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PPARG Modulated Lipid Accumulation in Dairy GMEC via Regulation of ADRP Gene

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ABSTRACT

Peroxisome proliferator-activated receptor-gamma (PPARG) is considered to be a central regulator of lipid metabolism in mammary cells. Adipose differentiation-related protein (ADRP), a member of PAT family (Perilipin, ADRP, TIP47 family), plays a key role in lipid accumulation in mammary gland. It has been found that PPARG significantly promoted lipid storage in goat mammary epithelial cells (GMEC), which was abolished after knockdown of ADRP gene. The results of real-time PCR, Western blot and luciferase reporter assay for goat *ADRP* promoter showed that ligand-activated PPARG up-regulated the *ADRP* gene expression and activity of *ADRP* promoter. Moreover, PPARG directly interacted with a PPRE (PPAR response element) spanning at −1003 to −990 on *ADRP* promoter. In this study, to our knowledge, we are the first to verify the function of PPARG on lipid storage on cellular level of goat mammary gland and our results revealed a novel pathway that PPARG may regulate lipid accumulation by controlling the expression of *ADRP* gene. J. Cell. Biochem. 116: 192–201, 2015.

KEY WORDS: GMEC; PPARG; LIPID; ADRP; PROMOTER

The mammary gland, as an active site of lipogenesis and secretion, is one of the central organs of lipid metabolism in the body. During lactation process, triacylglycerol (TG) are synthesized, lipid droplets (LDs) are formed which are then secreted into milk. Lipid droplets are dynamic cytoplasmic organelles which are not only serve as the storage sites for lipids, but also highly related to lipid metabolism, particularly connecting storage, hydrolysis and efflux of lipids [Olofsson et al., 2009]. Multiple functions of lipid droplets are characterized by specific proteins located on their surfaces. Perilipins (perilipin1, perilipin2/adipose differentiation-related protein (ADRP)/adipophilin, perlipin3/Tip47, perilipin4/S3–12, and perilipin5/OXPAT), also called PAT family (perilipin, ADRP, TIP47 family), are firstly identified specific lipid droplets marker proteins [Walther and Farese, 2012]. And ADRP, believed to be involved in the formation, stabilization, and metabolization of lipid droplets [Targett-Adams et al., 2003], is intrinsically associated with the surface of lipid droplets [Brasaemle et al., 1997] and can be secreted into milk associated with milk lipid globules [Heid et al., 1996; Cebo et al., 2010].

Peroxisome proliferator-activated receptor-gamma (PPARG) works as a nuclear receptor and is a member of PPARs family (PPAR alpha, beta, and gamma). It is expressed in numerous types of cells including adipocytes, macrophages, epithelial cells of the breast, colon, and lungs. Accumulating researches have reported that PPARG plays a crucial role in adipogenesis and glucose metabolism and seems to be a key regulator of fatty acid metabolism in mammary gland [Auwerx, 1999; Allred and Kilgore, 2005; Kawai and Rosen, 2010]. Although a regulatory network model has been built for bovine to reveal a central position of PPARG in milk fat synthesis [Bionaz and Loor, 2008], the molecular mechanisms by which PPARG regulates mammary lipid metabolism have not yet been well investigated, particularly in goat mammary gland.

Abbreviations: PPARG, peroxisome proliferator-activated receptor-gamma; ADRP, adipose differentiation-related protein; PAT family, perilipin, ADRP, TIP47 family; GMEC, goat mammary epithelial cells; PPRE, PPAR response element; TG, triacylglycerol; LDs, lipid droplets; ROSI, rosiglitazone; LCFA, long-chain fatty acids; Ad, adenovirus; MOI, multiplicity of infection; GFP, green fluorescent protein; NC, noneffective control; EMSA, electrophoretic mobility shift assay.

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Previous studies by Motomura et al. (2006) and Tobin et al. (2006) showed that the expression of *ADRP* was associated with PPARG activation and enhanced significantly after incubating with the ligands of PPARG, for example, rosiglitazone (ROSI) or long-chain fatty acids (LCFA). Furthermore, PPAR response element (PPRE) has been found on the promoter of *ADRP* gene in humans and mice [Fan et al., 2009]. In ruminants, however, the regulation of *ADRP* by PPARG remained to be confirmed to explore whether and how PPARG mediates lipid metabolism through controlling the expression of *ADRP* in the mammary gland. This study was designed to explore the role of PPARG in lipid accumulation and the regulatory relationship between PPARG and *ADRP* genes in mammary cells. The research could be useful to prove that PPARG is a key regulator of lipid storage in mammary cells and further determine about the regulatory network of lipid metabolism in ruminants. Our findings illustrated that PPARG could drive lipid accumulation through the direct regulation of *ADRP* by binding PPRE on ADRP promoter in goat mammary epithelial cells (GMEC), and ADRP was the main executor in the pathway of PPARG-induced lipid storage in mammary gland of goats.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

