Alleviation of Toxicity Caused by Overactivation of Pparα through Pparα-Inducible miR-181a2

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INTRODUCTION

Widely varied compounds, including certain plasticizers, hypolipidemic drugs (e.g., fenofibrate, clofibrate, WY-14643, and clofibrate), agrochemicals, and environmental pollutants, are peroxisome proliferators (PPs). Appropriate dose of PPs causes a moderate increase in the number and size of peroxisomes and the expression of genes encoding peroxisomal lipid-metabolizing enzymes. However, high-dose PPs cause varied harmful effects. Chronic administration of PPs to mice and rats results in hepatomegaly and ultimately carcinogenesis. Nuclear receptor protein peroxisome proliferator-activated receptor-α (Pparα) was shown to be required for this process. However, biological adaptations to minimize this risk are poorly understood. In this study, we found that miR-181a2 expression was induced by the Pparα agonist WY-14643. Moreover, exogenous expression of miR-181a-5p dramatically alleviated the cell toxicity caused by overactivation of Pparα. Further studies showed that miR-181a-5p directly targeted the Pparα 3’ untranslated region and depressed the Pparα protein level. This study identified a feedback loop between miR-181a-5p and Pparα, which allows biological systems to approach a balance when Pparα is overactivated.

Pparα is a nuclear receptor (NR) protein encoded by the Pparα gene. NRs are ligand-inducible transcription factors that alter transcription rates, as well as the subsequent expression levels of their target genes by contacting their promoter or enhancer sequences at specific recognition sites. Pparα is activated by PPs, and in response to PPs activation, Pparα heterodimerizes with retinoid X receptor α (Rxrα). Additionally, after the recruitment of co-activators, Pparα binds to the peroxisome proliferator response element (PPRE) in the promoter or enhancer region of those downstream target genes.

miRNAs are genomically encoded small non-coding RNAs that regulate the flow of genetic information through base pairing between the miRNA seed sequence (5’ nucleotides 2–8) and 3’ untranslated regions (3’ UTRs) of mRNAs, causing mRNA degradation, translation inhibition, or both. The human miR-181 family constitutes four members (miR-181a, miR-181b, miR-181c, and miR-181d). They are encoded by three different transcripts located on three different chromosomes. MiR-181a and miR-181b are clustered together on two genomic locations: the human miR-181-a1 and miR-181-b1 cluster being located on chromosome 1, and the miR-181a2 and miR-181b2 cluster being located on chromosome 9. The miR-181c and

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miR-181d cluster is located on chromosome 19. Recently, the transcriptional level regulation of the miR-181 family has received extensive concerns. Chemotherapeutic drug doxorubicin treatment significantly increased the expression of miR-181a1 in breast cancer cells, which enhanced breast cancer cell survival by targeting pro-apoptosis gene Bax. Induction of miR-181a upon cisplatin treatment was shown to enhance apoptosis in non-small-cell lung cancer A549 cells. Our work revealed that miR-181a2 expression was induced by high-dose Ppara agonist WY-14643.

In this study, we analyzed and evaluated the relationship among the overactivation Ppara, miR-181a-5p expression, and cytotoxicity. Our data showed that Ppara agonists induced miR-181a-5p transcription and that miR-181a-5p repressed Ppara expression through partial base pairing with its 3' UTR, forming a feedback loop in the regulation of high-dose WY-14643-induced cytotoxicity, and miR-181a-5p reduced the occurrence of high-dose WY-14643-induced cytotoxicity. The results provide clues regarding how the body maintains a balance after exposure to environmental PP toxins. Additionally, the toxicity and side effects of WY-14643 used for the treatment of metabolic disorders might be alleviated through inducing miR-181a-5p expression.

