

Published in final edited form as:

J Biol Chem. 2005 July 22; 280(29): 27466–27476. doi:10.1074/jbc.M503836200.

The Peroxisome Proliferator-activated Receptor- γ Regulates Murine Pyruvate Carboxylase Gene Expression *in Vivo* and *in Vitro**

Sarawut Jitrapakdee^{‡,§}, Marc Slawik^{‡,¶}, Gema Medina-Gomez[‡], Mark Campbell[‡], John C. Wallace^{||}, Jaswinder K. Sethi[‡], Stephen O’Rahilly[‡], and Antonio J. Vidal-Puig[‡]

[‡]Cambridge Institute of Diabetes, Endocrinology, and Metabolism and the Department of Clinical Biochemistry, University of Cambridge, Addenbrooke’s Hospital, Cambridge, CB2 2QR, United Kingdom

^{||}School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia

Abstract

Pyruvate carboxylase (PC) plays a crucial role in various metabolic pathways, including gluconeogenesis, lipogenesis, and glucose-induced insulin secretion. Here we showed for the first time that the PC gene is transcriptionally regulated by peroxisome proliferator-activated receptor- γ (PPAR γ) *in vitro* and *in vivo* in white and brown adipose tissue. PC mRNA and protein are markedly increased during differentiation of 3T3-L1 cells and HIB-1B, in parallel with the expression of the adipogenic transcription factors, CCAAT-enhancer binding protein α , PPAR γ 1, and PPAR γ 2. Tumor necrosis factor- α , a cytokine that blocks differentiation of 3T3-L1 cells, suppressed PC expression. Co-transfection studies in 3T3-L1 preadipocytes or HEK293T cells with a 2.3-kb promoter fragment of mouse PC gene linked to a luciferase reporter construct and with plasmids overexpressing retinoid X receptor α /PPAR γ 1 or retinoid X receptor α /PPAR γ 2 showed a 6–8-fold increase above the basal promoter activity. Furthermore, treatment of these transfected cells with the PPAR γ agonist doubled the promoter activity. Mutation of the putative PPAR-response element (–386/–374) of this 2.3-kb PC promoter fragment abolished the PPAR γ response. Gel shift and chromatin immunoprecipitation assays demonstrated that endogenous PPAR γ binds to this functional PPAR-response element of the PC promoter. Mice with targeted disruption of the PPAR γ 2 gene displayed ~50–60% reduction of PC mRNA and protein in white adipose tissue. Similarly, in brown adipose tissue of PPAR γ 2-deficient mice subjected to cold exposure, PC mRNA was 40% lower than that of wild type mice. Impaired *in vitro* differentiation of white adipocytes of PPAR γ 2 knock-out mice was also associated with a marked reduction of

*This work was supported in part by a Wellcome Trust Integrative Physiology grant (to A. V. P. and S. O’R.), a Biotechnology and Biological Sciences Research Council David Phillips fellowship (to J. K. S.), and a Medical Research Council Career Establishment grant (to A. V. P.).

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.

To whom correspondence should be addressed. Tel.: 662-201-5460; Fax: 662-354-7174; scsji@mahidol.ac.th.

[§]Supported by a Royal Society International Fellowship, UK. Performed work while on sabbatical leave from the Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand.

[¶]Supported by a fellowship from the Fritz Thyssen Foundation, Germany

PC mRNA. Our findings identified PC as a PPAR γ -regulated gene and suggested a role for PPAR γ regulating intermediary metabolism.

Adipose tissue not only plays a crucial role in lipid metabolism by storing circulating free fatty acids as triglycerides, but it is also a site where *de novo* fatty acid synthesis occurs (1). The differentiation of preadipocytes to mature adipocytes is a complex process involving biochemical and morphological changes. These changes are associated with the sequential activation of pro-adipogenic transcription factors, including the CCAAT-enhancer binding protein family, C/EBP β/δ ,¹ and ADD1/SREBP1 followed by C/EBP α and peroxisome proliferator-activated receptor- γ (PPAR γ) expression. This transcriptional activation cascade in turn switches on several lipogenic genes, resulting in preadipocyte growth arrest and development of mature adipocytes (2-4).

PPAR γ , a member of the PPAR subfamily of nuclear hormone receptors, heterodimerizes with the retinoid X receptor α (RXR α) and regulates the expression of pro-adipogenic genes (3). Binding of PPAR γ -RXR α to the PPAR-response element (PPRE) of the promoter recruits co-activators and releases corepressors that in turn results in transcriptional activation (5). PPAR γ consists of two isoforms, PPAR γ 1 and PPAR γ 2, the latter of which contains 30 additional amino acids at its N terminus as the result of alternative splicing at the 5'-end of the gene (6, 7). Although many tissues express PPAR γ 1 at low levels, PPAR γ 2 is restricted to white and brown adipose tissue (8), where each isoform represents 50% of the total PPAR γ .

Pyruvate carboxylase (PC), a member of the biotin-containing enzyme family, catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate (9). The level of PC is highest in gluconeogenic tissues, *i.e.* kidney cortex and liver (10, 11), but PC activity, protein, and mRNA are also highly expressed in adipose tissue as well as in differentiated adipocytes (12-16). The role of PC in lipogenesis is thought to provide substantial amounts of an acetyl group and NADPH required for *de novo* fatty acid synthesis (17). Acetyl-CoA is generated in the mitochondria by the oxidative decarboxylation of pyruvate, and after condensation with oxaloacetate, acetyl groups are transported to the cytoplasm as citrate, which undergoes ATP-dependent cleavage to yield acetyl-CoA and oxaloacetate. This pathway requires a continuous supply of oxaloacetate, which is provided by the activity of PC. Acetyl-CoA, a building block for long chain fatty acids, is then converted into malonyl-CoA by acetyl-CoA carboxylase. The cytoplasmic oxaloacetate generated from citrate is reduced by NADH to malate, which is decarboxylated to yield pyruvate and NADPH, with the latter being necessary for *de novo* fatty acid synthesis. Pyruvate carboxylation was shown to be necessary in hamster brown adipose tissue for maximal oxygen consumption in norepinephrine-stimulated respiration, even when drainage of the citric acid cycle for amino acid synthesis is eliminated, suggesting that the provision of oxaloacetate promotes the oxidation of acetyl-CoA from fatty acid degradation (18, 19).

¹The abbreviations used are: C/EBP α , CCAAT-enhancer binding protein α ; PC, pyruvate carboxylase; PPAR γ , peroxisome proliferator activated receptor- γ ; TNF α , tumor necrosis factor- α ; RXR α , retinoid X receptor- α ; PPRE, PPAR-response element; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; mPC, murine PC; E2F, elongation factor 2F.

Here we show that the levels of PC protein and mRNA are highly correlated with the expression of PPAR γ during adipocyte differentiation. Promoter analysis and transient transfection experiments with reporter constructs show that the 2.3-kb promoter fragment of the mouse PC gene contains a functional PPRE that directly mediates a PPAR γ response. Mutational analysis, EMSA, and ChIP assay also support our *in vitro* data. PPAR γ 2 null mice (20) also show a marked reduction of PC protein and PC mRNA both in white and brown adipose tissues.

EXPERIMENTAL PROCEDURES

Animals

PPAR γ 2^{-/-} male mice with a mixed background 129SV/C57Bl6 and their wild type littermates (18) were used in this study. After weaning (age of 3 weeks), the knock-out and the wild type mice were fed with chow or a high fat diet for 4 months before being sacrificed. Fasting and refeeding experiments were carried out as described previously (20). Epididymal fat pads were removed and snap-frozen in liquid nitrogen. For cold exposure, 4-month-old knock-out mice and their wild type littermates fed with chow diet were maintained at 4 °C or at room temperature (25 °C) for 12 days before they were sacrificed. Interscapular brown adipose tissues were removed and snap-frozen in liquid nitrogen before the RNA and protein were extracted.

Cell Culture

Murine 3T3-L1 preadipocytes were routinely grown in complete media (Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 100 units/ml penicillin, 100 μ g/ml streptomycin). For differentiation of preadipocytes to mature adipocytes, 2×10^5 cells were plated in 6-well plates and maintained in the above medium until they reached confluence. At day 2 post-confluence (designated as day 0), the cells were induced to differentiate with complete medium supplemented with 0.5 mM 3-isobutylmethylxanthine, 1 μ M dexamethasone, and 10 μ g/ml insulin. After 2 days in induction medium, 3T3-L1 cells were maintained in complete medium supplemented with 10 μ g/ml insulin. The medium was changed every 2 days. Human embryonic kidney (HEK293T) cells and brown adipocyte cell line (HIB-1B) (21) were also routinely grown in the complete medium above.

Preadipocyte Isolation and Culture

Preadipocyte isolation, culture, and differentiation were performed as described previously (20). At 0, 2, 4, and 8 days after differentiation in the absence or presence of 0.1 μ M rosiglitazone, cells were harvested, and RNAs were extracted.