Primary cultured dairy GMEC were isolated [Wang et al., 2010] and cultured as previously described by our group [Lin et al., 2013; Shi et al., 2013]. GMEC were transfected with plasmid DNA until 80–90% confluence by method of liposome transfection using X-treme GENE HP DNA Transfection Reagents (Roche, Switzerland). Recombinant adenovirus for over-expression of *PPARG* (Ad-PPARG) was obtained from our laboratorial stores and the RNAi of *ADRP* (Ad-sh511) recombinant adenovirus vectors were constructed as previously reported by our laboratory [Shi et al., 2013]. The sense sequence of the specific shRNA for goat ADRP gene sequence (HQ846827.1) was as follows: 5′-GATCCGATGAGCAGTGGAGTAGAAgagtactgTTCTACTCCACTGCTCATCTTTTTTC-3′, which included *Bam*H I and *Xho* I restriction sites suitable for the cloning process. The recombinant virus was generated as described elsewhere [Shi et al., 2013]. The GMEC at about 80% confluence were transduced with adenovirus supernatant at multiplicity of infection (MOI) of 200. The null adenovirus Ad-GFP (Green fluorescent protein) and shRNA negative control adenovirus Ad-NC (Noneffective Control) were used as negative controls. Transduced GMEC were cultured with ROSI (Biovision) or DMSO (Sigma) after 24 h of the initial culture and then harvested at 48 h (24 h later) for RNA or protein extraction. ROSI was diluted in DMSO and used at 50 μM working concentration.

ISOLATION OF RNA AND REAL-TIME PCR

The expression of *PPARG* and *ADRP* mRNA was determined by real-time PCR. GMEC were seeded into 12-well plates and treated with different loads of PPARG adenovirus for 48 h and ROSI for 24 h separately. A time-course test including 0, 6, 12, and 24 h of ROSI was also performed. Total cellular RNA was extracted using RNAprep pure cell kit (Tiangen Biotech, China) and prepared for reverse transcription using PrimeScript® RT reagent kit (Takara, Japan). Quantitative real-time PCR was performed with SYBR® Premix Ex Taq™ II (Takara, Japan) on a CFX96 Real-time System (Bio-Rad). Relative mRNA expression levels of the target genes were normalized with *GAPDH* gene as the internal control. The primers used were as follows: PPARGF, 5′-CCTTCACCACCGTTGACTTCT-3′, and PPARGR, 5′-GATACAGGCTCCACTTTGATTGC-3′; ADRPF, 5′-TTGCTGTTGCCAATACCT-3′, and ADRPR, 5′-TGCCACTGACCACGGACT-3′; GAPDHF, 5′-GCAAGTTCCACGGCACAG-3′, and GAPDHR, 5′-GGTTCACGCCCATCACAA-3′.

WESTERN BLOT

After harvested, GMEC were lysed with RIPA buffer with a protease inhibitor mixture (Solarbio, China). Protein concentrations were determined using a BCA protein assay (Pierce) according to the manufacturer's instruction. Western blot was performed as previously described [Miura et al., 2012]. The blots were incubated with a polyclonal antibody against ADRP (Santa Cruz Biotechnology, H-80, 1:200), and a monoclonal antibody against GAPDH (Cwbiotech, China, 1:1000). Secondary antibody used a goat anti-rabbit IgG (Transgen, 1:1000). Protein bands were detected using a chemiluminescent ECL Western blot detection system (Pierce) and visualized by autoradiography with a cold CCD camera (Bio-Rad).

OIL RED O STAINING

GMEC cultured in 60 mm dishes were incubated with Ad-PPARG and Ad-sh511 supernatant for 48 h. ROSI was added 24 h before harvesting the cells. Oil Red O staining was performed on GMEC by the method as previously described [Fujimoto et al., 2012].

QUANTIFICATION OF TOTAL CELLULAR TG

Cellular total TG was extracted after the same treatment with the Oil-Red O staining according to the GPO-Trinder triglyceride assay kit (Applygen Technologies, China). The concentration of TG was determined according to the manufacturer's instructions on a micro-titer plate reader (BioTek). TG content was normalized by total cellular protein assessed using a BCA protein assay (Pierce).

