

# Effects Of PPAR $\gamma$ 2 Pro12Ala Variant On Adipocyte Phenotype Dependent Of DHA

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Renhui Wan\*  
Zhengping Ding\*  
Sheng Xia  
Longyi Zheng  
Jin Lu 

Department of Endocrinology, Changhai Hospital, Second Military Medical University, Shanghai 200433, People's Republic of China

\*These authors contributed equally to this work

**Background:** Peroxisome proliferator-activated receptor  $\gamma$ 2 (PPAR $\gamma$ 2) plays a critical role in the regulation of adipocyte differentiation and adipocytokine production. The Pro12Ala variant is the most common mutation in the PPAR $\gamma$ 2 gene. Its effect appears to be sensitive to dietary factors, such as docosahexaenoic acid (DHA) level. The purpose of this study was to investigate the interaction effect between PPAR $\gamma$ 2 Pro12Ala variant and DHA on the phenotypes of adipocytes.

**Methods:** We generated stable 3T3-L1 cell lines expressing wild-type PPAR $\gamma$ 2 or PPAR $\gamma$ 2 Pro12Ala variant. These two cell lines were cultured with different concentrations of DHA (0, 50, 200  $\mu$ mol/L). Then Oil red O staining was used to observe cell differentiation and the degree of lipid accumulation, TUNNEL assay was used to detect cell apoptosis, and ELISA assays were used to detect the changes of TNF- $\alpha$ , resistin and adiponectin levels in cell culture supernatant.

**Results:** PPAR $\gamma$ 2 Pro12Ala variant reduced lipid droplet accumulation in 3T3-L1 preadipocytes treated with or without 50  $\mu$ mol/L DHA, but not with 200  $\mu$ mol/L DHA, compared to that of wild-type PPAR $\gamma$ 2. PPAR $\gamma$ 2 reduced resistin production and increased adiponectin production in 3T3-L1 adipocytes, whereas PPAR $\gamma$ 2 Pro12Ala variant diminished these effects. However, the absence of DHA blocked PPAR $\gamma$ 2 Ala12 variant-induced effects on adiponectin production. There was no significant difference in TNF- $\alpha$  secretion between wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala cells whether with or without DHA.

**Conclusion:** These results indicated that the effects of PPAR $\gamma$ 2 Pro12Ala variant were dependent on DHA concentration.

**Keywords:** peroxisome proliferator-activated receptor  $\gamma$ 2, Pro12Ala variant, docosahexaenoic acid, adipocyte, differentiation, adiponectin

## Introduction

Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), particularly the PPAR $\gamma$ 2 isoform, is a ligand-dependent nuclear receptor highly expressed in adipose tissue. Activation of PPAR $\gamma$ 2 regulates adipocyte differentiation, triglyceride storage, and some metabolic effects, including glucose homeostasis and insulin sensitivity.<sup>1,2</sup>

The Pro12Ala variant of the PPAR $\gamma$ 2 gene is common (3–14%)<sup>3</sup> and can cause a moderate decrease in its transcriptional activity and adipogenic potential.<sup>4</sup> Human population studies on PPAR $\gamma$ 2 gene Pro12Ala polymorphism showed the association between this variant and reduced weight gain and reduced risk of type 2 diabetes.<sup>5,6</sup> Moreover, the Pro12Ala variant appears to be sensitive to environmental effects, such as dietary factors.<sup>7–9</sup>

Long-chain n-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), have been reported to reduce adiposity by preventing fat accretion and

Correspondence: Jin Lu; Longyi Zheng  
Department of Endocrinology, Changhai Hospital, Second Military Medical University, No.168, Changhai Road, Shanghai 200433, People's Republic of China  
Email lujin-sh@139.com;  
longyizheng111@163.com

improve glycolipid metabolic disorders in high-fat diet-fed rats.<sup>10</sup> Additionally, a randomized clinical trial showed that DHA exhibited anti-inflammatory effects.<sup>11,12</sup>

Thus, in this study, we aimed to examine the gene-environment interactions between PPAR $\gamma$ 2 Pro12Ala variant and DHA in 3T3-L1 preadipocytes. We generated stable 3T3-L1 cell lines expressing wild-type PPAR $\gamma$ 2 or PPAR $\gamma$ 2 Pro12Ala variant. Then, the effects of wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala variant on the differentiation, apoptosis, and adipocytokine levels were investigated in 3T3-L1 preadipocytes treated with or without different concentrations of DHA.