RNA Isolation, cDNA Synthesis, and Real Time PCR

Total RNA was extracted from 3T3-L1 cells or primary cultures using an RNeasy kit (Qiagen) or from frozen adipose tissues with STAT-60 (AMS Biotechnology) and quantified by GeneQuant (Amersham Biosciences). The quality of extracted RNA was assessed by formaldehyde gel electrophoresis. cDNA synthesis was carried out at 37 °C for 1 h in a 20- μ l reaction volume containing 0.5 μ g of total RNA, 100 ng of random primers (Promega), 1 \times

reverse transcriptase buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol), 1 mM each of dNTP, and 200 units of Moloney murine leukemia virus-reverse transcriptase (Promega). PCR was carried out with real time PCR with a Taqman^R probe (Applied Biosystems). Real time PCR was carried out in a 96-well optical plate (Applied Biosystems). Each well contained 12 µl of reaction mixture consisting of 1× ABI master mix (Applied Biosystems), 0.25 µM each of forward and reverse primers, 0.125 µM fluorogenic probe in which its 5'- and 3'-ends were modified with 6-carboxy-fluorescein and 6-carboxy-tetramethyl-rhodamine, respectively, 2 µl of 1/10 dilution of cDNA. 18 S rRNA was also amplified as the internal control using 18 S ribosomal RNA control reagent (Applied Biosystems) with 1/200 dilution of cDNA samples. The primer and probe sets used to detect various mRNAs included mPC, mPPAR γ 1, mPPAR γ 2, and mC/EBP α . They were synthesized by Sigma Genosys and are shown in Table I. The amplification profile consisted of an initial incubation at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 1 min. The relative quantities of amplified cDNAs were analyzed by the SDS software (Applied Biosystems). The abundance of mRNA was normalized with 18 S rRNA, and the value was expressed as “relative gene expression.”

Reporter Construct and Mutagenesis

The 2.3-kb fragment of the lipogenic promoter of the mouse PC gene (22) was isolated from mouse genomic DNA by PCR. The PCR was carried out in a 50-µl reaction mixture containing 1× High Fidelity PCR buffer (Roche Applied Science), 0.25 mM each of dNTP, 100 ng of mouse genomic DNA, 0.25 µM of forward primer (5'-GGGCACGCGTGGGATGAATCCCCAGGTGGGACAG-3') and reverse primer (5'-GTGCTGCACACAGAGGACGTGATAGAG-3'), and 2.5 units of Expanded High Fidelity Polymerase (Roche Applied Science). The reaction was subjected to 35 cycles of amplification in which each cycle consists of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 3 min. The PCR product was gel-purified using a gel extraction kit (Qiagen) and ligated to pGEM-T Easy vector (Promega). The nucleotide sequence of the insert was verified by automated sequencing using Big Dye (Applied Biosystems) before being restricted with MluI and BamHI and subsequently ligated to MluI- and BglII-digested pGL-3 basic (Promega). This resulting construct was designated pGL-wt mPC. The mutation of putative PPRE on a wild type promoter fragment was carried out with XL-Quick change site-directed mutagenesis kit (Stratagene) with PPREmF1 (5'-CAGCCTTCAGGCTAATGCAGCTCTCACCTCCATCATGCCTGG-3') and PPREmR1 (5'-GTGCCAGGCATGATGGAAAGCAAAAATCACATTAGCCTGAAGG-3') as the mutagenic primers. The mutagenic reaction was performed following the manufacturer's instructions, and the correct mutagenic sequence was verified by DNA sequencing.

Transient Transfection and Transactivation Study

Briefly, 2×10^5 cells of 3T3-L1 cells or 1×10^5 cells of HEK293T were plated in 24-well plates in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum 1 day before transfection. Transfection was carried out the next day using Lipofectamine 2000 reagent (Invitrogen), with 0.5 pmol of firefly luciferase reporter construct alone or with equivalent amounts of pcDNA-PPAR γ 1, pcDNA-PPAR γ 2, or

pRSV-RXR α plasmids overexpressing PPAR γ 1, PPAR γ 2, and RXR α , respectively (23). *Renilla* luciferase reporter plasmids (1 ng) driven by the thymidine kinase promoter (pRL-TK) (Promega) were also included in all transfections as an internal control. The luciferase assay was performed with 20 μ g of cell lysate using the dual luciferase assay system (Promega) in a Berthold luminometer. The firefly luciferase activity was normalized with *Renilla* luciferase and expressed as “relative luciferase activity.”

EMSA

Six day differentiated 3T3-L1 or HEK293T cells transiently transfected with plasmids overexpressing RXR α /PPAR γ 1 or RXR α /PPAR γ 2 were used as the source of nuclear extract. Briefly, 2×10^6 cells were washed with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride before being scraped and centrifuged at 1,200 rpm at 4 °C. The cell pellet was subjected to crude nuclear protein preparation using a cytosolic and nuclear protein extraction kit (Pierce). EMSA was performed using LightShift chemiluminescent EMSA kit (Pierce). The probes were prepared by annealing oligonucleotides with their 3'-end labeled with biotin. The oligonucleotides include mouse aP2 PPRE-For (5'-GATCTGTGACCTTTGACCTAGTAAG-3'; underline indicates putative PPRE sequence), aP2 PPRE-Rev (5'-CTTACTAGGTCAAAGGTACAGATC-3') (24), mPC PPRE-For (5'-CTAATGTGACCCTTGCCCTCCATCA-3'), and mPC PPRE-Rev (5'-TGATGGAGGGCAAGGGTCACATTAG-3'). The DNA-protein binding assay was performed at room temperature for 20 min in a final volume of 20 μ l containing 1 \times binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol), 2.5% (v/v) glycerol, 5 mM MgCl₂, 1 μ g of poly(dI-dC), 0.05% (v/v) Nonidet P-40, 8 pmol of double-stranded biotinylated probe, and 10 μ g of nuclear extract. The DNA-protein complexes were separated by 5% PAGE in 0.5 \times TBE at 200 V at 4 °C for 2 h. DNA-protein complexes in gel were transferred to Hybond N+ nylon membrane (Amersham Biosciences) by electroblotting with 0.5 \times TBE at 350 mA for 1.5 h. DNA-protein complexes were fixed to the membrane by UV cross-linker and detected by a nonradioactive nucleic acid detection kit (Pierce). For the competition assay, 5 \times , 10 \times , or 20 \times more concentrated double-stranded DNAs were included in the binding reaction. For supershift EMSA, 2 μ l of PPAR γ monoclonal antibody (E8) or 2 μ l of RXR α polyclonal antibody (D20) (Santa Cruz Biotechnology) were preincubated in the binding reaction for 10 min before the probe was added.

ChIP Assay

Briefly, 2×10^5 cells of 3T3-L1 plated in a 35-mm well were used in the assay. The DNA and protein were cross-linked *in situ* with 0.5% (v/v) formaldehyde at 37 °C for 5 min. Soluble chromatin was prepared using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Inc.). The lysate was sonicated four times for 10 s at 4 °C. The lysates were precipitated with either 50 μ l of anti-E2F or 10 or 50 μ l of anti-PPAR γ (E8) mouse monoclonal antibodies (Santa Cruz Biotechnology) overnight before protein A-agarose beads were added. The proteins were removed from DNA by digesting with 10 μ g/ml proteinase K at 45 °C for 30 min. The DNA was further purified by a QIAquick PCR purification kit (Qiagen). The DNA was eluted in 50 μ l of sterile water. One microliter of eluted DNA was used to amplify a 250-bp amplicon with F1 (5'-

CCTTGCTTGTGTCTGGCAGTGC-3') and R1 (5'-GCTAGAAAGCAGCTGCAGACTT-3') primers that flanked the PPRE of the mPC or to amplify a 150-bp amplicon with the "negative control primers" F2 (5'-CCTGAACCTGAAGGAGCTGGAG-3') and R2 (5'-GGGTCCTTGAAGAAAGAGACGAG-3') that are located >3 kb downstream of the PPRE for the mPC. PCR was performed for 35 cycles for which linear amplification was obtained. Ten microliters of PCR products were analyzed by 2% agarose gel electrophoresis.

Protein Extraction and Western Analysis

Total protein lysates of 3T3-L1 from each well were extracted in 100 μ l of RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture (Roche Applied Science)) for 5 min on ice. The protein lysates were recovered by centrifugation at 13,000 rpm for 5 min at 4 °C. Aliquots (50 μ g) of protein lysates were subjected to reducing SDS-PAGE (25) (10% SDS-PAGE for PPAR γ 1, PPAR γ 2, and C/EBP α or 7.5% SDS-PAGE for PC). Proteins were transferred to polyvinylidene difluoride membranes by electroblotting. The blots were blocked in 4% (w/v) skim milk in PBS-T overnight. PC, PPAR γ , and C/EBP α bands were detected by incubating the blots with PC polyclonal antibody (26), PPAR γ monoclonal antibody (E-8), or C/EBP α polyclonal antibody (14AA) from Santa Cruz Biotechnology, respectively. The polyclonal rabbit anti-p85 α subunit of phosphoinositol-3-phosphate kinase was a gift from Professor K. Siddle, Department of Clinical Biochemistry, University of Cambridge. Immunoreactive bands were visualized upon adding goat anti-rabbit or goat anti-mouse polyclonal antibodies (Dako Cytomation) conjugated with horseradish peroxidase followed by ECL Western blot detection system (Amersham Biosciences).

