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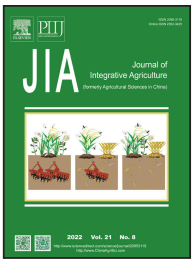

RESEARCH ARTICLE

PPAR gamma2: The main isoform of PPARγ that positively regulates the expression of the chicken *Plin1* gene

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Abstract

Perilipin1 (PLIN1) is a major phosphorylated protein that specifically coats the surface of neutral lipid droplets (LDs) in adipocytes and plays a crucial role in regulating the accumulation and hydrolysis of triacylglycerol (TG). Mammalian studies have shown that *Plin1* gene transcription is mainly regulated by peroxisome proliferator-activated receptor-gamma (PPARγ), the master regulator of adipogenesis. However, the regulatory mechanism of the chicken *Plin1* (*cPlin1*) gene is poorly understood. The present study aimed to investigate whether *Plin1* is regulated by PPARγ in chickens and identify its exact molecular mechanism. Reporter gene and expression assays showed that PPARγ2, but not PPARγ1, activated (P<0.01) the *cPlin1* gene promoter. An electrophoretic mobility shift assay and mutational analysis revealed that PPARγ2 bound to a special site in the *cPlin1* gene promoter to enhance its expression. In summary, our results show that PPARγ promotes the expression of the *cPlin1* gene and that PPARγ2 is the main regulatory isoform.

Keywords: chicken, PPAR gamma, isoform, *Plin1*, transcriptional regulation

1. Introduction

Perilipin1 (PLIN1) is a phosphoprotein found in rat epididymal adipocytes. It is phosphorylated by protein kinase A (PKA) (Lu *et al.* 2001) and is a major member of the PAT family of lipid droplet (LD) surface proteins. In mammals, PLIN1 is mainly expressed in white adipose tissue (WAT), with a small amount of brown adipose tissue (BAT) and myocardial liposarcoma. As a primary member of the phospholamban family, PLIN1 specifically coats the surface of lipid droplets in adipocytes (Castro-Chavez *et al.* 2003) and plays a crucial role in LD formation and triglyceride (TG) metabolism *in vivo*. Studies in

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mice and other mammals have shown that, in the basic (feeding) state, PLIN1 coats the surface of lipid droplets and acts as a protective barrier for neutral lipid droplets, inhibiting lipolysis by preventing triglyceride hydrolase from approaching the surface of lipid droplets (Brasaemle *et al.* 2000). Following the stimulation of catecholamine hormones, PLIN1 can be highly phosphorylated by PKA, which is beneficial for lipase to approach the lipid droplet surface, thus activating and promoting lipolysis.

Mammalian and livestock studies have shown that *Plin1* gene transcription is regulated by various transcription factors, proteins, and non-coding RNAs (Chen *et al.* 2016; Ghoshal *et al.* 2016; Mysore *et al.* 2016; Natori *et al.* 2017; Yao *et al.* 2018; Ju *et al.* 2019; Li 2019; Palhinha *et al.* 2019; Zhang *et al.* 2019; Lu *et al.* 2020; Mizunoe *et al.* 2020; Shijun *et al.* 2020; Yi *et al.* 2020; Zhou *et al.* 2020). Among these studies, the most thorough is regulatory study of the *Plin1* gene by peroxisome proliferator-activated receptor γ (PPARγ), the primary regulator of adipogenesis and lipogenesis. Arimura *et al.* (2004) found that a functional component of PPARγ (PPRE) in the 5′ flanking region of the mouse *Plin1* gene and an endogenous PPARγ2 protein recognize and bind to this site to play a transcriptional regulatory role in the *Plin1* gene.

Although PLIN1 has been extensively studied in mammals, the *cPlin1* gene has been studied only with regard to gene cloning, expression characteristics, and correlations between genetic polymorphisms and fat traits (Zhao *et al.* 2009; Pan *et al.* 2010; Zhang *et al.* 2015; Zhou *et al.* 2015). In particular, the transcriptional regulation mechanism of *cPlin1* remains unclear. Our previous studies showed that *cPlin1* is highly expressed in adipose tissue and involved in the PPAR signalling pathway. PPARγ are a family of ligand-dependent transcription factors belonging to the nuclear receptor superfamily regulating the transcription of various genes (Houseknecht *et al.* 2002; Surgucheva and Surguchov 2008). Additionally, retinoid X receptor α (RXRα), a key partner for PPARγ that regulates target genes, positively regulates *cPlin1* gene expression (Sun *et al.* 2020). This finding suggests that *Plin1* gene expression is also regulated by PPARγ in chickens. Additionally, we found that chicken PPARγ has two protein isoforms (cPPARγ1 and cPPARγ2) (Mueller *et al.* 2002), and their molecular functions are not exactly consistent (Mu *et al.* 2020).

Based on the above research results, we hypothesized: i) *cPlin1* might be the target gene of PPARγ; ii) two protein isoforms of PPARγ might have different regulatory effects on *cPlin1* gene expression; iii) PPARγ might promote its expression by binding to a specific site on the promoter of *cPlin1* gene. Therefore, the purpose of this study is to identify the distinction of the two protein isoforms of PPARγ on the regulation of *cPlin1* gene, and to clarify the molecular mechanism of PPARγ regulating *cPlin1* expression.

2. Materials and methods

2.1. Experimental design and treatments

The present study was divided into four parts, including nine experiments in a completely randomized design.

Experiment 1 was designed to study the effect of PPARγ agonist on mRNA expression levels of the *LPL*, *A-FABP*, *FAS*, *Plin1* and *PPARγ* genes with RT-qPCR technology. Two treatments, adding troglitazone and non-adding troglitazone, were taken on primary chicken preadipocytes.

Experiment 2 was designed to study the effect of PPARγ on the promoter activity of the *cPlin1* gene with luciferase reporter assay. Three treatments, cotransfection of pCMV-HA-PPARγ1 with pGL3-PLIN-1992/-11 plasmids, cotransfection of pCMV-HA-PPARγ2 with pGL3-PLIN-1992/-11 plasmids, and cotransfection of pCMV-HA with pGL3-PLIN-1992/-11 plasmids, were performed on DF1 and ICP1 cells with or without rosiglitazone.

Experiment 3 was designed to study the effect of PPARγ on the expression of the endogenous *cPlin1* gene with RT-qPCR technology. Three treatments, transfection of pCMV-HA-PPARγ1 plasmid, transfection of pCMV-HA-PPARγ2 plasmid, and transfection of pCMV-HA plasmid, were performed on DF1 and ICP1 cells.

Experiment 4 was designed to study the effect of PPARγ1 and PPARγ2 on the activity of the *cPlin1* gene promoter with different concentrations of rosiglitazone with luciferase reporter assay. Three treatments, cotransfection of pCMV-HA-PPARγ1 with pGL3-PLIN-1992/-11 plasmids, cotransfection of pCMV-HA-PPARγ2 with pGL3-PLIN-1992/-11 plasmids, and cotransfection of pCMV-HA with pGL3-PLIN-1992/-11 plasmids, were performed on DF1 and ICP1 cells with different concentrations of rosiglitazone.

Experiment 5 was designed to study the protein interaction of chicken PPARγ with RXRα with Co-IP assay. Five treatments, transfection of pCMV-Myc-RXRα plasmid, transfection of pCMV-HA-PPARγ1 plasmid, transfection of pCMV-HA-PPARγ2 plasmid, cotransfection of pCMV-Myc-RXRα with pCMV-HA-PPARγ1 plasmids, and cotransfection of pCMV-Myc-RXRα with pCMV-HA-PPARγ2 plasmids, were performed on DF1 cells.

Experiment 6 was designed to study the effect of the cooperation of PPARγ and RXRα on *cPlin1* gene

promoter activity with luciferase reporter assay. Three treatments, cotransfection of pCMV-HA-PPARγ1, pCMV-Myc-RXRα with pGL3-PLIN-1992/-11 plasmids, cotransfection of pCMV-HA-PPARγ2, pCMV-Myc-RXRα with pGL3-PLIN-1992/-11 plasmids, and cotransfection of pCMV-HA, pCMV-Myc-RXRα with pGL3-PLIN-1992/-11 plasmids, were performed on DF1 cells and ICP1 cells with or without rosiglitazone.

Experiment 7 was designed to study the effect of PPARγ2 on the activity of the serially truncated *cPlin1* gene promoter with luciferase reporter assay. Two treatments, cotransfection of pCMV-HA-PPARγ2 with luciferase reporter gene plasmids containing serially truncated *cPlin1* gene promoter sequences, and cotransfection of pCMV-HA with luciferase reporter gene plasmids containing serially truncated *cPlin1* gene promoter sequences, were performed on DF1 and ICP1 cells.