## Materials And Methods

### Cell Culture

HEK293T cells were obtained from the American type culture collection (ATCC). 3T3-L1 preadipocytes were obtained from the Chinese Academy of Sciences Cell Bank. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 mg/mL) in a humidified incubator with 5% CO<sub>2</sub> (v/v) at 37 °C.

### Construction Of Lentivirus Plasmids Containing PPAR $\gamma$ 2 Pro Or PPAR $\gamma$ 2 Ala Gene

Wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala genes were polymerase chain reaction (PCR)-amplified from human adipose tissue cDNA library. The PCR products were subcloned into the pMD18-T vectors (Takara), and the Pro12Ala variant was confirmed by sequencing (Shanghai Boshang Biotechnology Co. Ltd.). Then, the products were cloned into pLVTHM via restriction digestion (EcoRI/XhoI) and T4 DNA ligase (TOYOBO) to construct the pLVTHM-PPAR $\gamma$ 2 Pro and pLVTHM-PPAR $\gamma$ 2 Ala plasmids.

### Preparation Of Lentivirus

Lentiviral supernatants were produced in HEK293T cells by co-transfection of 20  $\mu$ g of transfer vectors harboring the indicated genes (empty vector, pLVTHM-PPAR $\gamma$ 2 Pro, or pLVTHM-PPAR $\gamma$ 2 Ala), 10  $\mu$ g of pRsv-REV, 15  $\mu$ g of pMDlg-pRRE, and 7.5  $\mu$ g of pMD2G, according to the manufacturer's protocol. Supernatants containing lentivirus were collected at 48 h after transfection. Cell debris was removed by centrifugation at 4000  $\times$ g for 10 min at 4 °C, followed by filtration through a 0.45  $\mu$ m polyethersulfone

membrane filter unit. Then, the virus was concentrated at 72,000  $\times$ g for 120 min at 4 °C.

### Generation Of Stable Cell Lines

3T3-L1 cells were seeded on a 24-well plate at a density of  $5 \times 10^4$  cells/well. Then, in the presence of polybrene (1  $\mu$ g/mL), cells were infected with pLVTHM-GFP lentivirus (LV-vector group), pLVTHM-PPAR $\gamma$ 2 Pro lentivirus (Lv-PPARG2 group), or pLVTHM-PPAR $\gamma$ 2 Ala lentivirus (Lv-PPARG2 P12A group). After 24 h incubation at 37 °C, the culture medium was replaced with a fresh complete medium without polybrene. At 72 h post-infection, stably transfected cell lines were selected using puromycin (Sigma-Aldrich; Merck Millipore) at a concentration of 1  $\mu$ g/mL for 7 days.

### Quantitative RT-PCR Assay

Total RNA was extracted using Trizol (Invitrogen), according to the manufacturer's instructions. cDNA was synthesized from 1  $\mu$ g of RNA using a cDNA reverse transcription kit with RNase inhibitor (MBI Fermentas). Real-time PCR was performed in a thermal cycler (ABI) using Quant qRT-PCR (SYBR Green I) kit (TIANGEN). The  $\Delta$ CT method was used to measure the relative expression. Results were normalized to that of a reference gene ( $\beta$ -actin). The primer sequences were as follows: PPAR $\gamma$ 2 (5'-GGAGCCCAAGTTTGAGTTTGC TGT-3', 5'-AGGGCTTGTAGCAGGTTGTCTTGA3') and  $\beta$ -actin (5'-TGTGATGGTGGGAATGGGTCAGAA-3', 5'-T GTGGTGCCAGATCTTCTCCATGT-3').

### Fatty Acid Treatments

On the first day of cell differentiation, the same number of 3T3-L1 cells were inoculated into 24-well plates. 3T3-L1 cells were treated with different concentrations of DHA (0, 50, or 200  $\mu$ mol/L) and MDI (Sigma) containing 0.5 mM 3-isobutyl-1-methylxanthine, 1  $\mu$ M dexamethasone, and 1  $\mu$ g/mL insulin.