RESULTS

Ppara Agonists Induce miR-181a2 Expression

To determine whether Ppara regulates miR-181a2 gene expression, HepG2 cells were treated with high-dose Ppara-specific agonist WY-14643, followed by quantitative real-time PCR analysis of Ppara, Pri-miR-181a2, Pre-miR-181a2, and mature miR-181a-5p expression. As positive controls, miRNA levels of the well-characterized Ppara target genes carnitine palmitoyltransferase 1A (Cpt1a) and microtubule-associated protein 1 light chain 3 alpha (Lc3a) were also measured. No significant difference in Ppara expression was observed between WY-14643 treatment group and vehicle control group (Figure S1A). The significant upregulations of Cpt1a (2.3-fold) and Lc3a (1.7-fold) expression demonstrated that Ppara was activated. After Ppara activation, mature miR-181a-5p (2-fold) expression was induced (Figure 1C). In addition to mature miR-181a-5p, we also detected the significant induction of Pri-miR-181a2 (2.2-fold) and Pre-miR-181a2 (2-fold) in response to WY-14643 treatment in HepG2 cells (Figures 1D and 1E). These data suggest that WY-14643 treatment induces miR-181a2 upregulation at the transcriptional level. Similar rises of Pri-miR-181b2 and Pre-miR-181b2 were observed in WY-14643-treated HepG2 (Figures S1B and S1C). However, the qPCR assay result of miR-181a2 host gene (miR-181a2hg) showed that Ppara activation had no effect on miR-181a2hg (Figure S1D). It suggests that miR-181a2 and miR-181b2 may be independent transcripts from miR-181a2hg. 18sRNA expression was normalized to β-actin to confirm that template amounts are equivalent from control group and WY-14643-treated group (Figure S1E). Treatment with another synthetic highly specific Ppara agonist, GW7647, led to a 2.2-fold induction of Cpt1a mRNA (Figure 1G) and a 1.5-fold induction of mature miR-181a-5p expression (Figure 1F). These data further indicate that Ppara activation induced miR-181a-5p expression.

Ppara Enhances the Transcriptional Activity of the Hsa-miR-181a2 Promoter

To clarify whether Ppara regulates hsa-miR-181a2 at transcriptional level, luciferase reporter assays were performed. The putative PPRE in hsa-miR-181a2 promoter region was predicted using an online algorithm (NUBIScan, http://www.nubiscan.unibas.ch/) (Figure 2A). Based on the finding of the prediction, luciferase reporters (pGL3-DR3-WT) containing PPRE(DR3) or mutated DR3 were constructed and separately transfected into HepG2 cells with the Renilla luciferase expression vector pRL-TK for luciferase assays. As shown in Figure 2B, the luciferase activity of WY-14643-treated pGL3-DR3-WT group was much higher than vehicle-treated pGL3-DR3-WT group, suggesting that this region may harbor a Ppara regulatory element. The mutation of DR3 within the construct pGL3-DR3 abolished the WY-14643-induced luciferase activity, indicating that hsa-miR-181a2 may be directly regulated by Ppara activation that converges on the DR3 acting as an enhancer within Pre-miR-181a2.

The direct interaction of Ppara with the DR3 element was further substantiated by electrophoretic mobility shift assay (EMSA). The sequences of DR3 and Mut DR3 probes are shown in Figure 2C. EMSA revealed that the interaction of labeled DR3 probe with the nuclear extracts of HepG2 cells yielded a DNA/protein shift band of the expected mobility. This binding was specific because it was competitively inhibited by the addition of excess unlabeled (cold) DR3 probe, but not cold Mut DR3 probe (Figure 2D). To further verify that Ppara binds to the enhancer DR3, we performed ChIP assays on soluble formaldehyde-cross-linked chromatin isolated from WY-14643-treated HepG2 with a monoclonal anti-Ppara antibody. As shown in Figure 2E, the anti-Ppara antibody precipitated the DNA fragment containing the DR3 element. Together, these results demonstrate that miR-181a2 is upregulated by Ppara activation through binding to the DR3 element.