Experiment 8 was designed to study the effects of mutation of the predicted PPARγ2-binding sites on PPARγ2-regulated activation of the *cPlin1* gene promoter with luciferase reporter assay. A 2×2 factorial arrangement of treatments was adopted in this experiment. Two reporter vector types were wild-type and mutant type, and 2 expression plasmids were the pCMV-HA-PPARγ2 and pCMV-HA, respectively. Four treatments, cotransfection of pCMV-HA-PPARγ2 with pGL3-PLIN-1307/-11 plasmids, cotransfection of pCMV-HA with pGL3-PLIN-1307/-11 plasmids, cotransfection of pCMV-HA-PPARγ2 with pGL3-Mut-PLIN-1307/-11 plasmids, and cotransfection of pCMV-HA with pGL3-Mut-PLIN-1307/-11 plasmids, were performed on DF1 and ICP1 cells.

Experiment 9 was designed to analyse the PPARγ2 binding sites in the –1126 bp/–1116 bp region of *cPlin1* gene promoter with EMSA assay. First, nuclear extracts were prepared from DF-1 cells transfected with the pCMV-HA-PPARγ2 plasmid. Second, six treatments, the nuclear extracts, incubation of the nuclear extracts with labelled probe, incubation of the nuclear extracts with labelled probe and different amounts of unlabelled probe, incubation of the nuclear extracts with labelled mutant probe, incubation of the nuclear extracts with labelled probe and PPARγ antibody, and incubation of the nuclear extracts with labelled probe and IgG antibody, were performed.

2.2. Plasmid construction (experiments 2–9)

The pGL3-basic and phRL-TK luciferase reporter vector and pCMV-HA-PPARγ1, pCMV-HA-PPARγ2, pCMV-Myc-RXRα, and pCMV-HA eukaryotic expression plasmids were all maintained in our laboratory.

The chicken *Plin1* promoter and its subsequent 5′ truncation mutants were generated by PCR from chicken genomic DNA using distinct forward primers and the same reverse primer (Table 1). Next, these PCR products were subcloned into the pGL3-Basic vector (Promega, Madison, WI, USA) to construct a series of reporter gene plasmids with truncated promoter mutations

Table 1 PCR primers used in this study

Primer	Application	Sequence (5′→3′)	Length (bp)
Plin1-1992	Cloning of the 5′ truncated mutation of the promoter	F: CGGGGTACCTGGGCTGTCTCAGCAAGTACAGTCT	1 982
Plin1-1834		F: GGGGTACCGCTGGGGGCTAGCAGTTAAATGTACC	1 824
Plin1-1307		F: CGGGGTACCGCAGAATGGTAAGTGAGATAAGTAATCT	1 297
Plin1-838		F: GGGTACCCTGGTGTCATGCCTGTTCACCGTGG	828
Plin1-689		F: CGGGGTACCGTTAATGCAGGGCTGTGGACAAG	679
Plin1-470		F: CGGGGTACCTGCTGGTCCAAGTGAGTAAG	460
Plin1-246		F: GGGTACCTCCTCCTCTTCTCCCTAGCCTTGGT	236
Plin1-11		R: CCTCGAGGTGTGGTGTTGGGGCACTACTACACC	
Plin1	qPCR	F: GCCAAGGAGAACGTGCT	142
		R: TCACTCCCTGCTCATAGACC	
PPARγ		F: GTGCAATCAAAATGGAGCC	170
		R: CTTACAACCTTCACATGCAT	
β-Actin		F: TCTTGGGTATGGAGTCCTG	331
		R: TAGAAGCATTTGCGGTGG	
A-FABP		F: ATGTGCGACCAGTTTGT	143
		R: TCACCATTGATGCTGATAG	
FAS		F: AAGGAGGAAGTCAACGG	196
		R: TTGATGGTGAGGAGTCG	
LPL		F: GCATTCACCATTCAGAGAGTCAG	535
		R: AACTGCTAAAGAGGAACTGATGG	
TBP		F: GCGTTTTGCTGCTGTTATTATGAG	122
		R: TCCTTGCTGCCAGTCTGGAC	

(e.g., pGL3-PLIN-1992/-11, pGL3-PLIN-1834/-11, and pGL3-PLIN-1307/-11). Site-directed mutagenesis of the promoter was performed utilizing a QuickMutation Site-Directed Mutagenesis Kit (Beyotime Institute of Biotechnology, Jiangsu, China). The site-mutated promoter was also cloned into the pGL3-Basic vector, and the pGL3-Mut-PLIN-1307/-11 plasmid was generated. All the primers used are listed in Table 1, and all the final constructs were confirmed by DNA sequencing.

2.3. Cell culture and differentiation

The immortalized chicken preadipocyte (ICP1) cell line (Wang *et al.* 2017) was maintained in our laboratory. Primary chicken preadipocytes were separated and cultured according to a previously reported protocol (Wang *et al.* 2017). ICP1 cells and primary chicken preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 5% foetal bovine serum (FBS) (Gibco) in a humidified incubator at 37°C and 5% CO_2. The chicken embryo fibroblast cell line DF-1, which was provided by the Harbin Veterinary Research Institute (Harbin, China), was maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS at 37°C in a humidified atmosphere of 5% CO_2.

When the passaged primary chicken preadipocytes had reached 50% confluence, fresh medium containing DMEM/F12 supplemented with 10% FBS and 160 μmol L^{-1} sodium oleate (Sigma-Aldrich, St. Louis, MO, USA) was replaced and removed every 24 h for cell differentiation. The preadipocytes were differentiated for 48 h and harvested for RT-qPCR every 24 h.

2.4. Transfection and luciferase activity assay (experiments 2, 4, 6, 7, and 8)

When the confluence of DF-1 and ICP1 cells reached 70–80%, series of plasmids or vectors and phRL-TK luciferase reporter vector were transiently transfected into these cells using Lipofectamine 2000 Reagent (Invitrogen, USA). After 48 h of transfection, the cells were collected, and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions with a FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany). Firefly luciferase (Fluc) activity was normalized to Renilla luciferase (Rluc) activity.

2.5. Western blotting (experiments 2 and 5)

Protein was extracted from DF-1 and ICP1 cells transfected with the pCMV-HA-PPARγ1 or pCMV-HA-PPARγ2 plasmid for 48 h using NE-PER Extraction Reagents (Pierce, Waltham, MA, USA). For Western blotting analysis, 40 μg of protein sample mixed with 6× loading buffer (TransGen Biotech, Beijing, China) was denatured at 100°C for 5 min and separated by 10% SDS-PAGE, followed by transfer to a nitrocellulose (NC) membrane (0.22 μm; Null, Millipore). Next, the membrane was blocked for 2 h and incubated overnight at 4°C on a shaker using the appropriate primary antibody (anti-HA; ZSGB-Bio, Beijing, China). The next day, after incubation for 1 h at room temperature on a shaker with an HRP-conjugated secondary antibody (ZSGB-Bio), specific protein bands were visualized using a BeyoECL Plus Kit (Beyotime) with the ImageQuant LAS 500 System (Sage Creation, Beijing, China).

2.6. RNA extraction and qPCR (experiments 1 and 3)

Total RNA of the DF-1 and ICP1 cells was extracted using a TRIzol Reagent Kit (Invitrogen) following the manufacturer's protocol. The expression levels of target genes were quantified by reverse transcription followed by qPCR. First-strand cDNA synthesis was performed using 1 μg of total RNA from each sample according to the manufacturer's directions of the ImPromII^{TM} Reverse Transcription System (TaKaRa, Dalian, China). qPCR was performed using the FastStart Universal SYBR Green Master Kit (Roche Life Science, Indianapolis, IN, USA) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR amplification conditions were: one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curves were analysed using MELTING CURVE 1.0 Software (Applied Biosystems, USA) for each PCR to detect and eliminate possible primer-dimer artefacts. The relative expression level of target genes was calculated using the $2^{-\Delta\Delta C_t}$ method. TATA-box binding protein (TBP) and β-actin were used as the reference genes. The sequences of primers used for qPCR are given in Table 1.