### Oil Red O Staining

After incubation with DHA for 6 h, the cells were washed three times with phosphate-buffered saline (PBS), fixed with 10% formaldehyde for 30 min, and then stained with 0.5% Oil Red O (Sigma) for 20 min at 20 °C. Red-stained adipocytes were observed under a phase-contrast microscope (Olympus). To quantify Oil Red O levels, 2.5 mL of 100% isopropanol was added to each well. After shaking at room temperature for 5 min, the optical density (OD) of samples was measured at 500 nm using a spectrophotometer (UV-765).

## TUNEL Assay

A one-step TUNEL cell apoptosis detection kit (Ggreen fluorescence) was purchased from Beyotime Biotechnology Research Institute. Briefly, cells cultured with DHA for 2 h were fixed with 4% paraformaldehyde for 30–60 min at room temperature. After washing with PBS, the cells were permeabilized with PBS containing 0.1% Triton X-100 on ice for 2 min. Then, they were stained with a freshly-prepared TUNEL detection solution (20  $\mu$ L of TdT enzyme, 480  $\mu$ L of the fluorescent labeling solution, and 500  $\mu$ L of the TUNEL detection solution) in dark at 37 °C for 60 min, followed by sealing using the antifluorescence quenching sealing fluid. Finally, apoptosis rates were evaluated using a fluorescence microscope.

## Enzyme-Linked Immunosorbent Assay (ELISA)

Adiponectin ELISA kit was purchased from B-Bridge International Corporation. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and resistin ELISA kits were obtained from Quantikine Corporation. Cytokine levels were measured by taking cell medium, according to the manufacturer's instructions.

## Statistical Analysis

Values are expressed as the means  $\pm$  standard error of the mean (SEM). Data were analyzed using SPSS package program version 22.0. One-way analysis of variance followed by Student-Newman-Keuls (SNK) *post-hoc* test was used to compare the responses among different groups. A *P*-value  $< 0.05$  was considered statistically significant.

## Results

### Generation Of Stable Cell Lines

To investigate the effects of PPAR $\gamma$ 2 genotypes in 3T3-L1 cells, we generated stable cell lines expressing wild-type PPAR $\gamma$ 2 (Lv-PPARG2) or PPAR $\gamma$ 2 Pro12Ala variant (Lv-PPARG2 P12A). Plasmids containing the Pro12Ala variant were confirmed by sequencing. The presence of the CCA $\rightarrow$ GCA mutation (Pro/Ala) at codon 12 of PPAR $\gamma$ 2 exon B without additional changes in the coding sequence was verified. The expression of PPAR $\gamma$ 2 in stable cell lines was verified by real-time PCR assay. The expression of PPAR $\gamma$ 2 in 3T3-L1 cells transfected with Lv-PPARG2 or Lv-PPARG2 P12A increased by 10 fold, compared to that in 3T3-L1 cells transfected with Lv-vector.