RESULTS

PC Is Expressed Concomitant with Adipogenic Transcription Factors during 3T3-L1 and HIB-1B Differentiation

It has been reported previously that PC activity and mRNA are induced during the conversion of 3T3-L1 preadipocyte to mature adipocyte by hormonal induction (1, 13, 15), suggesting PC may play a lipogenic role during terminal differentiation. However, the molecular mechanism controlling PC expression during adipocyte differentiation has yet to be elucidated. To address this, we correlated the window of PC expression with that of PPAR γ and C/EBP α , two major pro-adipogenic transcription factors, during differentiation of 3T3-L1. As shown in Fig. 1A, Western analysis clearly showed that PC expression is barely detectable in confluent preadipocytes but is rapidly increased at day 2 after cells were induced to differentiate. The level of PC reached a maximum by day 6 and remained unchanged thereafter. This expression pattern is also similar to those of PPAR γ 1, PPAR γ 2, and C/EBP α .

To determine whether PC induction is dependent on adipocyte differentiation, we used TNF α , a cytokine known to block adipocyte differentiation through a PPAR γ -dependent mechanism. As shown in Fig. 1A, TNF α not only markedly inhibited expression of C/EBP α ,

PPAR γ 1, and PPAR γ 2 by >90% but also dramatically suppressed PC expression. However, the suppression of PC expression was more directly correlated with the expression profile of PPAR γ , *i.e.* PC and PPAR γ levels were completely inhibited within the first 2 days of differentiation, and their expression was partially restored after day 4 and beyond. Real time RT-PCR analysis demonstrated that the induction of PC expression during differentiation occurred at the transcriptional level, *i.e.* the PC mRNA was increased ~20-fold 2 days after differentiation and reached maximum (35-fold) at day 6 (Fig. 2). The rise of PC mRNA was also closely correlated with that of PPAR γ 1, PPAR γ 2, and C/EBP α (Fig. 2).

The induction of PC expression during adipocyte differentiation was not restricted to this white adipocyte cell line as this effect was also seen in the brown adipocyte cell line HIB-1B. During confluence-induced spontaneous differentiation of HIB-1B, PC level was also markedly increased, reaching a maximal level at day 6 and remaining unchanged thereafter (Fig. 1B). This pattern was also closely related to those of PPAR γ 1, PPAR γ 2, and C/EBP α as seen during differentiation of 3T3-L1 cells.

PPAR γ but Not C/EBP α Transactivated the mPC Promoter

Because PC was expressed concomitant with C/EBP α and PPAR γ 1 and PPAR γ 2, we investigated whether PC was transcriptionally regulated by these transcription factors during adipogenesis. Therefore, we cloned a 2.3-kb DNA fragment upstream of the transcription start site of the mPC gene. This DNA fragment was extended ~1.5 kb further from the 5'-end of the 0.7-kb fragment than we have characterized previously (22). The 2.3-kb promoter fragment was cloned into the firefly luciferase reporter plasmid (PC-Luc) and was transiently co-transfected with the plasmids overexpressing C/EBP α , SREBP1c, PPAR γ 1 or PPAR γ 2, respectively, in HEK293T cells. As shown in Fig. 3A, the relative luciferase activity was unchanged when the PC-Luc construct was co-transfected with plasmids overexpressing C/EBP α or SREBP1c. However, the luciferase activity was increased 3-fold relative to the basal condition (cells transfected with PC-Luc alone), when PC-Luc construct was co-transfected with either PPAR γ 1 or PPAR γ 2 constructs. The luciferase activities were further increased to ~6-7-fold when the plasmid overexpressing RXR α , a heterodimer partner of PPAR γ , was co-transfected with either PPAR γ 1 or PPAR γ 2 constructs. Treating transfected cells with 0.1 μ M rosiglitazone (PPAR γ agonist) for 24 h doubled the luciferase activities of cells co-transfected with RXR α constructs together with PPAR γ 1 or PPAR γ 2 constructs to 12- or 14-fold, respectively. Similar results were obtained when the above assays were performed with 3T3-L1 preadipocytes (Fig. 3B). Taken together these data suggest the 2.3-kb mPC promoter fragment may contain a PPAR γ -response element (PPRE).

The mPC Promoter Contains Functional PPRE Element at Positions -386/-374

The 2.3-kb nucleotide sequence of the mPC promoter was searched for the putative transcription factor binding sites by the TESS data base (www.cbil.upenn.edu/tess/). As shown in Fig. 4A, we identified a putative PPRE (AGGGCAAGGGTCA, underline indicates two direct repeats (DR1 and DR2 respectively) separated by one nucleotide) on the opposite strand, at positions -386/-374 relative to transcription start site. This site was located upstream of the initiator site, HIP1, and two downstream GC boxes, potential

binding sites for the general transcription factor(s), Sp1/Sp3. The nucleotide sequence of this putative PPRE of PC appears to be conserved between mouse and rat (22, 27) genes (Fig. 4B). The PPRE of the mPC contains one nucleotide of each DR1 and DR2 that did not match the consensus PPRE site, *i.e.* AGGTCANAGGTCA (underline indicates DR1 and DR2) (5). Fig. 4C, shows a comparison of the putative PPRE of the mPC gene with other PPAR responsive genes that are involved in diverse metabolic pathway ranging from lipid metabolism to detoxification. The DR1 of PPRE for the mPC was identical to that of the glucose transporter 2 (GLUT2), cytochrome P450, and acyl-CoA synthase, whereas the DR2 appeared to be unique but identical to DR1 of the malic enzyme.

To determine whether the putative PPRE located at -386/-374 was functional, we mutated this site from AGGGCAAGGGTCA to AGGGTGAAGGCTA, creating the mutant construct designated as PPRE-Mut-Luc. This mutant reporter construct was co-transfected with RXR α /PPAR γ 1 or RXR α /PPAR γ 2 constructs into HEK293T cells. As shown in Fig. 5, mutation of PPRE (-386/-374) markedly reduced the PPAR γ response to 2- or 1.8-fold (*versus* 6- or 7-fold) when the cells were co-transfected with PPRE-Mut-Luc with RXR α /PPAR γ 1 or RXR α /PPAR γ 2 constructs, respectively. Treatment of cells transfected with PPRE-Mut-PC/RXR α /PPAR γ 1 or PPAR γ 2 constructs and rosiglitazone did not rescue the PPAR γ response to the same level as that of the wild type construct (PPRE-WT-Luc).

PPAR γ 1 and PPAR γ 2 Bind PPRE(-386/-374) of mPC Promoter *In Vitro* and *In Vivo*

To determine whether the PPAR γ or any other protein is capable of binding the -386/-374 region of the mPC promoter, we performed an electrophoretic mobility shift assay (EMSA) using the wild type (PC-PPRE(-386/-374)) as a probe. We also used the PPRE of mouse *aP2* gene (24) as the positive control (Fig. 6A). As shown in Fig. 6B, the wild type -386/-374 double-stranded probe, when incubated with nuclear extract from differentiated 3T3-L1, forms a single DNA-protein complex. The same pattern was observed when the PPRE of *aP2* was used as the probe, *i.e.* a single complex was formed and migrated to the same position as that seen with the PC-PPRE probe. Incubation of the 3T3-L1 nuclear extract with monoclonal antibody against PPAR γ or RXR α almost completely abolished the formation of the complex and also supershifted the complex to the higher molecular weight position (Fig. 6B, *asterisk*). Likewise, the complex bound to the *aP2*-PPRE probe was also supershifted to the same position when the antibody for PPAR γ was present in the binding reaction. This indicated that the above complex was formed between the PPRE and the RXR α -PPAR γ .

The binding specificity and affinity of the complex were determined in competition with excess unlabeled double-stranded wild type oligonucleotide (PC-PPRE). As shown in Fig. 6B, the PPRE-PPAR γ complex could be gradually competed off with 5, 10, and 20 \times excess amount of unlabeled wild type sequence. This PPRE-PPAR γ complex was partially competed off with a 5 \times excess amount of unlabeled double-stranded PPRE of *aP2* and was completely competed off with 10 and 20 \times excess amount of PPRE-*aP2* sequence indicating that PPAR γ binds the PPRE of *aP2* with higher affinity than that of the PC gene. In contrast, when a double-stranded oligonucleotide containing a mutated PPRE site was included as the

competitor in excess amounts of 5, 10, and 20 \times , they failed to abolish the formation of the PPRE-PPAR γ complex, indicating that its formation requires an intact PPRE sequence.

Similarly, PPRE-PPAR γ -RXR α complex was formed when PPRE of the mPC probe was incubated with nuclear extracts prepared from HEK293T overexpressing RXR α /PPAR γ 1 or RXR α /PPAR γ 2 (Fig. 6C). Incubation of nuclear extracts of these cells with antibody against PPAR γ supershifted the complex to the same position as that seen when 3T3-L1 adipocyte nuclear extract was used. In contrast incubation of probe with nuclear extract of mock-transfected HEK293T did not produce the corresponding PPRE-RXR α -PPAR γ complex.