2.7. Co-immunoprecipitation (Co-IP) (experiment 5)

To confirm the protein interaction of PPARγ with RXRα in chickens, co-IP was performed using Protein A/G Magnetic Beads (MedChem Express, Shanghai, China) following the manufacturer's instructions. First, 400 μL of 50 μg mL^{-1} of HA Taq antibody was added to an EP tube containing 40 μL of suspended magnetic beads. After incubation at 4°C for 150 min, the mixture of magnetic beads and HA antibody was collected using a magnetic frame. Second, 400 μL of protein sample extracted from

DF-1 cells transfected with the pCMV-HA-PPARγ plasmid for 24 h was added to the bead-antibody mixture. After incubation at 4°C for 150 min, the bead-antibody-antigen mixture was collected. Third, 40 μL of 1× SDS-PAGE loading buffer was added to the bead-antibody-antigen mixture and heated at 95°C for 5 min. The magnetic beads were extracted using a magnetic frame, and then the supernatant containing proteins precipitated by HA antibody was collected and SDS-PAGE and Western blotting were performed.

2.8. EMSA (experiment 9)

The nuclear extract of DF-1 cells was collected after transfection with the pCMV-HA-PPARγ2 expression plasmid for 48 h using NE-PER extraction reagents (Thermo Fisher Scientific, Shanghai, China). After incubation with a specific DNA probe (5′ terminal biotin labelling) of the *cPlin1* gene promoter at room temperature for 20 min, the DNA-protein complex was separated by 6% nondenatured polyacrylamide gel electrophoresis using 0.5× TBE loading buffer, followed by transfer to a nylon membrane (Pierce, Waltham, MA, USA). The membranes were then cross-linked in an ultraviolet ray cross linker for 1 min. The signal was detected using Chemiluminescent EMSA Kit (Beyotime) according to the manufacturer's instructions. In the competitive experiment, the nuclear extract was incubated with different amounts of unlabelled *cPlin1* gene promoter DNA probe at room temperature for 10 min, followed by the addition of labelled probe and incubation at room temperature for 20 min. In the supershift assay, the DNA-protein complex was incubated with specific PPARγ antibodies (Abcam, Cambridge, MA, USA) at room temperature for 30 min before electrophoresis. The sequences of the probes for EMSA are shown in Table 2.

2.9. Statistical analysis

The experimental data in this study were analysed using JMP Pro 16 Software and are presented as means±standard deviation (SD). Student's *t*-test was performed on the data of experiments 1 and 7; one-way ANOVA was performed on the data of experiments 2, 3, 4 and 6; 2×2 two-way ANOVA was performed on the data of experiment 8. The least significant difference (LSD) method was used to compare the differences between treatment means. Differences were considered statistically significant at $P<0.05$. For each experiment, each well was an experimental unit.

Table 2 Sequences of the probes used for EMSA

Primer	Sequence (5′→3′)	Length (bp)
-1126/-1116	F: TTTGTAGAGGAAAGGTGACTGAGG	24
	R: CCTCAGTCACCTTTCCTCTACAAA	
Mut-1126/-1116	F: TTTGTAAACCTGCTATGACTGAGG	24
	R: CCTCAGTCATAGCAGGTTTACAAA	

3. Results

3.1. Effect of PPARγ agonist on *cPlin1* gene expression (experiment 1)

To evaluate whether the *cPlin1* gene is the target gene of PPARγ, troglitazone (TGZ), a specific agonist of PPARγ (Baek *et al.* 2003; Li *et al.* 2005), was added to differentiated primary chicken preadipocytes. The mRNA expression of the *LPL*, *A-FABP*, *FAS*, *Plin1*, and *PPARγ* genes was detected at different time points after TGZ treatment. TGZ significantly increased ($P<0.05$) the mRNA expression levels of the *A-FABP* and *Plin1* genes at 24 and 48 h and the *LPL* and *FAS* genes at 48 h compared with those of the control group (Fig. 1). This finding suggests that *cPlin1* gene expression may be regulated by PPARγ.

3.2. Effect of PPARγ on the promoter activity and mRNA expression of the *cPlin1* gene (experiments 2, 3, and 4)

To determine the regulatory role of PPARγ on the *cPlin1* gene, the effect of two chicken PPARγ protein isoforms on the promoter activity of the *cPlin1* gene was analysed. After cotransfection of pCMV-HA-PPARγ1 or pCMV-HA-PPARγ2, eukaryotic expression plasmids that express two chicken PPARγ protein isoforms (Fig. 2-A), with pGL3-PLIN-1992/-11 plasmid into DF-1 and ICP1 cells, respectively, luciferase activity was detected in each group with or without rosiglitazone (Rosi), a PPARγ-specific agonist. In DF-1 and ICP1 cells, PPARγ2 overexpression significantly enhanced ($P<0.01$) the activity of the *cPlin1* gene promoter in the presence of Rosi, while PPARγ1 overexpression showed no enhanced activity in either cell line (Fig. 2-B–E).

To ensure that the promoter fragment (2.0 kb) cloned into the reporter gene vector was not too short to truly reflect the regulatory role of PPARγ1 on the *cPlin1* gene, two PPARγ isoforms were overexpressed in DF-1 and ICP1 cells. After transfection of the pCMV-HA-PPARγ1 or pCMV-HA-PPARγ2 plasmid for 48 h, the expression levels of the endogenous *cPlin1* gene were detected. PPARγ2, but not PPARγ1, promoted ($P=0.0749$ or $P<0.01$) the expression of the *cPlin1* gene in DF-1 and ICP1 cells

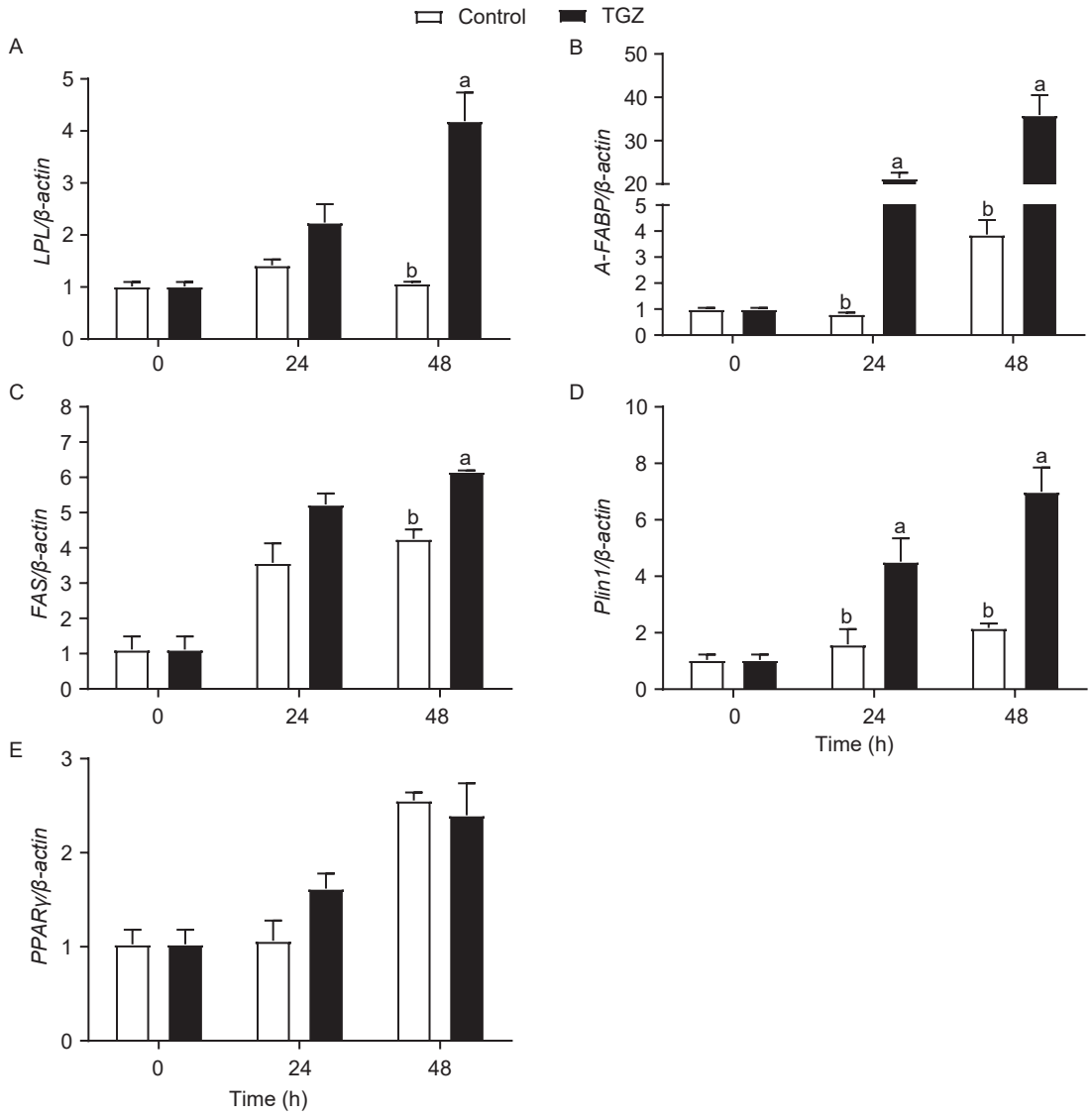

Fig. 1 Effect of troglitazone (TGZ) on the mRNA expression levels of the *LPL* (A), *A-FABP* (B), *FAS* (C), *Plin1* (D), and *PPARγ* (E) genes in primary chicken preadipocytes (experiment 1). After the primary chicken preadipocytes had differentiated for 24 h, 10 μmol L^{-1} TGZ (black column) and 0.1% (v/v) DMSO (white column, control) were added to the cells. The preadipocytes were then collected at different time points (0, 24, and 48 h), and the mRNA expression levels of the *LPL*, *A-FABP*, *FAS*, *Plin1* and *PPARγ* genes were measured by RT-qPCR. The chicken *β-actin* gene was used as the reference gene. The bar is SD, *n*=3. Different letters on the histogram indicate significant differences (P<0.05) in the mRNA expression of each group.