### Effects Of PPAR $\gamma$ 2 Pro12Ala Variant On Lipid Droplet Formation In DHA-Treated 3T3-L1 Adipocytes

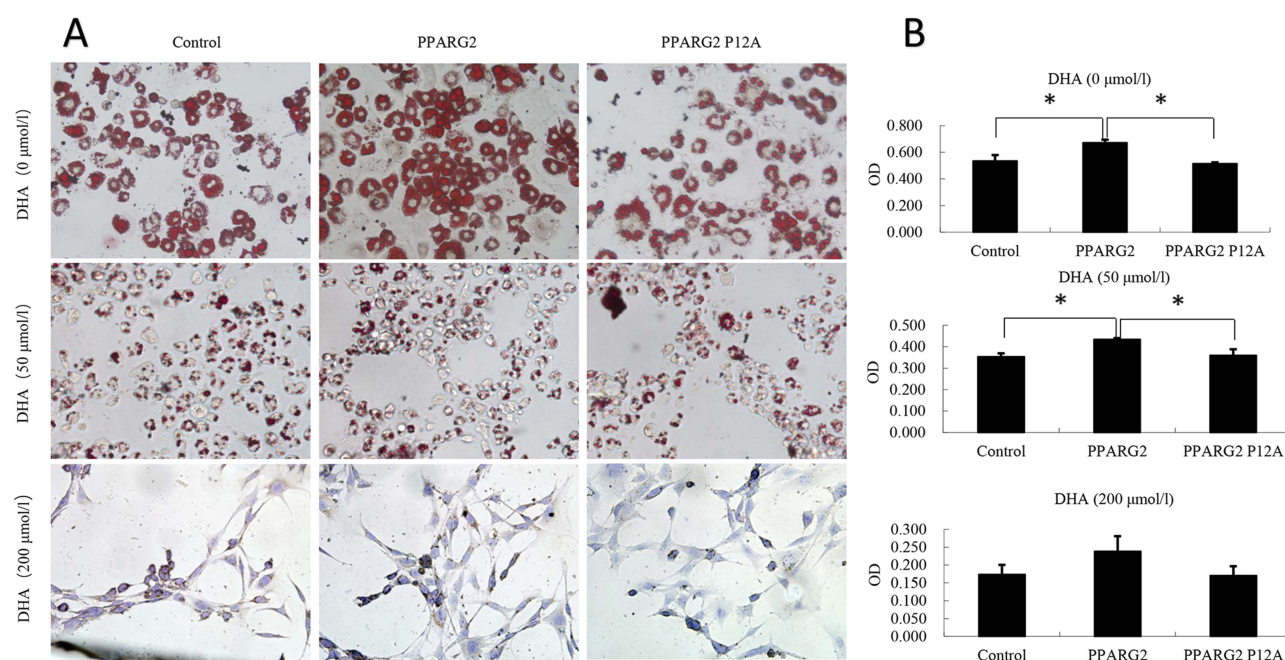
The effects of PPAR $\gamma$ 2 genotypes on 3T3-L1 preadipocyte differentiation were investigated. Lv-vector, Lv-PPARG2, or Lv-PPARG2 P12A-transfected 3T3-L1 cells were treated with different concentrations of DHA. Cells were stained with Oil Red O to evaluate the degree of lipid accumulation. As shown in Figure 1, overexpression of PPAR $\gamma$ 2 increased lipid accumulation in 3T3-L1 cells treated with 0 or 50  $\mu$ mol/L DHA, whereas overexpression of PPAR $\gamma$ 2 Pro12Ala variant reduced lipid accumulation, compared to that of PPAR $\gamma$ 2. However, at a concentration of 200  $\mu$ mol/L DHA, there was no statistical difference among the control, PPAR $\gamma$ 2 Pro12, and PPAR $\gamma$ 2 Pro12Ala-expressing adipocytes.

### Effects Of PPAR $\gamma$ 2 Pro12Ala Variant On Adipocyte Apoptosis In DHA-Treated 3T3-L1 Adipocytes

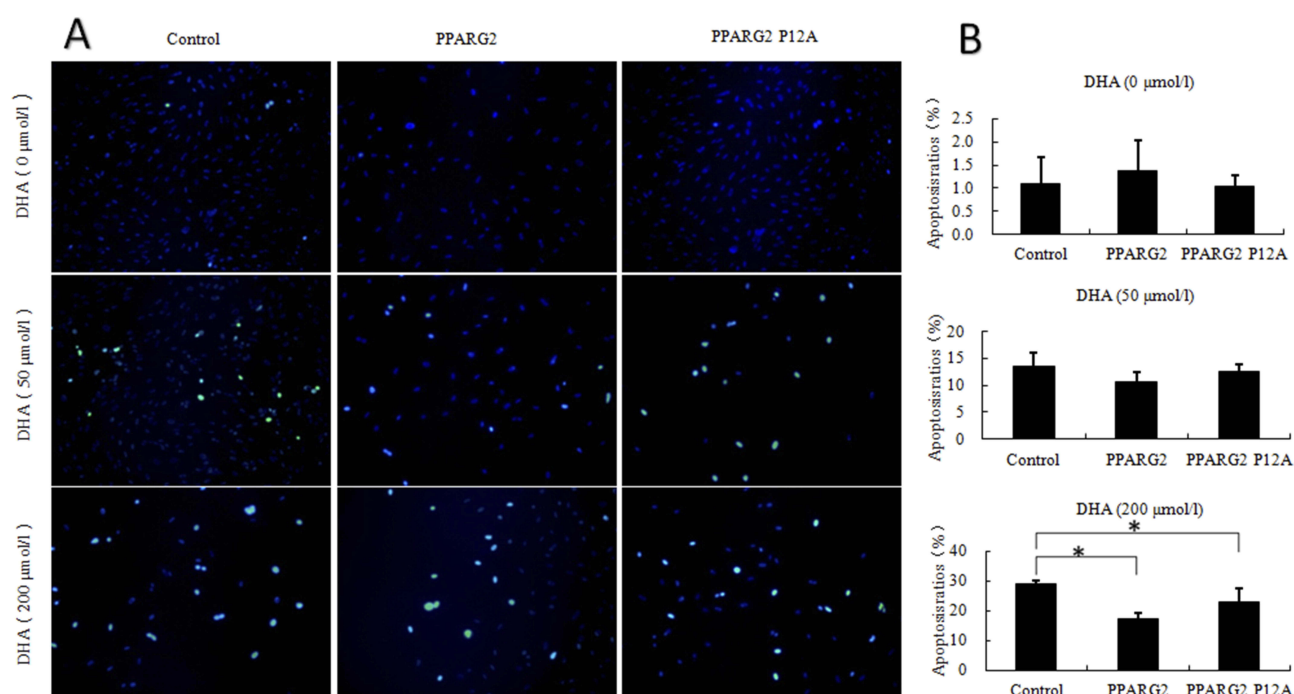
We then evaluated apoptosis ratios in different groups. As shown in Figure 2, overexpression of wild-type PPAR $\gamma$ 2 or PPAR $\gamma$ 2 Pro12Ala variant did not affect apoptosis in 3T3-L1 adipocytes treated with 0 or 50  $\mu$ mol/L DHA. However, in both wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala variant-expressing adipocytes treated with 200  $\mu$ mol/L DHA, apoptosis ratios were reduced, compared to that in the control group. At all DHA concentrations, there was no statistical difference in apoptosis ratios between 3T3-L1 adipocytes overexpressing wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala variant.