Ppara Is a Direct Target of miR-181a-5p

Bioinformatic analysis was performed to search potential target genes of miR-181a-5p. We selected potential target genes from Targetscan (http://www.targetscan.org), miRanda (http://www.microrna.org), and PicTar (http://pic.tar.mdc-berlin.de/). Interestingly, among the hundreds of predicted targets, Ppara is a potential miR-181a-5p target. Figure 3A displays the predicted miR-181a-5p-binding sites in the 3' UTR of Ppara. These results were further validated in 293A cells using luciferase reporter assay. The target sequence of Ppara 3' UTR (Figure 3B, WT) or the site-mutated sequence (Figure 3B, Mut) was cloned into the luciferase reporter vector PsCHECK2. Next, the wild-type (WT) or mutant (Mut) vector was co-transfected with miR-181a-5p mimic or negative control mimic into 293A cells. For the Ppara WT vector, significant repression of luciferase activity was induced by co-transfection with miR-181a-5p mimic compared with the negative control mimic co-transfection; however, for the Ppara Mut vector, the repression of luciferase activity was abolished (Figure 3C).
Using western analyses, we found that Ppara protein expression levels were significantly reduced by miR-181a-5p transfection in hepatic cell line LO2, human liver stellate cell line LX2, hepatocellular carcinoma cell lines Huh7, and HepG2 cells (Figure 3D). Exogenous Ppara expression vector was used to verify the specificity of Ppara antibody (the far left two bands) in HepG2 cells (Figure 3D). Altogether, these data indicate that Ppara is a critical functional target of miR-181a-5p.

Overactivation of Ppara Causes Cytotoxicity
Given that long-term administration of WY-14643 to mice and rats causes the carcinogenesis,6 we then analyzed the cytotoxicity caused by high-dose WY-14643 treatment in vitro cell lines. The western blotting results displayed that the expression of Prkcd (known miR-181a target) was significantly decreased by induced miR-181a2 (Figure 4A); however, the expression of Ppara had not been induced by high-dose WY-14643, which is different to previously reported moderate dose WY-14643 treatment.12,30 The reason for the comparable Ppara expression is that maybe it is suppressed by elevated miR-181a-5p, and pre-miR-181a2, while U6 snRNA was used as an internal control for the detection of mature miR-181a-5p. Data are presented as means ± SDs. *p < 0.05, **p < 0.01, determined by ANOVA.
However, the inflammatory factor Il6 expression had not been affected by high-dose WY-14643 exposure, which may be the pathways that regulates Il6 is different from that regulates Il1α and CyclinD1 (data not shown).

To examine the effects of high-dose WY-14643 on DNA damage, the phosphorylated form of histone H2a.x Ser 139 (γ-H2a.x), as a specific biomarker of DNA double-strand breaks (DSBs), was detected by immunoblot assay. High-dose WY-14643 treatment caused a clear increased DSBs, as indicated by increased γ-H2a.x in WY-14643-treated HepG2 cells relative to vehicle control, accompanied with the increased DSBs, the central homologous recombination repair protein, Rad51 expression was significantly induced (Figure 4D).

TUNEL assay was performed to assess the effects of high-dose WY-14643 on cell apoptosis. As shown in Figure 4E, the number of apoptotic HepG2 cells was significantly increased after overdose WY-14643 treatment. These results of ROS, DNA damage, inflammatory factor expression, and apoptosis directly demonstrate that Ppara overactivation causes cytotoxicity.
Exogenous Expression miR-181a-5p Attenuates the Cytotoxicity Caused by Ppara Overactivation

Since Ppara overactivation causes varied cytotoxic effects and miR-181a-5p can suppress Ppara at a post-transcriptional level, we then detected whether exogenous expression miR-181a-5p could alleviate the cytotoxicity caused by Ppara overactivation or not.

HepG2 cells or LO2 cells were transfected with miR-181a-5p mimic or control mimic for 6 hr and then were treated with high concentrations of WY-14643 for 48 hr; the protein expression of miR-181a-5p targets Ppara and Prkcd was analyzed by western blotting (Figure 5A). As illustrated in Figure 5A, Ppara and Prkcd were significantly reduced in miR-181a-5p mimic transfection group compared to the control. Also, as indicated in Figure 5B, exogenous expression of miR-181a-5p reduced the ROS level caused by overactivated Ppara. Next, we detected if exogenous expression of miR-181a-5p could affect inflammatory factors Il1a and Cyclind1 expression. HepG2 cells were transfected with miR-181a-5p mimic or control mimic for 6 hr and then were treated with high concentrations of WY-14643 for 48 hr. Quantitative real-time PCR analysis showed that Cyclind1

Figure 3. miR-181a-5p Target Gene Prediction, Verification, and the Regulation of miR-181a-5p on Ppara