(Fig. 3), a finding that was consistent with the results of the promoter activity analysis.

To further verify the main PPARγ protein isoforms that regulate *cPlin1* gene expression, we analysed the effects of PPARγ1 and PPARγ2 on the promoter activity of the *cPlin1* gene using different concentrations of Rosi in DF-1 and ICP1 cells. The promoting effect of PPARγ2 on the promoter activity of the *cPlin1* gene was enhanced (P<0.01) with increasing Rosi concentration in these two cell lines. However, even if the concentration of Rosi was raised to 20 μmol L^{-1}, PPARγ1 still did not affect the promoter activity of the *cPlin1* gene in either cell line (Fig. 4). This finding indicated that the regulatory activity of PPARγ2 was more sensitive to Rosi than PPARγ1 and that PPARγ2 was the main protein isoform that regulated *cPlin1* gene expression.

3.3. Effect of PPARγ and RXRα on the promoter activity of the *cPlin1* gene (experiments 5 and 6)

Mammalian studies have shown that PPARγ forms a heterodimer with RXRα after activation by ligands and then recognizes and binds to the specific DNA sequence upstream of the promoter to regulate *Plin1*

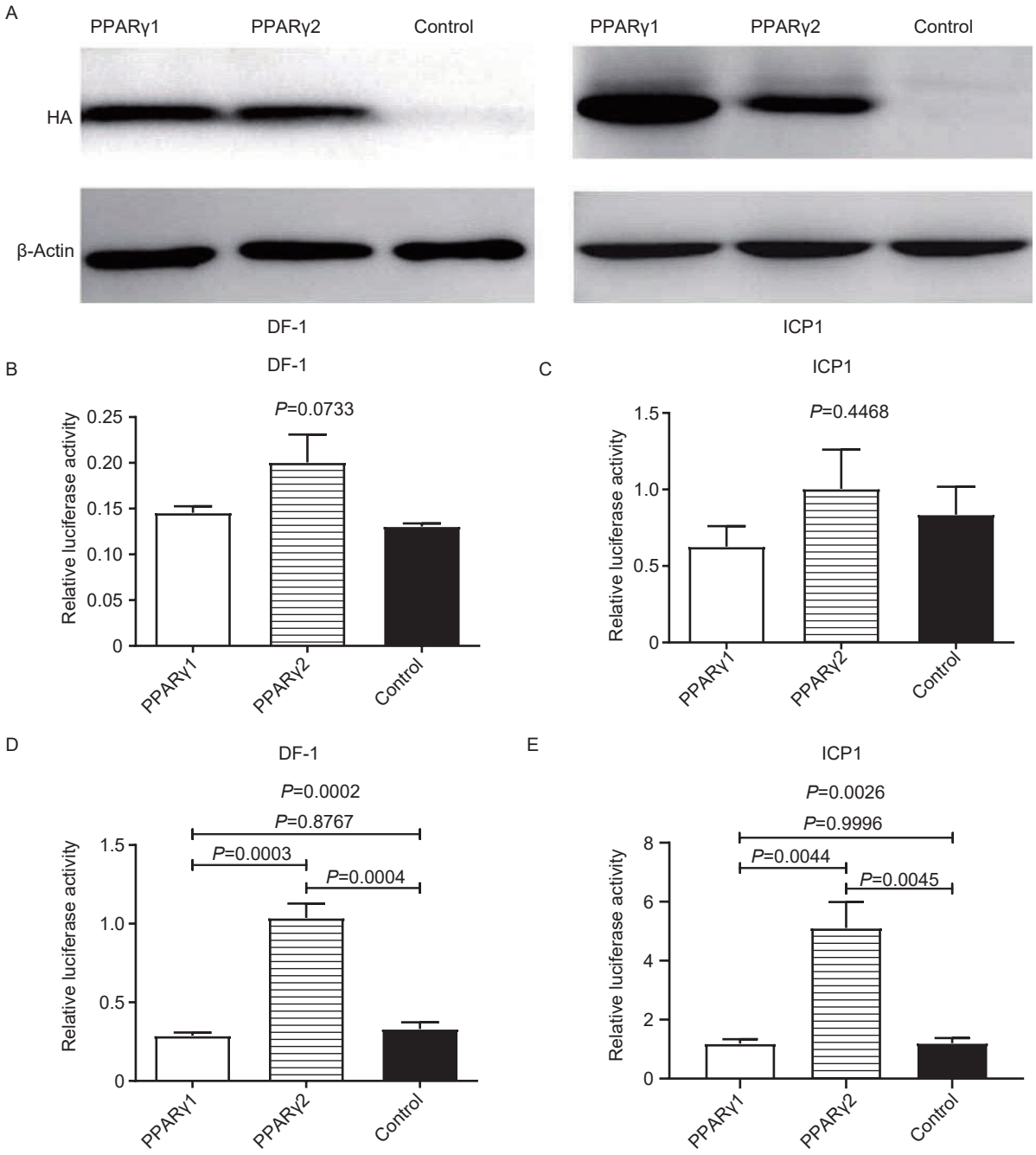

Fig. 2 Effects of PPARγ on the promoter activity of the *cPlin1* gene (experiment 2). A, Western blotting analysis of two chicken PPARγ protein isoforms in DF-1 and ICP1 cells transfected with pCMV-HA-PPARγ1, pCMV-HA-PPARγ2 and pCMV-HA plasmids. B and C, effect of PPARγ on the promoter activity of the *cPlin1* gene in DF-1 and ICP1 cells without Rosi respectively. D and E, effect of PPARγ on the promoter activity of the *cPlin1* gene in DF-1 and ICP1 cells with Rosi respectively. Reporter gene activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase activity (Fluc/Rluc). PPARγ1, PPARγ2 and control indicate transfection of the pCMV-HA-PPARγ1, pCMV-HA-PPARγ2 and pCMV-HA plasmids, respectively. The bar is SD, *n*=3. The *P*-value of the analysis of variance is marked above the graph.

gene expression (Lee *et al.* 2012). To verify the same regulation mode of PPARγ in the *Plin1* gene in chickens, we first performed Co-IP to detect the interaction of chicken PPARγ and RXRα. After cotransfection of pCMV-Myc-RXRα and pCMV-HA-PPARγ1 or pCMV-HA-PPARγ2 into DF-1 cells, the proteins bound to PPARγ were precipitated by a HA-specific antibody. Subsequent Western blotting with a Myc-specific antibody showed a protein interaction between RXRα and PPARγ, and RXRα showed no significant differences in its ability to combine