### Effects Of PPAR $\gamma$ 2 Pro12Ala Variant On Inflammatory Cytokine Levels In DHA-Treated 3T3-L1 Adipocytes

Furthermore, we examined the effects of PPAR $\gamma$ 2 genotypes on the secretion of inflammatory cytokines in 3T3-L1 adipocytes. As shown in Figure 3, overexpression of wild-type PPAR $\gamma$ 2 decreased the secretion of proinflammatory cytokines and resistin from 3T3-L1 adipocytes treated with or without DHA. Moreover, overexpression of PPAR $\gamma$ 2 Pro12Ala variant partially abolished these effects (Figure 3B). On the contrary, overexpression of wild-type PPAR $\gamma$ 2 increased the secretion of adiponectin from 3T3-L1 adipocytes treated with or without DHA. Similarly, overexpression of PPAR $\gamma$ 2 Pro12Ala variant in DHA-treated cells attenuated these effects. However, the absence of DHA blocked PPAR $\gamma$ 2 Ala12 variant-induced



**Figure 1** Effects of PPAR $\gamma$ 2 Pro12Ala variant on lipid droplet accumulation and 3T3-L1 preadipocyte differentiation after DHA treatment. Lv-vector, Lv-PPARG2 or Lv-PPARG2 P12A-transfected 3T3-L1 cells were pretreated and differentiated in the absence or presence of different concentrations of DHA. **(A)** Oil Red O staining was performed to examine cell morphology. **(B)** The lipid droplets were dissolved in 100% isopropanol, and the color intensity was measured at 500 nm using a spectrophotometer. OD values are expressed as the means  $\pm$  SEM of 3 samples. \* $p < 0.05$ .