(A) The seed sequence base pairing between miR-181a-5p and the 3′ UTRs of Ppara was predicted by TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/) and PicTar (http://pictar.mdc-berlin.de/). (B) Sketch of the construction of wild-type (WT) or mutant (Mut) Ppara 3′UTR vectors, where the mutant binding sequences are italicized. (C) Relative luciferase activity assays of luciferase reporters with wild-type or mutant Ppara 3′ UTR were performed after co-transfection with the miR-181a-5p (miR181a) mimic or control (Con) mimic. PsiCHECK 2-3′ UTR reporter plasmid in which the luciferase coding sequence had been fused to the 3′ UTR of Ppara was co-transfected into 293A cells with the control mimic or miR-181a-5p mimic. Renilla luciferase activity was normalized to that of Firefly luciferase. The 3′ UTR-Mut indicates the introduction of alterations into the seed complementary sites shown in (B). (D) LO2, LX2, Huh7, or HepG2 cells were transiently transfected with control mimic or miR-181a-5p mimic in vitro. Forty-eight hours later, cells were collected, and the protein level of Ppara was detected by western blotting (upper) and calculated by ImageJ (lower). Gapdh was used as loading control. HepG2 cells were transfected with Ppara expression construct or a control vector to verify the specificity of Ppara antibody (the far left two bands). The data are shown as means ± SDs. *p < 0.05, **p < 0.01, determined by ANOVA.
and IIa mRNA levels were decreased in exogenous of miR-181a-5p treatment group (Figure 5C).

The effect of exogenous expression miR-181a-5p on DNA damage caused by Ppara overactivation was examined, too. Exogenous expression of miR-181a-5p significantly reduced γ-H2a.x level that was due to overactivated Ppara (Figure 5D). Rad51 expression induced by high-dose WY-14643 was obviously alleviated by the exogenous expression miR-181a-5p (Figure 5D). The TUNEL assay showed decreased numbers of TUNEL-positive HepG2 cells after the overexpression of miR-181a-5p (Figure 5E). All these results demonstrated that exogenous expression of miR-181a-5p dramatically attenuated the cytotoxicity caused by Ppara overactivation.

**DISCUSSION**

Recently, the potential significance of miRNAs in the process of carcinogenesis caused by long-term exposure of WY-14643 to mice has been highlighted by accumulating studies.14,31,32 Here, we found that short-term exposure of HepG2 cells to high-dose Ppara-specific agonist WY-14643-induced miR-181a-5p expression may play a
crucial role in balancing the cytotoxic effects caused by Ppara overactivation.

The NR Ppara, a multi-functional transcription factor, has received numerous attentions as a therapeutic target for its lipid-lowering action.\(^3\)–\(^5\) Normally, Ppara is moderately activated and participates in a diverse range of biological functions, including control of fatty acid transport and catabolism, atherosclerosis, oxidative stress, anti-inflammation, immunomodulation, and autophagy.\(^1\)–\(^3\),\(^17\)–\(^38\) Previous studies demonstrated that Ppara can sequester the p65 subunit of the Nfκb complex and prevent Nfκb-dependent regulation of genes involved in pro-inflammatory responses.\(^39\)–\(^40\) However, cardiac-specific overexpression of Ppara in mice contributes to the development of cardiac dysfunction. It is partially because of the generation of ROS produced by increased mitochondrial flux.\(^19\) Excessive production of ROS may inflict various biological responses, ranging from a transient
growth arrest, permanent growth arrest or senescence, apoptosis, and necrosis. It has been reported that higher levels of ROS can induce IIa secretion in macrophages from Atg5−/− mice. We found that high-dose WY-14643 treatment induced significantly elevated ROS production, and increased expression of inflammatory factors Cyclin1d and IIa was observed. The elevated ROS levels caused by Ppara overactivation may be the major role that induces IIa and Cyclin1d expression in high-dose WY-14643-treated HepG2 cells. Cytotoxicity always elicits an inflammatory response, too. Damage caused by ROS is considered the most common type of DNA lesion. Our results showed that Ppara overactivation induced DNA damage and the central homologous recombination repair protein Rad51 expression. Distinct roles of Ppara have been reported in regulating apoptosis depending on cell types, ligand types, ligands dose, et al. Ppara antagonist NXT629 can induce apoptosis and inhibit proliferation of chronic lymphocytic leukemia cells in vitro and in vivo. Ppara agonist fenofibrate inhibits aldosterone-induced apoptosis in adult rat ventricular myocytes via stress-activated kinase-dependent mechanisms. However, Ppara activators can also induce apoptosis in activated macrophages and then contribute to the treatment of atherosclerosis. Ppara serves an E3 ubiquitin ligase to induce Bcl2 ubiquitination and degradation leading to cell apoptosis in response to chemotherapeutic drugs. Ppara agonist fenofibrate induces effective apoptosis in mantle cell lymphoma by inhibiting the Tnfα/Nfκb signaling axis. Here, we found that Ppara overactivation can significantly induces cells apoptosis.