We next investigated whether PPAR γ binds PPRE of the mPC *in vivo* by CHIP assay. A chromatin preparation of 3T3-L1 adipocytes was cross-linked with PPAR γ , sheared, and immunoprecipitated with (i) elongation factor 2F (E2F) monoclonal antibody, (ii) 10 μ l of PPAR γ monoclonal antibody, or (iii) 50 μ l of PPAR γ monoclonal antibody. The immunoprecipitates containing PPAR γ -bound PPRES were subjected to PCR using two sets of primers that flanked either the mPC PPRES or another site located greater than 3 kb downstream of the PPRES (see Fig. 7A). By using F1/R1 primers in the PCR, the 250-bp amplicon was detected from input DNA (Fig. 7A, lane 1) and from a DNA fraction that was immunoprecipitated with 50 μ l of PPAR γ antibody (lane 4) but not from a DNA fraction that was immunoprecipitated with 10 μ l of PPAR γ antibody (lane 3) or with E2F monoclonal antibody (lane 2). In contrast, with primer F2/R2, the 150-bp amplicon was only detected from input DNA but not from other fractions (Fig. 7C). These results indicated that endogenous PPAR γ of 3T3-L1 adipocytes were associated with the PPRES of the mPC *in vivo*.

Down-regulation of PC Expression in PPAR γ 2 Knock-out Mice

We recently generated PPAR γ 2 knock-out mice (20). Microarray data analysis of RNAs prepared from white adipose tissues of these mice indicated that PC expression was affected by the lack of PPAR γ 2 expression (data not shown). This result was confirmed by PCR and Western analysis of white adipose tissue of the wild type mice and of the PPAR γ 2 knock-out mice fed with chow diet or with high fat diet. As shown in Fig. 8A, PPAR γ 2 knock-out mice displayed a reduction in the level of white adipose tissue PC mRNA by ~60% relative to the wild type mice. This decrease in PC expression correlates with the decrease in total PPAR γ gene expression (50% approximately) (20) as a result of the specific deletion of the PPAR γ 2 isoform.

During fasting, where PPAR γ 2 expression is known to decrease (42), PC was also down-regulated by 50% in wild type mice. However, in the knock-out mice, fasting did not cause a significant change in PC mRNA. Refeeding caused a 2-fold increase of PC mRNA expression in the wild type mice but nonsignificant increase in the knock-out mice. These slight changes of PC mRNA during fasting and refeeding conditions in the knock-out mice suggest that PPAR γ 1, which is still intact in the knock-out mice, responds to the nutritional changes to a lesser extent than does PPAR γ 2, in agreement with our previous report (42). PPAR γ 2 knock-out mice also showed a reduction of PC mRNA and PC protein by ~50%, relative to the wild type mice when fed a high fat diet (Fig. 8B). Under the high fat diet

conditions, where PPAR γ 2 is 50% elevated (42), PC mRNA and PC protein appeared to increase 2-fold higher than under chow diet conditions (Fig. 8B).

PC is thought to play a crucial role in brown adipose tissue by supplying substantial amounts of oxaloacetate to be coupled with acetyl-CoA being generated by β -oxidation (18, 19), in order to completely oxidize this acetyl-CoA in the Krebs cycle and generate ATP. We therefore examined whether the level of PC was affected in brown adipose tissue of PPAR γ 2 knock-out mice when maintained at room temperature (25 °C). PPAR γ 2 knock-out mice had PC mRNA and protein levels that were not significantly different from those of the wild type mice fed with either chow diet or high fat diet (Fig. 8C). However, as shown in Fig. 8D in wild type mice challenged with cold exposure, PC mRNA was increased 12-fold concomitant with a 5-fold increase of PC protein compared with room temperature exposure. However, PC expression of PPAR γ knock-out mice was increased to only 7-fold concomitant with a 2.5-fold increase of PC protein upon cold exposure. These data indicated that the lack of PPAR γ 2 expression in brown adipose tissue was associated with the dysregulation of PC in brown adipose tissue during cold exposure. The alteration of PC expression level in PPAR γ 2 knock-out mice appeared to be specific in adipose tissue as no change of PC mRNA level was observed in the liver of the knock-out and the wild type mice fed with either chow or high fat diet (Fig. 8E).

Impaired *In Vitro* Differentiation of Adipocytes Is Associated with the Reduction of PC Expression in PPAR γ 2 Knock-out Mice

The lack of PPAR γ 2 expression results in the impaired differentiation of preadipocytes to mature adipocytes *in vitro* (20, 43). To examine the tight regulation between the lack of PPAR γ and PC expression, we prepared primary cultures of preadipocytes isolated from wild type and PPAR γ 2 knock-out mice, and we induced these preadipocytes to differentiate to mature white adipocytes *in vitro*. As shown in Fig. 9, PC expression gradually increased during differentiation of primary cultures obtained from wild type mice, becoming 8-fold higher after 8 days compared with day 0. Treatment of cultures with rosiglitazone further increased the level of PC mRNA (13-fold). In sharp contrast, PC expression only increased by 2-fold in cultures prepared from PPAR γ 2 knock-out mice. Treatment of these cultures with rosiglitazone only marginally increased expression of PC mRNA. Taken together, these data indicate that the marked reduction of PC mRNA expression was associated with the lack of expression of PPAR γ 2, which is necessary for *in vitro* differentiation.

DISCUSSION

PPAR γ is a key transcription factor that regulates adipogenesis, lipogenesis, and insulin sensitivity. Expression of PPAR γ during early adipogenesis not only facilitates differentiation but also results in transcriptional activation of downstream genes whose products are involved in lipid deposition. This in turn causes the growth-arrested preadipocytes to become morphologically distinct with cytosolic fat accumulation. *De novo* fatty acid synthesis in differentiating adipocytes starts with the reaction catalyzed by acetyl-CoA carboxylase, which converts acetyl-CoA to malonyl-CoA, a building block of long chain fatty acids. However, this pathway relies solely on the export from mitochondria of

acetyl groups as citrate produced by the combined activities of PC and citrate synthase. The observed increase of PC activity during adipocyte differentiation support a “lipogenic role” for this enzyme in adipose tissue, in addition to its gluconeogenic role in liver and kidney (44). The recent microarray and proteomic analyses of adipocytes of *ob/ob* mice treated with rosiglitazone showed the up-regulation of several mitochondrial proteins, including PC (45), suggesting PC could be transcriptionally regulated by PPAR γ . Our finding that PC is a novel target of PPAR γ provides a mechanistic and functional insight for the above reports. We have demonstrated in two independent mouse cell lines, 3T3-L1 and HIB-1B, that PC expression was highly correlated with the expression of PPAR γ 1 and PPAR γ 2 during differentiation. TNF α , an agent that inhibits adipocyte differentiation through the reduction of PPAR γ expression (46), also suppressed PC expression, providing further support for the role of PPAR γ regulating the expression of PC.

Bioinformatic analysis and transactivation studies identified the presence of a functional PPRE, located at positions -386/-374, with respect to the transcription initiation site. This PPRE appears to be invariant between the mouse and rat PC gene, suggesting that the regulation by PPAR γ in adipocytes is well preserved in these two mammalian species. Unlike PPAR γ , other known adipogenic/lipogenic transcription factors such as C/EBP α and ADD1/SREBP1c did not transactivate the mPC reporter construct in either HEK293T cells or 3T3-L1 preadipocytes, suggesting that the mPC gene is a specific target of PPAR γ .

The PPRE of the mPC was further characterized by EMSA and ChIP assay. Although the PPRE of the mPC is similar but not identical to the canonical PPRE, a stable PPRE-RXR α -PPAR γ complex was observed in the EMSA. This complex was also supershifted by antibodies against PPAR γ and RXR α . The binding affinity of PPAR γ to PPRE of the mPC appeared to be weaker than that of the PPRE of *aP2* gene as demonstrated by competition assay. This is perhaps because of one nucleotide mismatch within the DR1 and DR2 sites of PPRE of the mPC gene.

PPAR γ is present in two isoforms, PPAR γ 1 and PPAR γ 2. These two isoforms are generated by alternative splicing of the first coding exon, with PPAR γ 1 thus lacking the first 30 N-terminal amino acids that are found in PPAR γ 2. This distinct N-terminal region of both PPAR γ 1 and PPAR γ 2 appears to exhibit different activation capacities (47). Unlike PPAR γ 1, which is widely expressed, PPAR γ 2 is restricted to white and brown adipose tissue and is preferentially affected by nutritional status (42). The PPRE of PC did not show a preference for transcriptional activation of an mPC promoter-luciferase reporter construct with PPAR γ 1 or PPAR γ 2 expression plasmids in both 3T3-L1 preadipocytes and HEK293T cells.

Total disruption of PPAR γ is embryonic lethal (48), whereas selective disruption of PPAR γ in muscle results in insulin resistance in muscle (49, 50), and the selective disruption of PPAR γ in adipose tissue causes a marked reduction of fat mass and subsequent progressive lipodystrophy (51). As PPAR γ consists of PPAR γ 1 and PPAR γ 2, it was uncertain whether the functional redundancy of these two isoforms exists *in vivo*. PPAR γ 2 knock-out mice have been generated recently by our group (20) and another group (43). These knock-out mice displayed global changes in the expression of genes involved in lipid metabolism. In

support of our *in vitro* data, here we present *in vivo* evidence that disruption of PPAR γ 2 also affected PC expression in both white and brown adipose tissues but not in the liver. In white adipose tissue, PPAR γ 2 knock-out mice, either fed with a chow or a high fat diet, exhibited 50–60% reduction of PC mRNA and protein that correlates with the total PPAR γ gene dosage represented by the PPAR γ 2 isoform. The remaining PC mRNA in this tissue probably resulted from transcriptional activation of the PC gene by PPAR γ 1. However, because PPAR γ 2 is the isoform preferentially regulated by nutrition (42), it is likely that nutritional regulation of PC in adipose tissue *in vivo* depends mainly on the alteration of PPAR γ 2 gene expression.