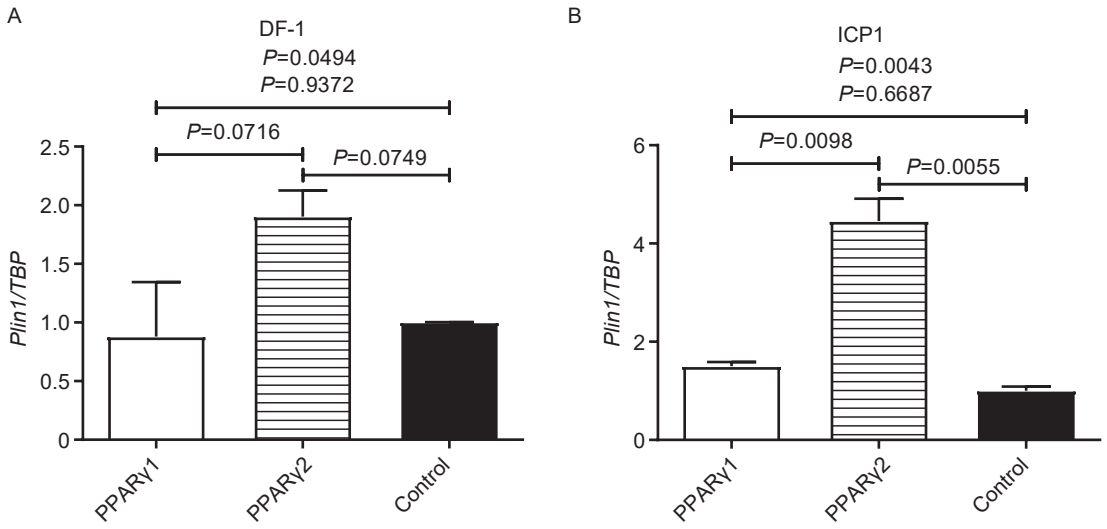

Fig. 3 Effect of PPARγ on the expression of the endogenous *cPlin1* gene (experiment 3) in DF-1 cells (A) and ICP1 cells (B). After transfection of the pCMV-HA-PPARγ1 and pCMV-HA-PPARγ2 plasmids into ICP1 and DF-1 cells for 48 h, respectively, the cells were collected and RT-qPCR was performed. The *TBP* gene was used as the reference gene. PPARγ1, PPARγ2 and control indicate transfection of the pCMV-HA-PPARγ1, pCMV-HA-PPARγ2 and pCMV-HA plasmids, respectively. The bar is SD, *n*=3. The *P*-value of the analysis of variance is marked above the graph.

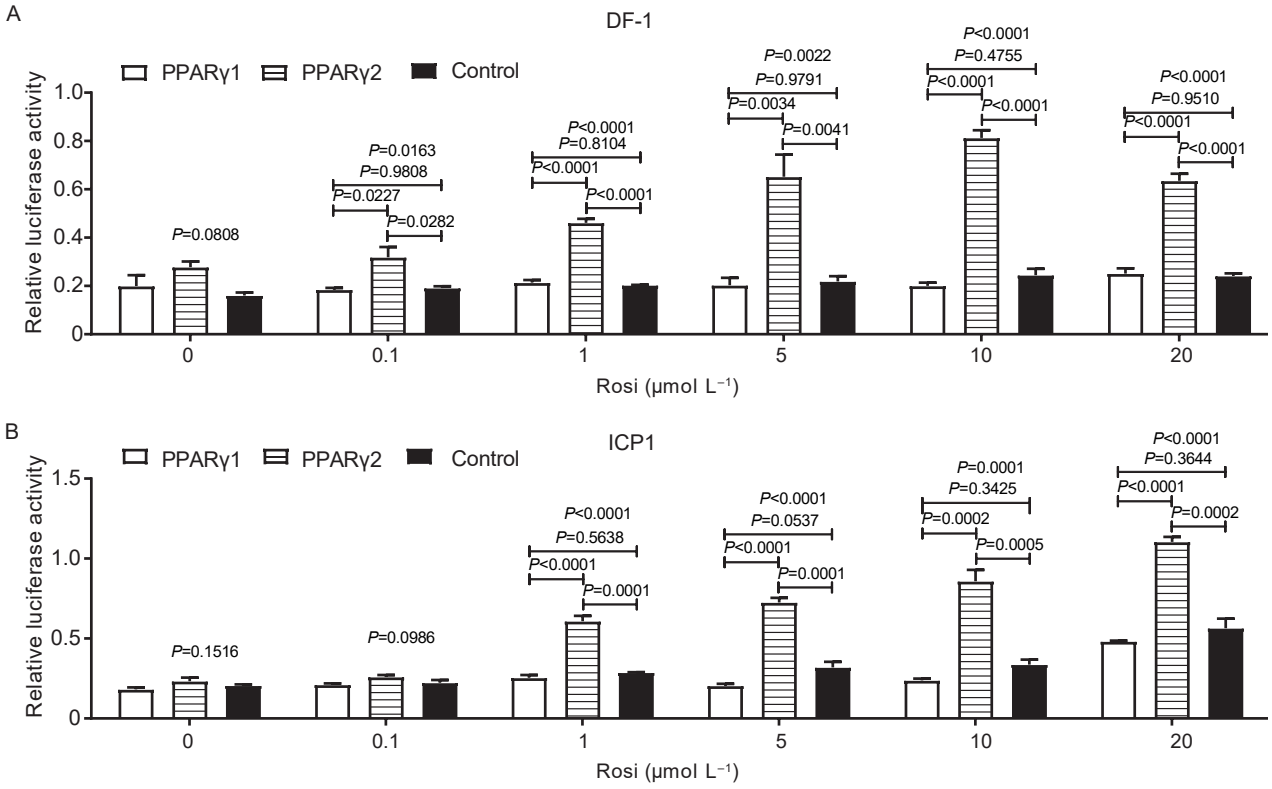

Fig. 4 Effect of PPARγ1 and PPARγ2 on the activity of the *cPlin1* gene promoter in DF-1 cells (A) and in ICP1 cells (B) with different concentrations of Rosi (experiment 4). We cotransfected pCMV-HA-PPARγ1 and pCMV-HA-PPARγ2 with the pGL3-PLIN-1992/-11 plasmid into DF-1 and ICP1 cell lines, added different concentrations of rosiglitazone (Rosi) for 48 h, and then detected luciferase activity. Reporter gene activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase activity (Fluc/Rluc). PPARγ1, PPARγ2 and control indicate transfection of the pCMV-HA-PPARγ1, pCMV-HA-PPARγ2 and pCMV-HA plasmids, respectively. The bar is SD, *n*=3. The *P*-value of the analysis of variance is marked above the graph.

with PPARγ1 or PPARγ2 (Fig. 5).

Next, to confirm the cooperation between PPARγ and RXRα in promoting *cPlin1* gene expression, we detected the effect of PPARγ and RXRα coexpression on the promoter activity of the *cPlin1* gene. In DF-1 and ICP1 cells, PPARγ2 and RXRα coexpression significantly enhanced (P<0.01) the promoter activity of the *cPlin1* gene compared with RXRα expression alone. However, PPARγ1 and RXRα coexpression did not show a similar phenomenon (Fig. 6). Thus, both PPARγ1 and PPARγ2 bind to RXRα in chickens, although *cPlin1* gene transcription is mainly regulated by PPARγ2, not PPARγ1.

3.4. Identification of binding sites for PPARγ2 that regulate *cPlin1* gene expression (experiments 7, 8, and 9)

To determine the regulatory region of the *cPlin1* gene promoter by PPARγ2, DF-1 and ICP cells were cotransfected with luciferase reporter gene plasmids containing serially truncated *cPlin1* gene promoter sequences and pCMV-HA-PPARγ2 or control (pCMV-HA) plasmids. In DF-1 cells, PPARγ2 overexpression significantly promoted (P<0.05) *cPlin1* gene promoter

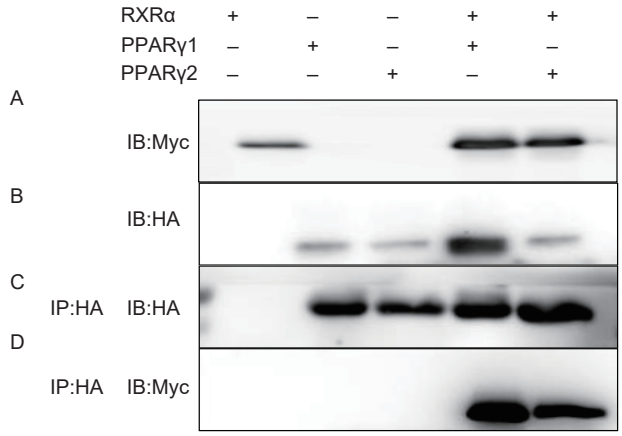

Fig. 5 Protein interaction of chicken PPARγ with RXRα (experiment 5). We transfected pCMV-Myc-RXRα, pCMV-HA-PPARγ1, pCMV-HA-PPARγ2, pCMV-HA-PPARγ1/pCMV-Myc-RXRα and pCMV-HA-PPARγ2/pCMV-Myc-RXRα plasmids into DF-1 cells. After 48 h of transfection, the expression and interactions of the RXRα, PPARγ1 and PPARγ2 proteins were detected by Western blotting and Co-IP. A, Western blotting of RXRα protein expression using the Myc Taq antibody. B, Western blotting of PPARγ1 or PPARγ2 protein expression using the HA Taq antibody. C, Co-IP was performed using the HA Taq antibody, and then PPARγ1 or PPARγ2 protein expression was detected by western blotting using the HA Taq antibody. D, Co-IP was performed using the HA antibody, and then RXRα protein expression was detected by Western blotting using the Myc Taq antibody.

activity with different fragments except the –838 bp/–11 bp region. Additionally, with the truncation of the promoter segment from –1 307 to –838 bp, the regulatory effect of PPARγ2 on *cPlin1* changed from promotion to inhibition (Fig. 7-A). A similar trend was observed in ICP1 cells (Fig. 7-B). These results suggest that sites positively regulated by PPARγ2 may exist in the –1 307 bp/–838 bp regions of the *cPlin1* gene promoter.