Accumulating evidence suggests that transcription factors can bind to the promoters or enhancers of miRNAs and regulate their expression. Previous reports demonstrated that 27 miRNAs were significantly regulated following WY-14643 treatment. These implied that miRNA dysregulation caused by WY-14643-induced Ppara activation may play a pivotal role in Ppara agonist-induced cytotoxic effects. It has been widely reported that miR-181a expression is frequently regulated by genotoxic agent treatment. Stimulation with genotoxic agents, such as chemotherapeutic drugs doxorubicin or cisplatin, was found to induce miR-181a expression in breast cancer cells or non-small-cell lung cancer A549 cells. Our results revealed that Ppara-specific agonist WY-14643 can induce miR-181a2 expression accompanied by the induction of miR-181b2, but not miR-181a2hg. A previous report demonstrated that ~35% of intronic miRNAs are independent of host gene transcription, and miR-181a2 and miR-181b2 may be independent transcription from miR-181a2hg. The reported miR-181a1 promoter lies in upstream region of Pre-miR-181a1, not miR-181a1hg. Therefore, the promoter of miR-181a2 is more likely located in the upstream region of Pre-miR-181a2, not miR-181a2hg. NUBIScan prediction and luciferase reporter assay results verified that Ppara directly binds to the DR3 and regulates miR-181a2 expression. Then the increased miR-181a-5p can in turn repress its reported target Prkcd expression. Decreased Prkcd level may partially inhibit apoptosis, but the final outcome of Ppara overactivation is to promote apoptosis.

The miR-181 family plays diverse roles in regulating key aspects of cellular growth, development, inflammatory response, differentiation, and autophagy. Bioinformatic analysis and our results indicated that miR-181a-5p targets Ppara. We found that the intracellular DNA damage and apoptosis induced by overdose WY-14643 treatment were significantly reduced by the ectopic expression of miR-181a-5p. Our results displayed that exogenous expression of miR-181a-5p significantly reduced the intracellular ROS level, and inflammatory factors IIa and Cyclin1d levels caused by Ppara overactivation. It is anticipated that suppression of Ppara signaling can decrease the cytotoxic effects caused by a high concentration of WY-14643. A feedback loop regulation between miR-181a-5p and Ppara was revealed by our experiments.

As we know, PPs are widely distributed in the environment. It is inevitable for human to contact with known or unknown PPs. Previously, extensive studies focused on the risk put to human by exposure to these components. However, the body comes into contact with the PP toxic substances in the environment frequently. Thus, how does the body reduce harm to itself to keep healthy, and by which mechanism? Our experiments show that the feedback loop regulation between miR-181a-5p and Ppara plays an important role in facilitating a balance in the cytotoxicity caused by high concentrations of WY-14643 (Figure 6). Inducible miR-181a2 by Ppara overactivation plays critical roles in this protective process, and the induced miR-181a-5p depressed the excess Ppara levels. Such a balancing mechanism would assist the establishment of meaningful human
risk assessments and also noted that there are many potential targets of miR-181a-5p that could also contribute to the effect. Further studies are needed to provide a comprehensive picture to understand the miR-181a-5p alleviation of the toxic effects caused by high concentration of WY-14643.

MATERIALS AND METHODS

Reagents
The Ppara agonists WY-14643 and GW7647 were purchased from Cayman Chemical Company (Michigan, USA). For ex vivo experiments, WY-14643 and GW7647 were dissolved in DMSO at 0.1%–0.2% final concentration. The PrimeScript RT Reagent Kit with gDNA Eraser was purchased from TaKaRa, Japan. The dual luciferase assay system was from Promega (Madison, WI). The EMSA Assay kit and ChIP Assay kit were from Beyotime (Shanghai, China). Antibodies against γ-H2ax (sc-25775) and Prkd (sc-9616) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Ppara (sc-398394), β-actin (sc-47778), Rad51 (sc-398587), goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) (sc-2004), and goat anti-mouse IgG-HRP (sc-2005) were purchased from Santa Cruz Biotechnology (CA, USA). Gapdh antibody (BM-1623) was from Boster Biological Technology (Wuhan, China). Lmnb1 antibody (A1910) was purchased from Abclonal (MA, USA).