In brown adipose tissue of PPAR γ 2 knock-out mice raised at ambient temperature (25 °C), no change of PC expression was observed. However, under cold exposure challenge, ablation of PPAR γ 2 resulted in a 50% reduction of cold-induced PC expression. This response is consistent with the physiological roles of PC in brown adipose tissue, *i.e.* (i) to ensure a continuous supply of oxaloacetate for the complete oxidation of fatty acids during thermogenesis; (ii) to promote indirectly thermogenesis by enabling *de novo* synthesis of long chain fatty acids in brown adipose tissue for subsequent oxidation. Cold-induced thermogenesis has also been reported to increase the expression of mRNAs for ACC1 and citrate lyase accompanied by a marked increase in *de novo* fatty acid synthesis in brown adipose tissue (52).

Our finding that PC expression was unaffected in liver of PPAR γ 2 knock-out mice further indicated that the regulation of PC expression in liver and adipose tissue are highly independent. This distinct regulation is at least in part because of the restriction of PPAR γ 2 expression to adipose tissue.

Although our *in vitro* data (Fig. 3) and *in vivo* data (Fig. 8) indicate that the transcription of the mPC gene could be regulated equally by PPAR γ 1 or PPAR γ 2, the regulation of PC expression during *in vitro* differentiation of preadipocytes to mature adipocytes of PPAR γ 2 knock-out mice provides a conflicting result. Although PPAR γ 1 was still intact (20, 43), the lack of PPAR γ 2 resulted in a complete failure to drive the differentiation of white adipocytes *in vitro* (20, 43). This was associated with the marked reduction of PC mRNA, suggesting that PC expression is mainly regulated by PPAR γ 2 under this *in vitro* condition. This suggests that important compensatory mechanisms must be operated *in vivo* to allow PPAR γ 1 to regulate efficiently the PC expression when PPAR γ 2 is absent. However, this unknown mechanism could not completely adjust the expression level of PC to the normal level. Nevertheless, the complete loss of PC expression concomitant with impaired *in vitro* differentiation of adipocytes from PPAR γ 2 knock-out mice strengthens the regulation of PC by PPAR γ during adipogenesis.

The transcriptional control of PC in mammals is complex, involving the alternative usage of two promoters, namely the proximal promoter and the distal promoter (22, 27, 53). Alternative transcription of these two promoters results in the production of different primary transcripts that undergo differential splicing in the 5'-noncoding regions. Although the proximal promoter regulates the production of transcript that is only expressed in gluconeogenic tissues and the adipose tissue, the distal promoter appears to be active in most

tissues (10, 27). Although both gluconeogenic (liver and kidney) and lipogenic tissues (adipose) use the proximal promoter to control transcription of PC, it was unclear until now what signal(s) control transcription in these two different anabolic pathways. The identification of PPAR γ as a positive transcriptional regulator of the proximal promoter of the mPC gene further explains the selective activation of this promoter in adipose tissue where PPAR γ 1 and PPAR γ 2 are highly expressed.

In summary, we provide evidence that PC is regulated *in vitro* and *in vivo* in white and brown adipose tissues. The identification of PC as a target of PPAR γ identifies a new class of PPAR γ target genes involved in a metabolic switch controlling fuel partitioning toward either gluconeogenesis or lipogenesis. Thus far, the only other PPAR γ target gene that falls into this category is phosphoenolpyruvate carboxykinase, a well known gluconeogenic enzyme that is also involved in glycerogenesis in adipose tissue (for a review see Reshef *et al.* (54)).

Acknowledgment

We thank Dr. Justin Rochford for critically reading the manuscript.

REFERENCES

1. Mackall JC, Student AK, Polakis SE, Lane MD. *J. Biol. Chem.* 1976; 251:6462–6464. [PubMed: 10298]
2. Cowherd RM, Lyle RE, McGehee RE Jr. *Semin. Cell. Dev. Biol.* 1999; 10:3–10. [PubMed: 10355023]
3. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. *Genes Dev.* 2000; 14:1293–1307. [PubMed: 10837022]
4. Rosen ED, Spiegelman BM. *Annu. Rev. Cell Dev. Biol.* 2000; 16:145–171. [PubMed: 11031233]
5. Rosen ED, Spiegelman BM. *J. Biol. Chem.* 2001; 276:37731–37734. [PubMed: 11459852]
6. Fajas L, Auboef D, Raspe E, Schoonjans K, Lefebvre AM, Salaadin R, Najlb J, Laville M, Fruchart JC, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J. *J. Biol. Chem.* 1997; 272:18779–18789. [PubMed: 9228052]
7. Zhu Y, Qi C, Korenberg JR, Chen XN, Noya D, Rao MS, Reddy JK. *Proc. Natl. Acad. Sci. U. S. A.* 1995; 92:7921–7925. [PubMed: 7644514]
8. Braissant O, Foulle F, Scotto C, Dauca M, Wahli W. *Endocrinology.* 1996; 137:354–366. [PubMed: 8536636]
9. Jitrapakdee S, Wallace JC. *Biochem. J.* 1999; 340:1–16. [PubMed: 10229653]
10. Jitrapakdee S, Walker ME, Wallace JC. *Biochem. Biophys. Res. Commun.* 1996; 223:695–700. [PubMed: 8687459]
11. Wexler ID, Du Y, Lisgaris MV, Manda LSK, Freytag SO, Yang BS, Liu TC, Kwon M, Patel MS, Kerr DS. *Biochim. Biophys. Acta.* 1994; 1227:46–52. [PubMed: 7918683]
12. Mackall JC, Lane MD. *Biochem. Biophys. Res. Commun.* 1977; 79:720–725. [PubMed: 597301]
13. Freytag SO, Utter MF. *J. Biol. Chem.* 1983; 258:6307–6312. [PubMed: 6343373]
14. Lynch CJ, McCall KM, Billingsley ML, Bohlen LM, Hreniuk SP, Martin LF, Witters LA, Vannucci SJ. *Am. J. Physiol.* 1992; 262:E608–E618. [PubMed: 1375435]
15. Zhang J, Xia W-L, Ahmad F. *Biochem. J.* 1995; 306:205–210. [PubMed: 7864811]
16. Jitrapakdee S, Gong Q, MacDonald MJ, Wallace JC. *J. Biol. Chem.* 1998; 273:34422–34428. [PubMed: 9852109]
17. Ballard FJ, Hanson RW. *J. Lipid Res.* 1967; 8:73–79. [PubMed: 14564711]
18. Cannon B, Nedergaard J. *Physiol. Rev.* 2004; 84:277–359. [PubMed: 14715917]