Consistent with the above results, we analysed the possible PPARγ-specific binding sites (PPREs) in the –1 307 bp/–838 bp region of the *cPlin1* promoter using JASPA (http://jaspar.genereg.net/) and identified a putative PPARγ-specific binding site in the –1 126 bp/–1 116 bp region. To verify the authenticity of this site, some bases of the predicted PPARγ-binding site were mutated by site-directed mutagenesis using DNA synthesis, and mutant reporter constructs were generated. Subsequent cotransfection and luciferase assays showed that, in DF-1 and ICP1 cells, PPARγ2 overexpression significantly increased (P<0.05) the promoter activity of the wild-type and mutant *cPlin1* genes (Fig. 8). However, compared with the wild-type, the enhancement effect of PPARγ2 on the promoter activity of the mutator was decreased to a significant level in DF-1 cell and ICP1 cells, respectively (Fig. 8), indicating that this PPARγ-binding site (–1 126 bp/ –1 116 bp) is required for PPARγ2-mediated positive activation of the *cPlin1* promoter.

To confirm that the predicted –1 126 bp/–1 116 bp sites are directly recognized by PPARγ2, we generated a probe corresponding to the –1 126 bp/–1 116 bp sequence and the Mut-1126/-1116 mutated probe containing a *GAGGAAAGGT* to *AACCTGCTAT* mutation in the putative binding site. We used these two probes in the electrophoretic mobility shift assay (EMSA) with recombinant nuclear PPARγ2 protein. A distinct shift was observed in the DNA–protein complex in the presence of the -1126/-1116 probe and PPARγ2 nucleoprotein (Fig. 9-A, lane 2). The signal of the DNA-protein complex gradually disappeared in the presence of a 40-, 60- and 80-fold molar excess of unlabelled probes (Fig. 9-A, lanes 3–5), indicating that the DNA binding was sequence-specific. Notably, complex signals were still observed after incubation with the Mut-1126/-1116 probe and PPARγ2 nuclear extract (Fig. 9-A, lane 7). This finding indicates that, although these base mutations weakened the combination of DNA and protein, they failed to completely eliminate the combination, consistent with the results of the reporter gene experiment (Fig. 8).

To confirm that PPARγ2 specifically binds to the predicted binding site, a supershift assay was performed using the PPARγ-specific antibody. In contrast with

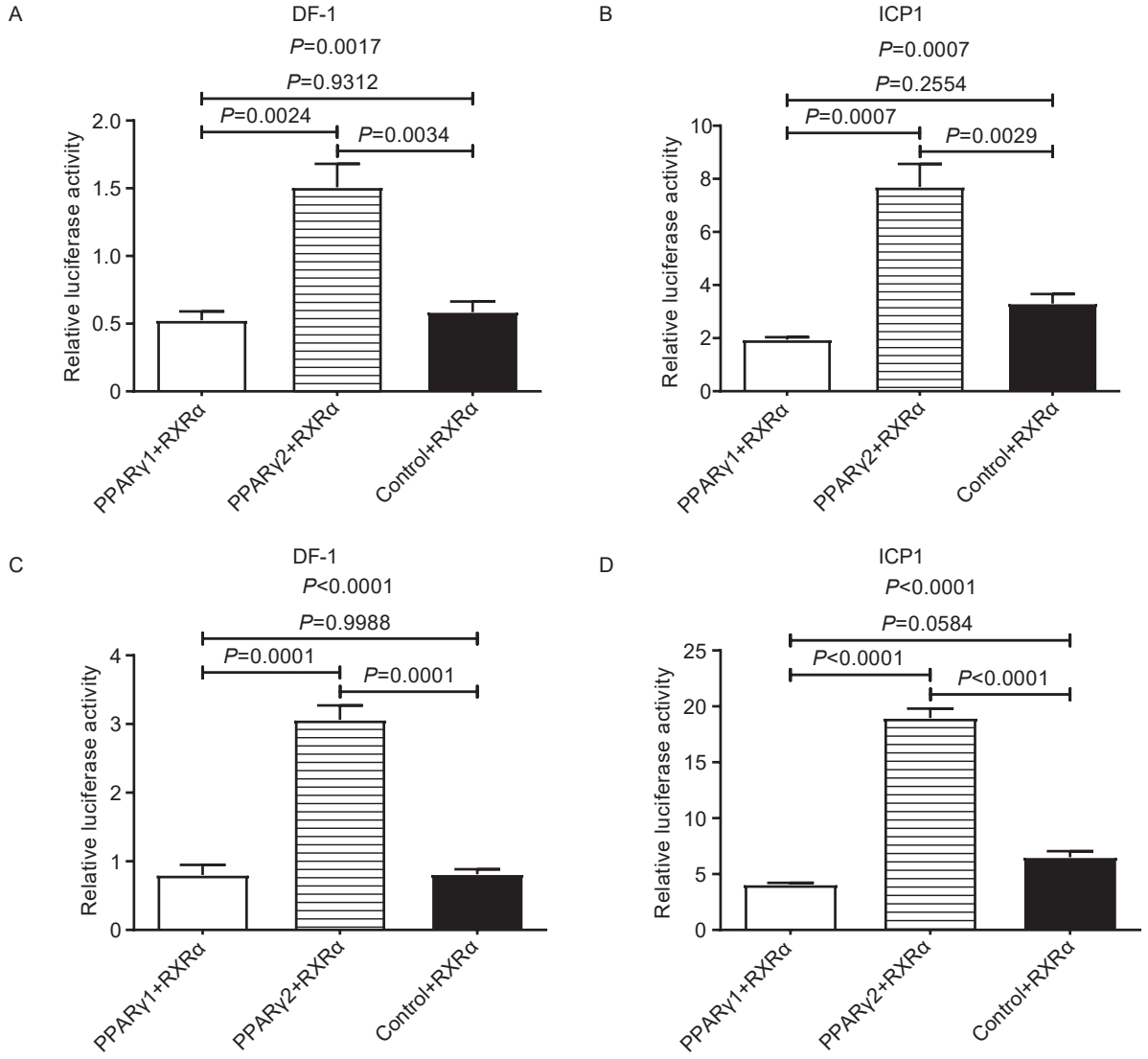

Fig. 6 Effect of the cooperation of PPARγ and RXRα on *cPlin1* gene promoter activity (experiment 6). We cotransfected pCMV-HA-PPARγ1 or pCMV-HA-PPARγ2 with pCMV-Myc-RXRα and pGL3-PLIN-1992/-11 into DF-1 and ICP1 cells, respectively. After 48 h of treatment with or without Rosi, luciferase activity was detected. A and B, effect of PPARγ and RXRα on the promoter activity of the *cPlin1* gene in DF-1 and ICP1 cells without Rosi, respectively. C and D, effect of PPARγ and RXRα on the promoter activity of the *cPlin1* gene in DF-1 and ICP1 cells with Rosi, respectively. Reporter gene activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase activity (Fluc/Rluc). The bar is SD, n=3. The P-value of the analysis of variance is marked above the graph.

the addition of IgG antibody (negative control), addition of PPARγ antibody resulted in a retarded band that corresponded to DNA-protein-antibody complexes (Fig. 9-B). These results suggest that the PPARγ2 protein could specifically recognize and bind to site –1 126 bp/–1 116 bp of the *cPlin1* gene promoter.