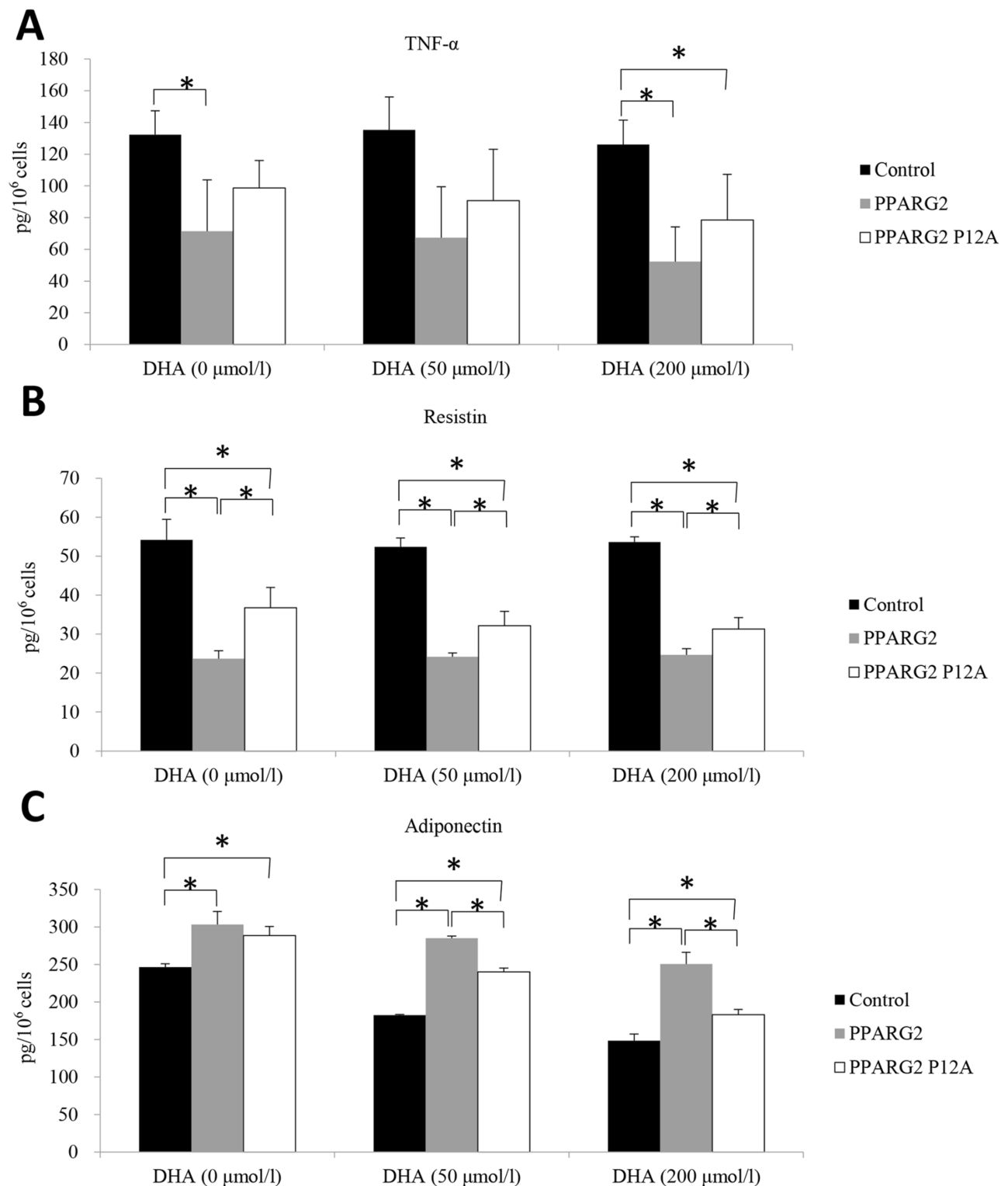


**Figure 2** Effects of different concentrations of DHA on apoptosis of adipocytes of different genotypes. 3T3-L1 preadipocytes of different genotypes were prepared in the absence or presence of different concentrations of DHA. **(A)** TUNEL detection was performed to observe adipocyte apoptosis level. The excitation wavelength ranged from 450 to 500 nm, whereas the emission wavelength ranged from 515 to 565 nm (green fluorescence). **(B)** Adipocyte apoptosis ratios were calculated. Values are expressed as the mean  $\pm$  SEM of 3 samples. \* $p < 0.05$ .

effects on adiponectin production (Figure 3C). However, there was no significant difference in TNF- $\alpha$  secretion

between wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala-expressing adipocytes, regardless of DHA treatment (Figure 3A).





**Figure 3** Effects of different genotypes on the secretory functions of adipocytes. The samples were analyzed for determination of the protein levels of TNF- $\alpha$  (A), resistin (B), and adiponectin (C) by ELISA. Values are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ .

## Discussion

PPAR $\gamma$ 2 is a nuclear receptor involved in lipid metabolism, adipocyte differentiation, and proliferation.<sup>13</sup> A missense

mutation (CCGPro-GCGAla) at codon 12 is a common variant of PPAR $\gamma$ 2 gene. Meta-analyses showed that PPAR $\gamma$ 2 Pro12Ala variant correlated with improved insulin

sensitivity.<sup>14,15</sup> Another clinical study verified that PPAR- $\gamma$ 2 Pro12Ala variant had modest protective effects against the development of type 2 diabetes.<sup>16</sup>

In our study, overexpression of wild-type PPAR $\gamma$ 2 gene resulted in increased lipid droplet accumulation and adipocyte differentiation. PPAR $\gamma$ 2 Pro12Ala variant with or without 50  $\mu$ mol/L DHA treatment attenuated these effects, suggesting that PPAR $\gamma$ 2 Pro12Ala variant might correlate with lower risk of obesity. However, there was no significant difference in lipid droplet accumulation between wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala1-expressing adipocytes treated with 200  $\mu$ mol/L DHA, indicating that the effects of PPAR $\gamma$ 2 Pro12Ala variant was dependent on the concentration of DHA to some extent. Meantime, our results showed that DHA can induce apoptosis of adipocytes and this effect becomes more obvious with the increase of DHA concentration. This may also be the reason why there is no statistically significant difference in 200 $\mu$ mol/L DHA group.

However, overexpression of PPAR $\gamma$ 2 did not affect apoptosis of adipocytes except when incubated with high concentration (200 $\mu$ mol/L) of DHA. And PPAR $\gamma$ 2 Pro12Ala variant showed no effect on apoptosis of adipocytes.

It has been reported that some adipocytokines, such as resistin, TNF $\alpha$ , and adiponectin, are under the transcriptional control of PPAR $\gamma$ 2. Resistin belongs to the family of cysteine-rich proteins, called resistin-like molecules.<sup>17</sup> The expression of resistin positively correlated with body fat, insulin, glucose, and triglyceride levels in high-fat diet-fed mice.<sup>18</sup> Additionally, a PPAR $\gamma$  agonist was shown to improve insulin sensitivity via downregulation of resistin expression.<sup>19</sup> PPAR $\gamma$  also regulates the circulating levels of adiponectin, where the effects of PPAR $\gamma$  on adiponectin production are partially attributed to the direct activation of adiponectin gene transcription.<sup>20</sup> Adiponectin expression and its signaling pathway are directly regulated by PPAR $\gamma$ .

In line with the results of previous studies, we showed that overexpression of PPAR $\gamma$ 2 gene reduced TNF- $\alpha$  and resistin production and increased adiponectin production. However, overexpression of PPAR $\gamma$ 2 Pro12Ala variant attenuated these effects. It is noteworthy that a significant difference in adiponectin level between wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala-expressing cells was only observed in cells treated with DHA (50 and 200  $\mu$ mol/L). There was no significant difference in adiponectin level between wild-type PPAR $\gamma$ 2 and PPAR $\gamma$ 2 Pro12Ala-expressing DHA-untreated cells. Additionally, PPAR $\gamma$ 2 Pro12Ala

variant showed no effect on TNF- $\alpha$  secretion in adipocytes, compared to that of wild-type PPAR $\gamma$ 2.

In summary, our study showed that overexpression of PPAR $\gamma$ 2 gene in preadipocytes resulted in increased lipid droplet accumulation and adipocyte differentiation, and exhibited anti-inflammatory effects by reducing proinflammatory cytokine (TNF- $\alpha$  and resistin) secretion and increasing anti-inflammatory cytokine (adiponectin) production. Most of these effects, except for TNF- $\alpha$  secretion, were attenuated owing to the overexpression of PPARG2 Pro12Ala variant. Moreover, the effects of PPAR $\gamma$ 2 Pro12Ala variant varied at different concentrations of DHA treatment. The effects of PPAR $\gamma$ 2 Pro12Ala variant on adiponectin secretion were dependent on the presence or absence of DHA treatment. Additionally, the effect of PPAR $\gamma$ 2 Pro12Ala variant on lipid accumulation disappeared with the increase in DHA concentration. Thus, our results showed that the effects of PPAR $\gamma$ 2 Pro12Ala variant were dependent on gene-environment interactions. Several clinical studies also found that the regulation of body fat by PPAR $\gamma$ 2 Pro12Ala variant was affected by dietary conditions.<sup>21,22</sup> However, further studies are needed to clarify the exact effects of PPAR $\gamma$ 2 Pro12Ala variant under different dietary conditions.

## Abbreviations

ATCC, American type culture collection; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; SNK, Student-Newman-Keuls; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

## Author Contributions

JL and LYZ designed the study. RHW, ZPD and SX performed the experiments. RHW analyzed the data and wrote the manuscript. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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