19. Cannon B, Nedergaard J. *Eur. J. Biochem.* 1979; 94:419–426. [PubMed: 428395]
20. Medina-Gomez G, Virtue S, Lelliott C, Boiani R, Campbell M, Christodoulides C, Perrin C, Jimenez-Linan M, Blount M, Dixon J, Zahn D, Thresher RR, Aparicia S, Carlton M, Colledge WH, Kettunen MI, Seppanen-Laakso T, Sethi JK, O'Rahilly S, Brindle K, Cinti S, Burcelin R, Vidal-Puig A. *Diabetes.* 2005; 54:1706–1716. [PubMed: 15919792]
21. Ross SR, Choy L, Graves RA, Fox N, Solevjeva V, Klaus S, Ricquier D, Spiegelman BM. *Proc. Natl. Acad. Sci. U. S. A.* 1992; 89:7561–7565. [PubMed: 1323843]
22. Jitrapakdee S, Petchamphai N, Sunyakumthorn P, Wallace JC, Boonsaeng V. *Biochem. Biophys. Res. Commun.* 2001; 287:411–417. [PubMed: 11554743]
23. Meirhaeghe A, Crowley V, Lenaghan C, Lelliott C, Green K, Stewart A, Hart K, Schinner S, Sethi JK, Yeo G, Brand MD, Cortright RN, O'Rahilly S, Montague C, Vidal-Puig AJ. *Biochem. J.* 2003; 373:155–165. [PubMed: 12678921]
24. Tontonoz P, Graves RA, Budavari AI, Erdjument-Bromage H, Lui M, Hu E, Tempst P, Spiegelman BM. *Nucleic Acids Res.* 1994; 22:5628–5634. [PubMed: 7838715]
25. Laemmli UK. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
26. Rohde M, Lim F, Wallace JC. *Arch. Biochem. Biophys.* 1991; 290:197–201. [PubMed: 1898090]
27. Jitrapakdee S, Booker GW, Cassady AI, Wallace JC. *J. Biol. Chem.* 1997; 272:20520–20528.
28. Zhang J, Xia WL, Brew K, Ahmad F. *Proc. Natl. Acad. Sci. U. S. A.* 1993; 90:1766–1770. [PubMed: 8446588]
29. Kim H-I, Kim J-W, Kim S-H, Cha J-Y, Kim K-S, Ahn Y-H. *Diabetes.* 2000; 49:1517–1524. [PubMed: 10969836]
30. Bardot O, Aldridge TC, Latruffe N, Green S. *Biochem. Biophys. Res. Commun.* 1993; 192:37–45. [PubMed: 8386511]
31. Tontonoz P, Hu E, Devine J, Beale EG, Spiegelman BM. *Mol. Cell. Biol.* 1995; 15:351–357. [PubMed: 7799943]
32. Girunn GD, Domann FE, Moore SA, Robbins ME. *Mol. Endocrinol.* 2002; 16:2793–2801. [PubMed: 12456800]
33. Castelein H, Gulick T, Declercq PE, Mannaerts GP, Moore DD, Baes MI. *J. Biol. Chem.* 1994; 269:26754–26758. [PubMed: 7929410]
34. Arimura N, Horiba T, Imagawa M, Shimizu M, Sato R. *J. Biol. Chem.* 2004; 279:10070–10076. [PubMed: 14704148]
35. Rodriguez JC, Gil-Gomez G, Hegardt FG, Haro D. *J. Biol. Chem.* 1994; 269:18767–18772. [PubMed: 7913466]
36. Tugwood JD, Issemann I, Anderson RG, Bundell KR, McPheat WL, Green S. *EMBO J.* 1992; 11:433–439. [PubMed: 1537328]
37. Schoonjans K, Watanabe M, Suzuki H, Mahfoudi A, Krey G, Wahli W, Grimaldi P, Staels B, Yamamoto T, Auwerx J. *J. Biol. Chem.* 1995; 270:19269–19276. [PubMed: 7642600]
38. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, Staels B, Auwerx J. *EMBO J.* 1996; 15:5336–5348. [PubMed: 8895578]
39. Frohnert BI, Hui TY, Bernlohr DA. *J. Biol. Chem.* 1999; 274:3970–3977. [PubMed: 9933587]
40. Sears IB, MacGinnitie MA, Kovacs LG, Graves RA. *Mol. Cell. Biol.* 1996; 16:3410–3419. [PubMed: 8668156]
41. Mascaro C, Acosta E, Ortiz JA, Marrero PF, Hegardt FG, Haro D. *J. Biol. Chem.* 1998; 273:8560–8563. [PubMed: 9535828]
42. Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE. *J. Clin. Investig.* 1996; 97:2553–2561. [PubMed: 8647948]
43. Zhang H, Fu M, Cui T, Xiong C, Xu K, Zhong W, Xiao Y, Floyd D, Jiang J, Li E, Song Q, Chen YE. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:10703–10708. [PubMed: 15249658]
44. Jitrapakdee, S.; Wallace, JC. *Encyclopedia of Biological Chemistry.* Lennarz, WJ.; Lane, MD., editors. Vol. 3. Elsevier Science Publishing Co., Inc.; Oxford: 2004. p. 606-610.
45. Wilson-Fritch L, Nicoloso S, Chouinard M, Lazar MA, Chul PC, Leszyk J, Straubhaar J, Czech MP. *J. Clin. Investig.* 2004; 114:1281–1289. [PubMed: 15520860]

46. Zhang B, Berger J, Hu E, Szalkowski D, White-Carrington S, Spiegelman B, Moller D. *Mol. Endocrinol.* 1996; 6:1457–1466. [PubMed: 8923470]
47. Werman A, Hollenberg A, Solanes G, Bjorbaek C, Vidal-Puig AJ, Flier JS. *J. Biol. Chem.* 1997; 270:20230–20235. [PubMed: 9242701]
48. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM. *Mol. Cell.* 1999; 4:585–595. [PubMed: 10549290]
49. Hevener A, He W, Barak Y, Le J, Bandyopadhyay B, Olson P, Wilkes J, Evans RM, Olefsky J. *Nat. Med.* 2003; 9:1491–1497. [PubMed: 14625542]
50. Norris AW, Chen L, Fisher SJ, Szanto I, Ristow M, Jozsi AC, Hirshman MF, Rosen ED, Goodyear LJ, Gonzalez FJ, Spiegelman BM, Kahn CR. *J. Clin. Investig.* 2003; 112:608–618. [PubMed: 12925701]
51. He W, Barak Y, Hevener A, Olson P, Liao D, Le J, Nelson M, Ong E, Olefsky JM, Evans RM. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100:15712–15717. [PubMed: 14660788]
52. Yu X-X, Lewin DA, Forrest W, Adams SH. *FASEB J.* 2002; 16:155–168. [PubMed: 11818363]
53. Carbone MA, MacKay N, Ling M, Cole DE, Douglas C, Rigat B, Feigenbaum A, Clarke JTR, Haworth JC, Greenberg CR, Seargeant L, Bobinson BH. *Am. J. Hum. Genet.* 1998; 62:1312–1319. [PubMed: 9585612]
54. Reshef L, Olswang Y, Cassuto H, Blum B, Croniger CM, Kalhan SC. *J. Biol. Chem.* 2003; 278:30413–30416. [PubMed: 12788931]

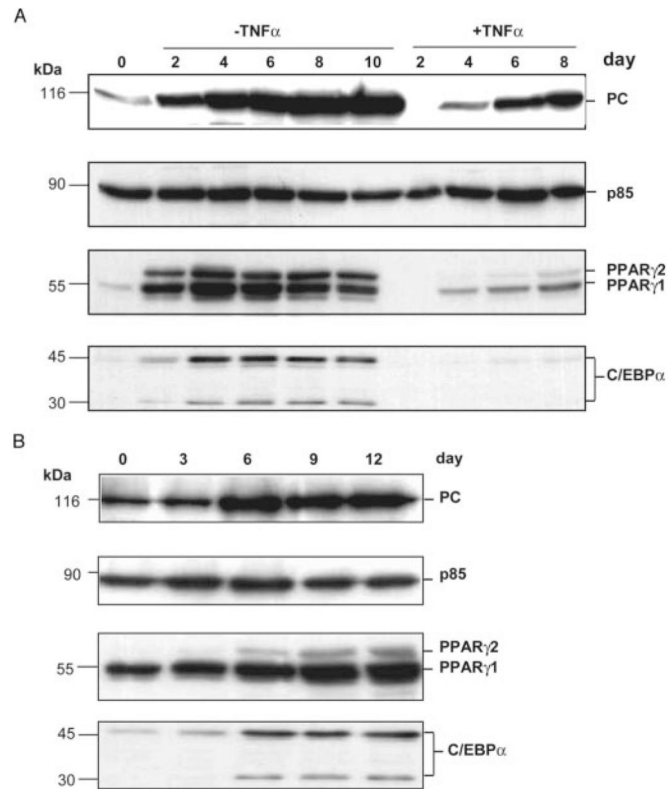


FIG. 1. Expression of PC protein with adipogenic transcription factors during differentiation of adipocytes

A, Western analysis of PC, PPAR γ 1, PPAR γ 2, and C/EBP α during differentiation of 3T3-L1 preadipocytes to mature adipocytes over 8 days in the absence or presence of TNF α . *B*, same analysis with brown adipocyte cell line, HIB-1B, differentiated for 12 days. Membranes were stripped and reprobed for anti-p85 α subunit of phosphoinositol-3-phosphate kinase as a loading control.

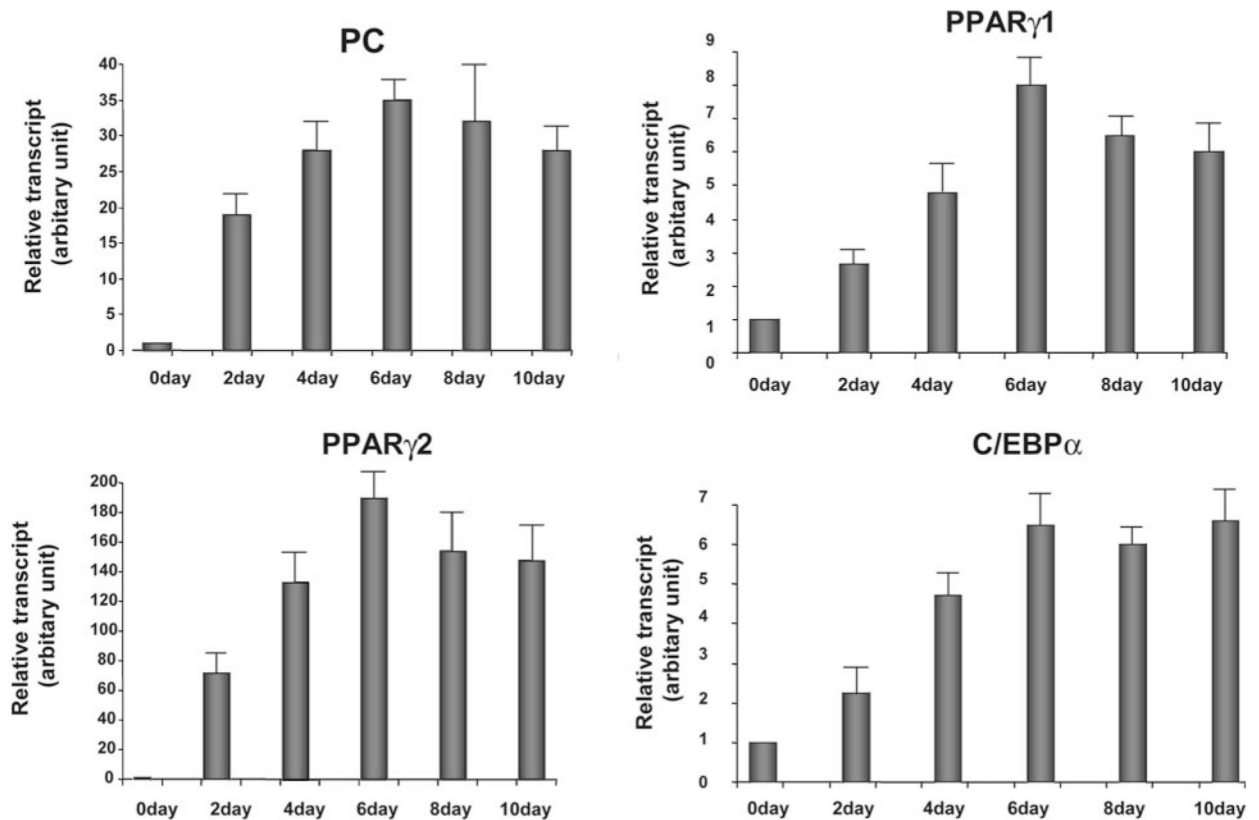


FIG. 2. Real time PCR analysis of expression of PC, PPAR γ 1, PPAR γ 2, and C/EBP α mRNAs in 3T3-L1 during differentiation at indicated times (day)