4. Discussion

PPARγ is a ligand-dependent transcription factor in the nuclear receptor superfamily and a major regulator in the growth and development of adipose tissue (Martin and Parton 2006; Puri *et al.* 2007). In mammals, PPARγ could regulate the transcription of *Plin1* gene by binding the functional response elements (PPREs) of PPARγ located in the 5´ flanking region of the *Plin1* gene (Arimura *et al.* 2004; Dalen *et al.* 2004; Nagai *et al.* 2004). However, the transcriptional regulatory effect of PPARγ on the *Plin1* gene in chickens and its molecular mechanism have not been reported. In this study, our hypothesis that a certain PPARγ protein isoform would activate the transcription of *cPlin1* gene by binding to a specific site located on

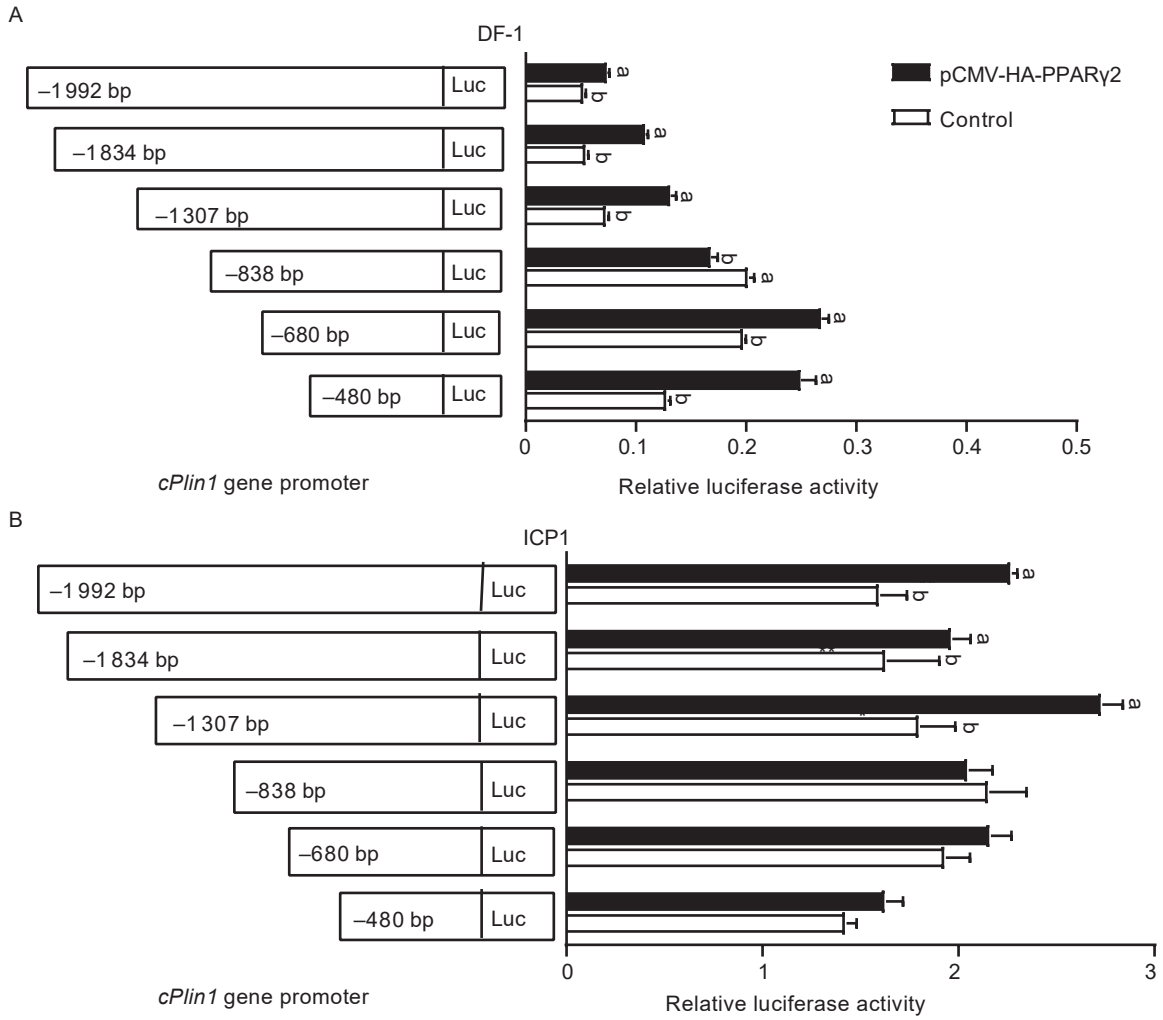

Fig. 7 Effect of PPARγ2 on the activity of the serially truncated *cPlin1* gene promoter (experiment 7). We cotransfected pCMV-HA-PPARγ2 using a series of *cPlin1* gene truncated promoter reporter gene plasmids into DF-1 and ICP1 cells and detected luciferase reporter activity 48 h later. A and B, regulatory effects of PPARγ2 on *cPlin1* promoter truncation mutants in DF-1 and ICP1 cells respectively. Reporter gene activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase activity (Fluc/Rluc). Control indicates transfection of the pCMV-HA plasmid. The bar is SD, n=3. Different letters on the histogram indicate significant differences (P<0.05) in the reporter gene activity of each group.

the promoter of *cPlin1* gene has been supported by the results of the present study. First, we found that a specific agonist of PPARγ could enhance the mRNA expression of *cPlin1* gene, suggesting the *cPlin1* gene is the target gene of PPARγ. Second, the results of luciferase reporter assay and expression analysis showed that PPARγ2, but not PPARγ1, played a major regulatory role on *cPlin1* gene. Finally, the results of promoter truncation and mutation analysis and EMSA assay showed that PPARγ2 could bind to the –1 126 bp/–1 116 bp sites in the promoter of *cPlin1* gene to enhance its expression. Our study provided a strong sense of the molecular mechanism of chicken PPARγ on the transcriptional regulation of *Plin1* gene, and provided better understanding of the molecular genetic basis of chicken adipose tissue growth and development.

In the current study, we first examined the effect of TGZ, a ligand that induces the activation of PPARγ (Nazim *et al*. 2017), on *Plin1* expression and PPARγ target genes in chicken adipocytes. TGZ significantly increased (P<0.05) the mRNA expression of the *LPL*, *A-FABP*, *FAS*, and *Plin1* genes during the differentiation of chicken adipocytes. Studies have shown that *A-FABP* (Chen *et al.* 2016), *LPL* (Han *et al.* 2021) and *FAS* (Wang *et al.* 2018) are downstream target genes of PPARγ, suggesting that *cPlin1* gene expression is also regulated by PPARγ.

The PPARγ gene is a multipromoter regulator. PPARγ has two protein isoforms in both humans (Villapol 2018; Skat-Rordam *et al.* 2019) and mice (Viccica *et al.* 2010). Studies in mammals have shown that the two

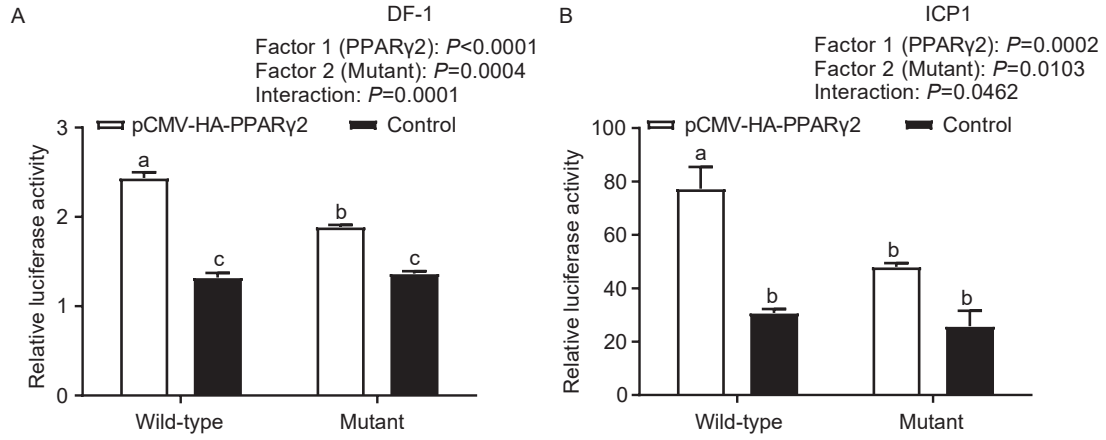

Fig. 8 Effects of mutation of the predicted PPARγ2-binding sites on PPARγ2-regulated activation of the *cPlin1* gene promoter (experiment 8). DF-1 and ICP1 cells were cotransfected with wild-type (pGL3-PLIN-1307/-11) or mutant (pGL3-Mut-PLIN-1307/-11) plasmids and the pCMV-HA-PPARγ2 or pCMV-HA plasmid. After 24 h of transfection, the cells were collected and luciferase activity was detected. A and B, luciferase reporter assay in DF-1 and ICP1 cells respectively. Reporter gene activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase activity (Fluc/Rluc). Control indicate transfection of the pCMV-HA plasmid. The bar is SD, *n*=3. Different letters on the histogram indicate significant differences (P<0.05) in the reporter gene activity in each group.

