ligand-activated PPARα increased autophagy of AML12 cells or livers via PPARα-mediated autophagy-associated gene expressions such as LC3a and LC3b in liver cells or liver tissues [13]. However, the effect of PPARα on cancer cell autophagy is still unclear. Here we found that PPARα significantly induced cancer cell autophagy, while it was independent of its transcription activity. Similarly, the agonist enhanced cancer cell autophagy in a PPARα-dependent manner, which was also independent of PPARα transcription activity. These findings revealed a new mechanism of PPARα-mediated cancer cell autophagy. As an antiapoptotic protein, Bcl2 interacts with Beclin-1 leading to disruption of Beclin-1/VPS34 complex and autophagy suppression [14]. As a proto-oncogene, Bcl2 inhibits cell apoptosis in the cancer

development that is widely expressed in various malignancies such as lung, breast, prostate, and colorectal cancer [15], which plays a critical role in maintenance of normal tissues homeostasis and uncontrolled cell proliferation [12, 16]. Our previous findings show that PPARα was independent of its transcriptional activity to induce Bcl2 ubiquitination and degradation [6]; here we found that PPARα reduced cytoplasmic Bcl2 protein levels and increased LC3-II levels. Further analysis showed that PPARα-mediated Bcl2 degradation led to increasing the Beclin-1/VPS34 complex formation, which promoted autophagy progression [14]. We further detected the relationship of PPARα/Bcl2/autophagy signaling on tumor progression. Agonist (clofibrate) significantly decreased Bcl2 protein levels and increased autophagy and inhibition of tumor progression in a PPARα-dependent manner, which suggests that PPARα could be a potential drug target for cancer treatment. More importantly, chemotherapy drugs (camptothecin, taxol, etoposide, and cisplatin) were PPARα dependent-induced autophagy formation; similar results observed that agonist/PPARα/cisplatin signaling enhanced autophagy and subsequently promoted cancer cell chemotherapy sensitivity and tumor suppression.

Taken together, PPARα/Bcl2/autophagy signaling promoted autophagy and enhanced tumor suppression and chemotherapy sensitivity to cancer cells (Figure 7(g)).

4. Materials and Methods

4.1. Cell Culture, Reagents. The human embryonic kidney cell line HEK393T (ATCC® CRL-11268™), human colon cancer cell line SW480 (ATCC® CCL-228™), and human cervix cancer cell line Hela (ATCC® CCL-2™) were purchased from ATCC. These cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco). Clofibrate was purchased from Toronto Research Chemical Inc. Taxol was purchansed from Ruibio. Cisplatin was purchased from Tokoyo Chemical industry. Etoposide and camptothecin were purchased from Hefei Bomei Biotechnology of China. Puromycin was purchased from Life Technologies.

4.2. Plasmids. Human PPARα plasmid was described previously [7], which was mutated by the site-directed mutagenesis method. Plasmids were transfected by turboFect transfection reagent according to the manufacturer's instructions (Thermo Scientific). PPARα shRNA plasmid was described previously [7].

4.3. Western Blot and Antibodies. LC3b antibody was purchased from Novus Biologicals. Actin, GAPDH, Bcl2, and PPARα were purchased from Sangon Botech (Shanghai, China). Secondary antibodies were purchased from Jackson Immunoresearch. Western blot method was described previously [7, 17]. Data are triplicates from three independent experiments.

4.4. Quantitative Real Time PCR. Total RNA from SW480 cells was extracted by RNeasy kit (Sangon Biotech). The mRNA expressing levels were determined by Real-Time PCR analysis kit (Takara). The expression levels of relative mRNA were normalized against β-actin. Fold change over control was assayed by using the ΔCt method.

4.5. Transmission Electron Microscopy (TEM). WT or PPARα shRNA silenced SW480 cells were fixed in 2.5% glutaraldehyde for overnight at 4°C. Subsequently, samples were treated with 1% osmium tetroxide, embedded in resin. And then, the samples were cut into 70 nm sections for TEM analysis (Analytical and Testing Center of Nanjing Medical University, NJMU).

4.6. Xenograft Mice Model. The xenograft tumor model was described previously [17]. NU/NU nude mice (eight weeks, female) were obtained from SLAC Laboratory Animal Cooperation (Shanghai, China). The stable PPARα silenced SW480 cells were selected by puromycin. SW480 cells ($1x10^6$) were injected subcutaneously in the nude mice. Tumor volume was measured every week by using a digital caliper during four weeks. In addition, SW480 cells ($1x10^6$) were injected subcutaneously in nude mice. After two weeks, mice were treated without or with Clo (20mg/kg/day) or Clo+cisplatin (3mg/kg) for another two weeks by intraperitoneal injection. Tumor volume = $1/2(length \times width^2)$. All studies were carried out with the approval of the Jiangsu University Animal Care Committee.

4.7. Statistical Analysis. Data are expressed as the mean ± SEM. Statistical comparison was carried out with student's t test or one way analysis of variance (ANOVA) and Dunnett's test as appropriate.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Mengli You, Jiaming Gao, and Jianhua Jin equally contributed to this work.

Acknowledgments

Supplementary Materials

SFigure.1. Silenced PPARα had no effect on the autophagy-associated gene expressions. SW480 cells were transfected control shRNA or PPARα shRNA for 36 h. mRNA was extracted for qPCR analysis. Results are expressed as means ± SEM (n=4). SFigure.2. Overexpression of PPARα had no effect on the autophagy-associated gene expressions. SW480 cells were transfected with vector or PPARα plasmid for 36 h. mRNA was extracted for qPCR analysis. Results are expressed as means ± SEM (n=4). SFigure. 3. PPARα-induced Bcl2 degradation had no effect on apoptosis. SW480 cells were transfected with control vector or Flag-PPARα for 36 h. One group was treated with MG132 (20μM) for 6 h before cell lysis. The other group was treated with cisplatinum (30μM) for 6 h before cell lysis. Cell lysates were subjected to Western blot. SFigure.4. Clo had no effect on the autophagy-associated gene expressions. SW480 cells were treated with or without Clo for 12 h. mRNA was extracted for qPCR analysis. Results are expressed as means ± SEM (n=4). *(Supplementary Materials)*

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