Total RNA was prepared, reverse-transcribed to cDNA, and analyzed by real time PCR with Taqman as described under "Experimental Procedures." The abundance of each mRNA is normalized with 18 S and is shown as relative gene expression \pm S.D. of three independent experiments. The relative gene expression detected at day 0 was arbitrarily set as 1.

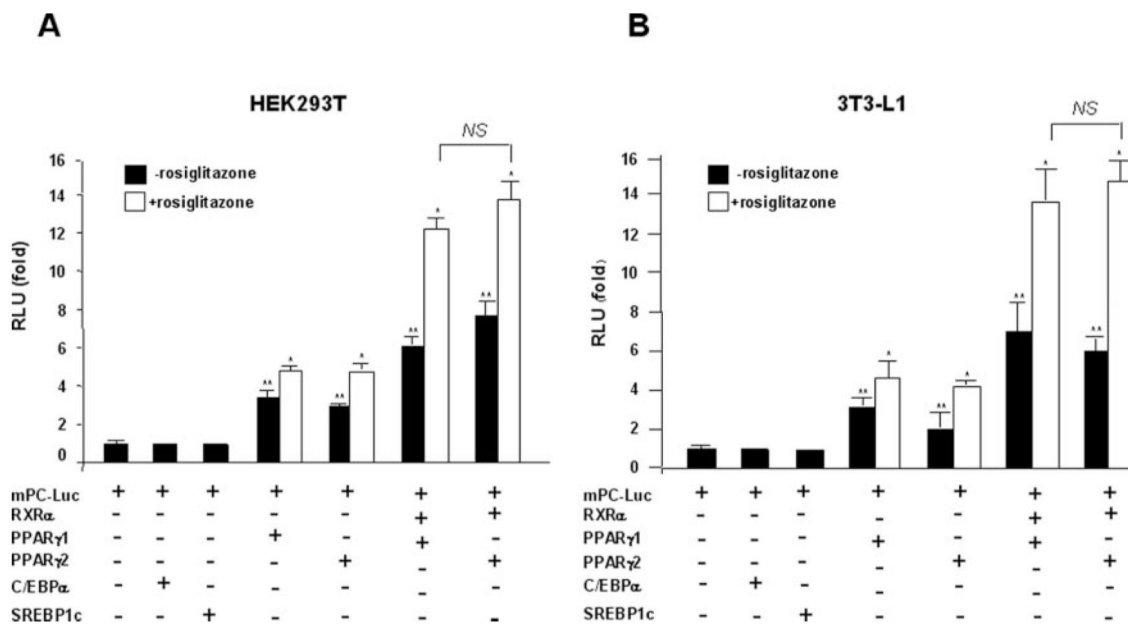
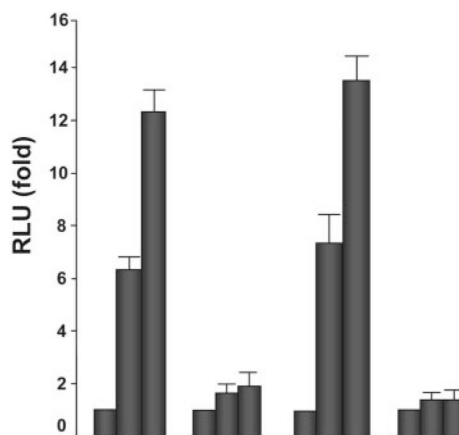
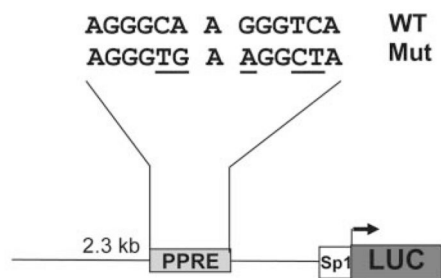


FIG. 3. Transactivation of the mPC promoter-luciferase reporter construct (PC-Luc) by PPAR γ 1 and PPAR γ 2 in 3T3-L1 and in HEK293T cells

The mPC promoter-luciferase reporter plasmid was transiently co-transfected with plasmids expressing C/EBP α , SREBP1c, or RXR α together with PPAR γ 1 or PPAR γ 2 into HEK293T (A) or 3T3-L1 (B). At 24 h post-transfection, cells were incubated with 0.1 μ M rosiglitazone (open bar) or vehicle (solid bar) and further incubated for the next 24 h before being harvested and assayed for luciferase activity. The relative luciferase activities (RLU) shown are the means \pm S.D. of three independent experiments, each in triplicate. Luciferase activity measured from the cells transfected with mPC-Luc alone (basal condition) in the absence of rosiglitazone was arbitrarily set as 1. ** represents $p < 0.001$ compared with mPC-Luc; * represents $p < 0.05$ compared with transfected cells treated with no rosiglitazone. NS, nonsignificance.

fatty acid transporter (39); *UCPI*, uncoupling protein 1 (40); *CP*, muscle-type carnitine palmitoyltransferase (41).



PPRE-WT-Luc	+	+	+	-	-	-	+	+	+	-	-	-
PPRE-Mut-Luc	-	-	-	+	+	+	-	-	-	+	+	+
RXRα/PPARγ1	-	+	+	-	+	+	-	-	-	-	-	-
RXRα/PPARγ2	-	-	-	-	-	-	-	+	+	-	+	+
rosiglitazone	-	-	+	-	-	+	-	-	+	-	-	+

FIG. 5. Mutational analysis of putative PPRE(-386/-374) in the mPC promoter

The wild type mPC promoter-luciferase (*PPRE-WT-Luc*) or its mutant (*PPRE-Mut-Luc*) construct alone or both were co-transfected with plasmids expressing either RXRα/PPARγ1 or RXRα/PPARγ2 into HEK293T cells. At 24 h post-transfection, cells were incubated with 0.1 μM rosiglitazone or vehicle and incubated for a further 24 h before being harvested and assayed for luciferase activity. The relative luciferase activities (RLU) shown are the means ± S.D. of three independent experiments, each in triplicate. Luciferase activity measured from the cells transfected with mPC-Luc alone (basal condition) in the absence of rosiglitazone was arbitrarily set as 1.

PPAR γ . *Lane 1*, probe alone; *lane 2*, probe with nuclear extract of HEK293T overexpressing PPAR γ 1. *Lane 3*, same as *lane 2* but with PPAR γ antibody. *Lane 4*, probe with nuclear extract of HEK293T overexpressing PPAR γ 2. *Lane 5*, same as *lane 4* but with PPAR γ antibody. *Lane 6*, probe with nuclear extract of mock-transfected HEK293T. NS, nonspecific binding. +/-, with or without nuclear extract (NE).

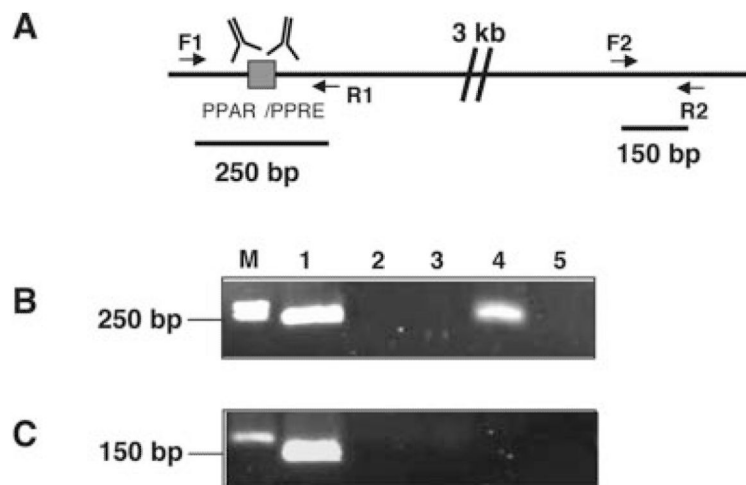


FIG. 7. Chromatin immunoprecipitation assay of PPAR γ bound to PPRE of mPC promoter in 3T3-L1 adipocytes

Soluble chromatin was prepared from 6-day differentiated 3T3-L1 described under “Experimental Procedures.” The PPAR γ -associated DNA fragments were immunoprecipitated (*IP*) with mouse monoclonal antibodies against E2F or PPAR γ . *A*, the positions of primers (*arrows*) relative to the PPRE of the mPC promoter (*shaded box*) are shown. *B*, the PPAR γ -associated PPRE of the mPC promoter was PCR-amplified with F1 and R1 primers before immunoprecipitation (“input”, *lane 1*) and after immunoprecipitation with anti-E2F (*lane 2*) or with 10 (*lane 3*) or 50 μ l (*lane 4*) of anti-PPAR γ . *Lane 5*, negative control DNA template. *Lane M*, DNA marker. *C*, PCR was performed with negative control primers (F2 and R2) that are located >3 kb downstream of the PPRE.