protein isoforms of PPARγ (PPARγ1 and PPARγ2) are essential for adipocyte differentiation and adipose tissue development (Mueller *et al.* 2002). The expression patterns and effects of PPARγ1 and PPARγ2 on lipogenesis are different. PPARγ1 is expressed in various tissues and cells, while PPARγ2 is mainly expressed in adipose tissue under normal physiological conditions (Vidal-Puig *et al.* 1996); additionally, PPARγ2 is more potent than PPARγ1 in inducing adipogenesis (Ren *et al.* 2002). In chickens, two types of PPARγ protein isoforms are observed (Duan *et al.* 2015). PPARγ1 is widely distributed in the body and mainly involved in energy metabolism balance, including lipid storage. PPARγ2 is mainly distributed in adipocytes and liver cells and regulates insulin resistance and adipose tissue development. PPARγ1 and PPARγ2 in chickens also show certain differences in regulating preadipocyte proliferation, apoptosis, and differentiation (Mu *et al.* 2020). These findings suggest that distinct expression regulation mechanisms may exist between PPARγ isoforms in chickens.

To test this hypothesis, the transcriptional activity and endogenous expression level of the *cPlin1* gene were detected after the overexpression of PPARγ1 or PPARγ2. In ICP1 cells, PPARγ2 overexpression significantly enhanced (P<0.01) the promoter activity and mRNA expression level of the *cPlin1* gene (Figs. 2-E and 3-B), and the promoting effect of PPARγ2 on the activity of the *cPlin1* gene promoter increased (P<0.01) as the Rosi concentration increased (Fig. 4); however, PPARγ1 overexpression did not show a similar promoting effect on the *cPlin1* gene. Mammalian studies revealed that ligand-activated PPARγ preferentially forms heterodimers with RXRα, thus playing a transcriptional regulatory role in the *Plin1* gene (Zou *et al.* 2010). We also found that when RXRα was co-transcribed and activated, the transcriptional regulation of the *cPlin1* gene by PPARγ2 was significantly higher (P<0.01) than that by RXRα alone, although the coexpression of RXRα with PPARγ1 and expression of RXRα alone showed no differences in promoting *cPlin1* gene expression (Fig. 6). Based on the above results, we conclude a synergistic effect exists between PPARγ and RXRα in chickens, the transcriptional activation activity of PPARγ2 is higher than that of PPARγ1, and the transcription of the *cPlin1* gene is mainly regulated by PPARγ2, consistent with the findings in mammalian studies (Nagai *et al.* 2004). However, our previous research showed that RXRα positively regulates expression of the chicken *Plin1* gene in a PPARγ-independent manner (Sun *et al.* 2020), which is quite different to the studies in mammals (Arimura *et al.* 2004). These results indicate the transcriptional regulation mechanism of *Plin1* gene in chicken is not exactly same to that in mammals.

To further determine the molecular mechanism by which PPARγ2 regulates *cPlin1* gene expression, we implemented a truncation strategy at the 5′ end of the promoter to identify the main regulatory regions. The regulatory effect of PPARγ2 on the expression of the *cPlin1* gene changed dramatically (P<0.05) as the promoter segment was truncated from –1 307 to –838 bp, indicating a potential PPARγ2-binding site in this region.

JASPA analysis identified a potential PPARγ binding site, GAGGAAAGGTGA, in the core binding region, which is highly conserved in humans and mice (Nagai *et al.* 2004), with sequence homologies of 69.23 and 76.92%, respectively. The decreased promoting effect of PPARγ2 on the reporter gene activity of the mutant plasmid also indicates that the binding site –1 126 bp/–1 116 bp is necessary for PPARγ2 to promote the transcription of the *cPlin1* gene. The subsequent EMSA confirmed this hypothesis. In the competition experiment, these special DNA-protein complex bands gradually disappeared with the addition of excess unlabelled probe (Fig. 9-A). Additionally, after adding of a specific PPARγ antibody, a retarded band corresponding to the DNA-protein-antibody complex appeared above the single-shifted DNA-protein complex band (Fig. 9-B, lane 3), which confirms that PPARγ2 binds specifically to the predicted binding site. These results indicate that the DNA probe specifically recognizes and binds to the cell protein that overexpresses PPARγ2. Thus, the predicted binding site of –1 126 bp/–1 116 bp in the promoter is indeed a real binding site for PPARγ2-associated regulation of *cPlin1* gene expression.

5. Conclusion

In summary, similar to that in mammals, chicken PPARγ has a positive regulatory effect on the transcription of the *cPlin1* gene, and the PPARγ2 inform is the main regulator that promotes *cPlin1* gene expression by binding to the conserved site located in the –1 126 bp/–1 116 bp promoter region. These results indicate that the mechanism of *Plin1* regulation by PPARγ is evolutionarily conserved between mammals and birds.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Ethical approval

All animal work was conducted in accordance with the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of China (approval no. 2006–398) and was approved by the Institutional Biosafety Committee of Northeast Agricultural

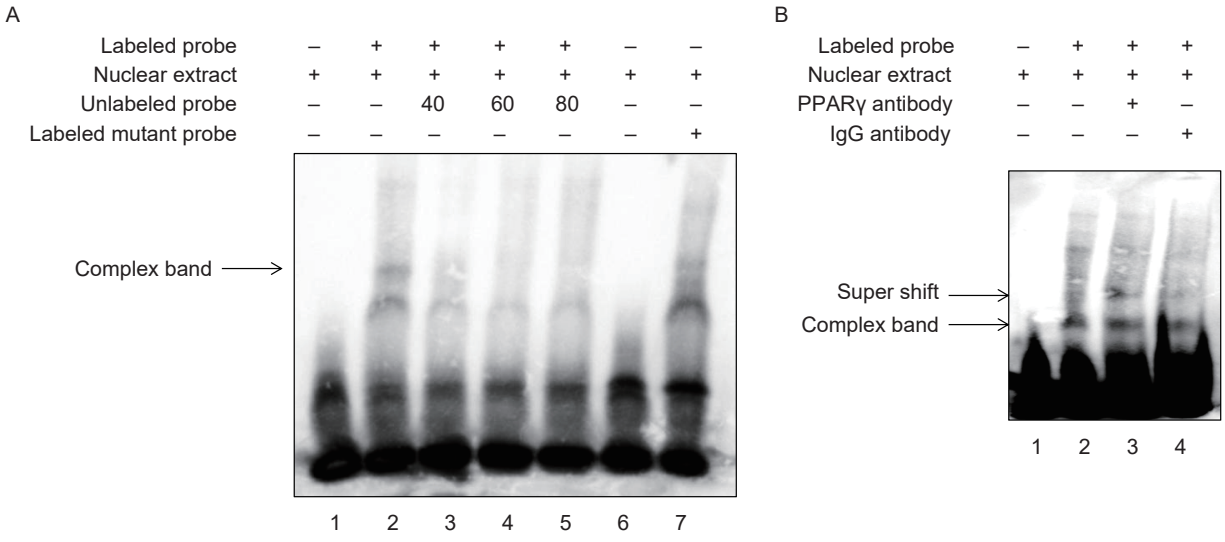

Fig. 9 Analysis of PPARγ2 binding sites in the –1 126 bp/–1 116 bp region using EMSA (experiment 9). A, analysis of the binding affinity of the –1 126 bp/–1 116 bp site for the PPARγ2 protein. The probe corresponding to –1 126 bp/–1 116 bp was synthesized and labelled with biotin. Nuclear extracts were prepared from DF-1 cells transfected with the pCMV-HA-PPARγ2 plasmid. Competition EMSA was performed using a 40-, 60-, and 80-fold molar excess of unlabelled probe (lanes 3–5). The Mut-1126/-1116 probe, whose binding site was mutated from *GAGGAAAGGT* to *AACCTGCTAT*, was incubated with PPARγ2 nuclear extract (lane 7). B, identification of protein binding to the –1 126 bp/–1 116 bp site. A supershift assay was performed using an PPARγ antibody, which identified overexpressed PPARγ2 protein (lane 3). IgG antibody was used as a negative control.

University (Harbin, China). Plasmid construction and transfection were performed according to the directions of the Regulation on Safety Administration of Agricultural Genetically Modified Organisms (RSAGMO) established by China (revised version 2017).

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