Cell Culture and miRNA Transfection
The cell lines HepG2, 293A, LX2, Huh7, and LO2 were cultured in DMEM (Life Technologies, Carlsbad, USA) with 10% fetal bovine serum (FBS), streptomycin (100 U/mL), and penicillin (100 U/mL) at 37°C in a 5% CO2 humid incubator. miRNA mimics were transfected at a concentration of 40 nM using RNAimax (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. The mimic sequences sets for siRNA transfection are listed in Table S1.

Quantitative Real-Time PCR
A specific stem loop primer was utilized for the reverse transcription of mature miR-181a-5p. For examination of the miRNA and mRNAs, total RNA was extracted with Trizol reagent (TaKaRa, Japan), and then the first-strand cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). Real-time PCR was performed using the SYBR Green Real-Time PCR Master Mix (Toyobo, Japan). The relative levels of the mRNAs were normalized to that of β-actin mRNA. The relative levels of the miRNA were normalized to that of U6 small nuclear RNA (snRNA). Relative expression of these genes was calculated using the ∆∆CT method. The primer sets for quantitative real-time PCR are listed in Table S2.

Western Blotting
Nuclear extracts were prepared using the Active Motif Nuclear Extract Kit (catalog nos. 40010 and 40410) according to the manufacturer’s instructions. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer according to the manufacturer’s instructions (Beyotime, Jiangsu, China) to prepare whole-protein extracts. Protein lysates were separated by 10% or 12% SDS-PAGE (20 μg each lane). Next, the gel was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% skimmed milk or 5% BSA in Tris-buffered saline/Tween-20 (TBST), the membranes were incubated with the primary antibodies overnight at 4°C, and then with the respective HRP-conjugated secondary antibodies at room temperature for 1.5 hr. Finally, the membranes were visualized with enhanced chemiluminescence (ECL) (Bio-Rad, USA). The signal was analyzed by ImageJ. Each experiment was performed in triplicate except for Figure 5D Rad51.

Plasmid Construction, Transfection, and Luciferase Reporter Assay
Putative PPREs in the Hsa-miR-181a2 promoter region were predicted using an online algorithm (NUBIScan: http://www.nubiscan.unibas.ch/). Based on this prediction (Figure 2A), the Hsa-miR-181a2 promoter region was amplified by PCR using LO2 cell genomic DNA as a template (the primer sequences are listed in Table S3). The fragments were then separately inserted between the KpnI and XhoI sites of the pGL3-basic vector (Promega, USA), and the resulting plasmids were named as follows with the fragment of the Hsa-miR-181a2 promoter region specified: pGL3-DR3(−319 to +105) (also named pGL3-DR3-WT); pGL3-DR3-Mut, derived from pGL3-DR3-WT, contained mutations in the DR3 element (AGGGCTATC to AGGGCTATC (also named BSB). The mutated bases are underlined). For luciferase reporter assays, the above plasmids were separately co-transfected with the Renilla luciferase expression vector pRL-TK (Promega, USA) into HepG2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. After 6 hr of incubation, the cells were treated with vehicle DMSO or WY-14643 (200 μM) for 36 hr. The cells were then harvested for detection of luciferase activity using the dual-luciferase assay kit (Promega, USA) according to the manufacturer’s instructions. The enzymatic activity of luciferase was measured using a Fluoroskan Ascent FL (Thermo Scientific, USA). Firefly luciferase activity was normalized to that of Renilla luciferase activity.

PsiCHECK-2 vectors containing WT or mutated miR-181a-5p Mut response elements (MREs) from the Ppara 3’ UTR were co-transfected with miR-181a-5p mimic or control mimic into 293A cells. Forty-eight hours later, cells were lysed. Firefly and Renilla luciferase activities were measured using a dual-luciferase reporter assay system (Promega, USA). The results were normalized Renilla luciferase activity to that of Firefly luciferase activity.