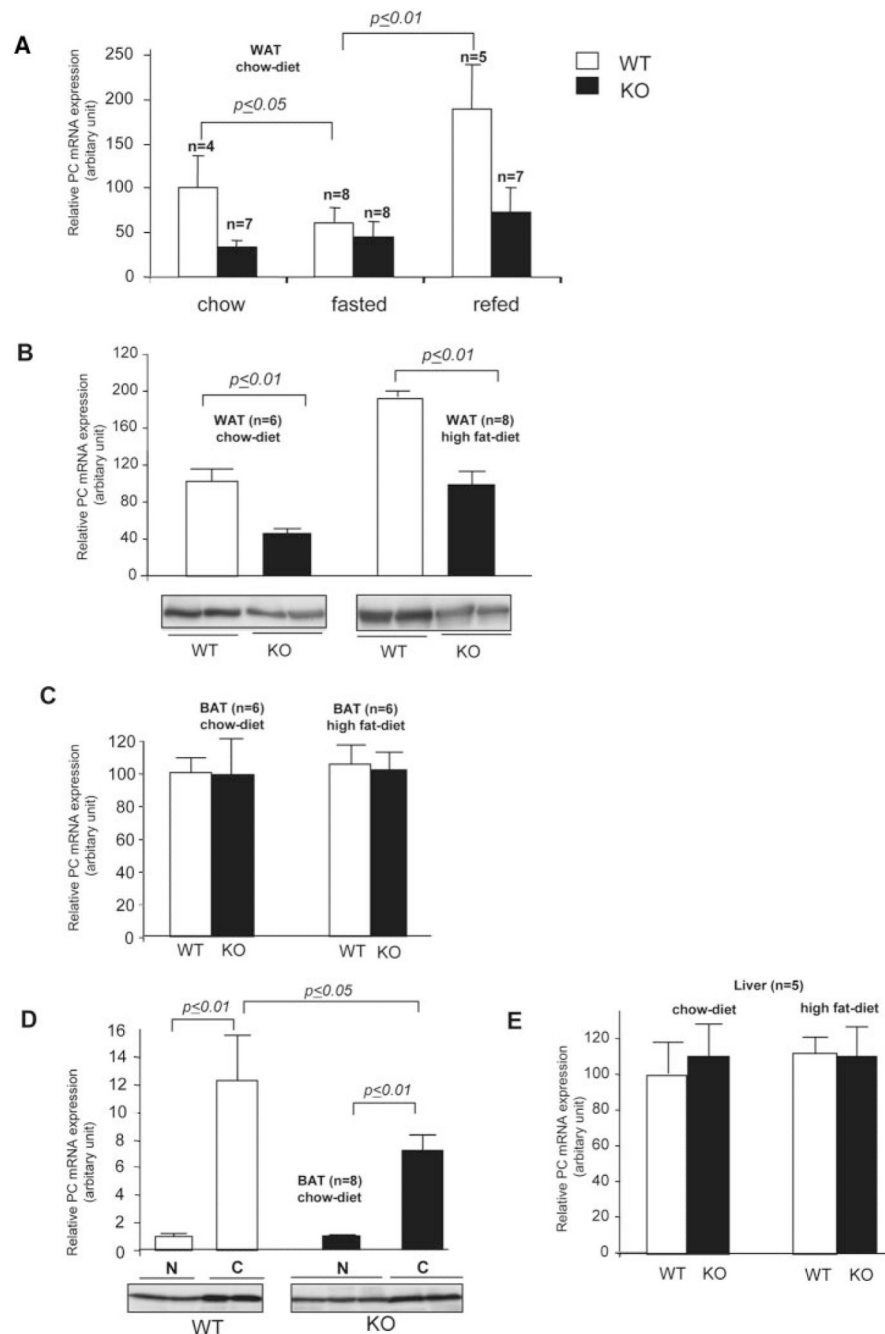


FIG. 8. Down-regulation of PC mRNA and protein in white adipose tissue and brown adipose tissue of PPAR γ 2 knock-out mice

A, white adipose tissues (WAT) were collected from wild type (WT) or PPAR γ 2 knock-out (KO) mice fed, fasted, or re-fed with chow diet. B, white adipose tissues collected from wild type or knock-out mice fed with a high fat diet were compared with those fed with a chow diet. Western analysis of each group of animals is shown *below* the graph. C, brown adipose tissues (BAT) were collected from wild type and knockout mice fed with chow or high fat diet. D, BAT were collected from wild type and knock-out mice fed with chow diet and maintained at room temperature (25 °C) or cold temperature (4 °C). Western analysis of

each group of animals is shown *below* the graph. *E*, livers were collected from wild type or knock-out fed with chow or high fat diet. Total RNAs were extracted, reverse-transcribed to cDNA, and analyzed by real time PCR with PC primer and Taqman probe. The abundance of PC mRNA is normalized with 18 S and is shown as relative gene expression \pm S.D. The relative PC mRNA expression detected in tissues from wild type mice for each treatment was arbitrarily set as 100%. Total cell lysates were also prepared from these tissues, and 50 μ g of total protein was subjected to Western blot analysis with PC antibody. *N*, room temperature; *C*, cold exposure. *n* = number of mice in each experiment.

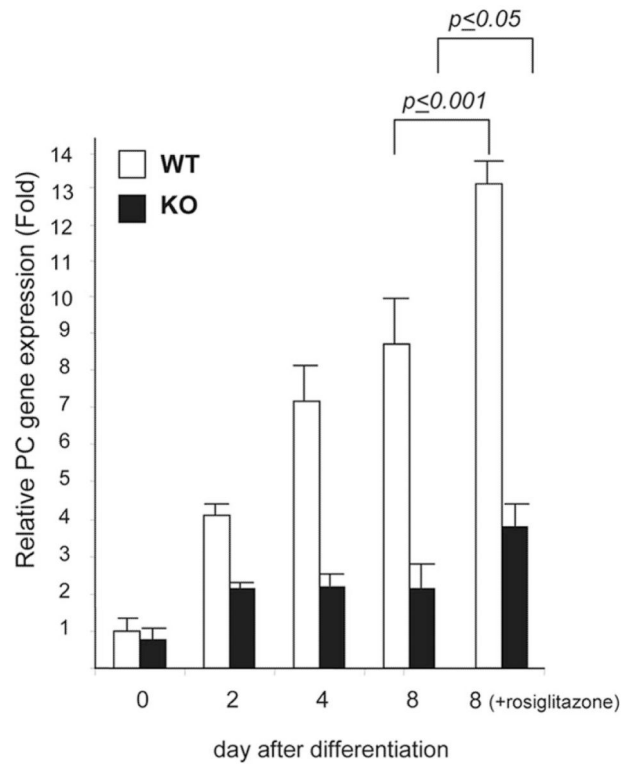


FIG. 9. Impaired PC mRNA expression in primary cultures of white adipose tissue of wild type and knock-out mice

Preadipocytes isolated from epididymal fat of wild type (*WT*, *open bars*) and *PPAR* γ 2 knock-out (*KO*, *filled bars*) mice were differentiated *in vitro* in the presence or absence of 0.1 μ M rosiglitazone. Total RNAs obtained at the time points indicated were analyzed by real time PCR as described under “Experimental Procedures.” The abundance of PC mRNA was normalized with 18 S. Results shown are mean of independent experiments. The relative gene expression detected at day 0 of wild type mice was arbitrarily set as 1.

Table I

Primers and fluorogenic probes used for real time PCR

Gene name	Forward primer	Reverse primer	Fluorogenic probe
mPC	5'-GATGACCTCACAGCCAAGCA-3'	5'-GGGTACCTCTGTGTCCTCAAAGGA-3'	5'-CCCTGGTGGCCTGTACCAAAGGG-3'
mPPAR γ 1	5'-TTTAAAAACAAGACTACCCTTTACTGAAATT-3'	5'-AGAGGTCCACAGAGCTGATTCC-3'	5'-AGAGATGCCATTCTGGCCCACTT-3'
mPPAR γ 2	5'-GATGACTGCCTATGAGCACTT-3'	5'-AGAGGTCCACAGAGCTGATTCC-3'	5'-AGAGATGCCATTCTGGCCCACTT-3'
mC/EBP α	5'-GGAACAGCTGAGCCGTGAAC-3'	5'-GCGACCCGAAACCATCCT-3'	5'-AAGGCCATGACTGCGCGTGAGGCG-3'