Plasmids of PcDNA3.1 or PcDNA3.1-Ppara from Lisheng Zhang’s laboratory were transfected with lipofectamine 2000 into HepG2 cells according to the manufacturer’s protocol.

EMSA
Nuclear extracts were prepared from WY-14643-treated HepG2 cells with or without exogenous expression Ppara using the Active Motif Nuclear Extract Kit (Active Motif, CA, USA, nos. 40010 and 40410), and the protein concentrations were determined using the BCA protein assay kit (Beyotime, Jiangsu, China). Double-stranded
oligonucleotides (Sangon, Shanghai, China) corresponding to the DR3 within the Hsa-miR-181a2 promoter were synthesized and annealed into double strands. The DNA binding activity of Ppara was detected by a chemiluminescent EMSA Kit. The binding reactions were performed separately in a 10 μL reaction mixture containing 5× gel shift binding buffer and 10 μg of nuclear proteins. For supershift assays, 1 μL of antibody against Ppara (Santa Cruz, CA, USA, sc-398394) was mixed with nuclear proteins in supershift assay and exogenous expression Ppara assay and incubated on 25°C for 20 min. For competition experiments, unlabeled (cold) DR3 or Mut DR3 probe was added to the reaction mixture at 2 × excess concentrations over the labeled probe. The mixtures were then incubated at room temperature for 10 min. Subsequently, 5 pmol of labeled probe was added to each reaction mixture and incubated at room temperature for 10 min. All of the reaction products were analyzed by electrophoresis in a 6.6% non-denaturing polyacrylamide gel in 0.5× Tris-borate-EDTA. The nylon was visualized using ECL (Bio-Rad, USA). The DNA-binding reaction system and double-stranded oligonucleotides are listed in Table S4 and Figure 2C, respectively. The reactions were analyzed by electrophoresis in a non-denaturing 6.6% polyacrylamide gel, followed by development.

ChIP
ChIP assays were performed using the ChIP Assay kit (Beyotime, Jiangsu, China) according to the manufacturer’s instructions. In brief, HepG2 cells were treated with 200 μM WY-14643 for 24 hr, and then incubated with formaldehyde at a final concentration of 1% (v/v) for 10 min at 37°C to cross-link the nuclear proteins to DNA. Subsequently, cells were sonicated and then immunoprecipitated with the antibody against Ppara, taking IgG as a negative control and no antibody (no anti-Ppara and IgG in the reaction) as mock control. The captured chromatin was eluted and un-cross-linked, and the DNA was recovered. The ChIP-isolated DNA was subjected to PCR amplification using the primer pair spanning the PPRE/DR3 in miR-181a2 promoter region (the primer sequences are listed in Table S2).

ROS Assay
After treatment with WY-14643 or vehicle with or without the transfection of miR-181a-5p or control mimics, cells were washed three times in PBS and were fixed in freshly prepared 4% paraformaldehyde in PBS for 1 hr at room temperature, followed by washing with PBS and permeabilization in 0.1% Triton X-100 in 0.1% sodium citrate. Cells were then incubated with TUNEL reaction mixture (Roche) for 60 min at 37°C in a humidified atmosphere in the dark. After washing twice with PBS, the cells were counterstained by DAPI (Beyotime, Jiangsu, China) and evaluated by fluorescence microscopy.

Statistical Analysis
All experiments (except for Figure 5D Rad51) were performed in triplicate or higher. Statistical analysis was performed using ANOVA. Data were presented as the means ± 2SDs, and the level of statistical significance was set at *p < 0.05, **p < 0.01.

SUPPLEMENTAL INFORMATION
Supplemental Information includes one figure and four tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2017.09.008.

AUTHOR CONTRIBUTIONS
L.Z. and G.L. conceived and designed the experiments, wrote the manuscript, and contributed to funding acquisition; Y.C. performed the experiments, analyzed the data, and wrote the manuscript. Z.W. conducted the experiments and performed statistical analyses. S.X. designed the experiments and revised the article. Y.P. conducted the experiments and revised the article. Y.Y., D.Q., and S.L. conducted the experiments. Y.X. performed statistical analyses and revised the article. All authors reviewed the manuscript.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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