GOAT *ADRP* GENE PROMOTER CLONING AND BIOINFORMATIC ANALYSIS

Based on the *ADRP* gene sequence (GenBank: HQ846827.1) and bovine genome (Chromosome 8;26,595,455 to 26,604,143 bp), the specific primers for goat *ADRP* promoter were designed (PomoterF:5′-TATGAGGGATACATTTTTGCCACGG-3′, PromoterR:5′-ATGTCACTTTAGTGTCCCCAACGGC-3′). *ADRP* promoter was amplified from the goat blood genomic DNA using PrimerSTAR HS DNA Polymerase (Takara, Japan) (−1868 to +509 bp, 2378 bp. Transcriptional factor sites of the promoter were analyzed using online software TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and PPRESearch (http://www.classicrus.com/PPRE/).

VECTORS CONSTRUCTION

The promoter was subcloned with *Hind*III/*Kpn*I (Takara, Japan) restriction enzyme into pGL3-basic vectors containing firefly luciferase reporter gene (Promega) to generate pGL3-*ADRP*

promoter vector. According to the predicted PPRE sequence, overlapping PCR was used to generate PPRE nested mutation and deletion mutants of *ADRP* promoter. The primers were designed to mutate the latter "AGG" in original PPRE to "GTC," and to delete the "CA" from the original one. Primers used in first PCR besides the ones used for promoter cloning described above were as follows: PPRE-Mut-AS(5′-TCCCCTTCTGACCCTGCCCCCAAT-3′), PPRE-Del-AS(5′-CTTCTCCTCCCCCCCAATCCCTC-3′), PPRE-Mut-S (5′-ATTGGGGG-CAGGGTCAGAAGGGGA-3′), PPRE-Del-S(5′- GAGGGATTGGGGG-GGAGGAGAAG-3′) . The second PCR was performed employing the first PCR fragments as the template with the promoter cloning primers. All the PCR products were subsequently subcloned into pGL3-basic vectors.

LUCIFERASE ASSAY

GMEC were seeded into 48-well plates and co-transfected with 200 ng plasmids of pGL3-*ADRP* promoter or pGL3 vectors with mutational PPRE and 4 ng pRL-TK which contains the Renilla luciferase reporter gene as an internal control for the luciferase analysis. Cells were harvested and lysed after 48 h. The relative luciferase activity was analyzed using Dual-Luciferase Reporter Assay (Promega) based on the manufacturer's instructions.

PREPARATION OF NUCLEAR EXTRACTS AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts from normal GMEC and Ad-PPARG infected GMEC were prepared following the protocol of Nuclear and Cytoplasmic Extraction Kit (Cwbiotech, China). The concentration of nuclear protein was determined by the BCA protein assay. EMSA was performed using LightShift® Chemiluminescent EMSA Kit (Pierce). The probes were labeled by biotin (Invitrogen). Nuclear extracts and probes were incubated at room temperature for half hour and loaded to 6% (w/v) non-denaturing polyacrylamide gels in 0.5 × TBE buffer. For the competition assays, 200-fold unlabeled probe competitor was added into the reaction mixture before addition of the nuclear extract.

DATA ANALYSIS

All the treatments were executed in triplicates and all the experimental data are presented as means ± SEM. One-way ANOVA was used for statistical comparisons in the effects among the treatments by IBM SPSS Statistics 20, and P values <0.05 were considered as significant ($P < 0.05$).

RESULTS

ROSI OR OVER-EXPRESSION OF PPARG UP-REGULATED EXPRESSION OF *ADRP* GENE

To acquire evidence that the expression of *ADRP* gene can be regulated by PPARG and its agonist, the time course and dose responsive relationship of the expression of *ADRP* mRNA with ROSI and Ad-PPARG in GMEC was shown in Figure 1A. Figure 1B shows the representative image of GFP fluorescence after GMEC was infected by Ad-PPARG for 48 h. PPARG induced an increase of *ADRP* mRNA level by about fivefold compared with control, and ROSI enhanced *ADRP* mRNA level by about 23-fold (Fig. 1C). The increase of ADRP protein level was statistically significant when *PPARG* was over-expressed with or without ROSI (Fig. 1D). However, over-expressing PPARG with incubation of ROSI leaded to an increase of 23.5% in ADRP protein level which was significantly greater ($P < 0.05$) than that without the addition of ROSI (12.9% increase). Thus, it could be inferred that PPARG promoted the expression of *ADRP* in GMEC, which was enhanced by PPARG agonist treatment.

PPARG INCREASED LIPID ACCUMULATION IN GMEC

Ad-PPARG was used to elevate the expression level of PPARG in GMEC. As shown in Figure 2A, over-expression of *PPARG* remarkably mounted lipid accumulation in GMEC which was further strengthened by ROSI, whereas the treatment with ROSI alone did not have a significant effect on lipid accumulation. Ad-sh511 was used to infect GMEC (Fig. 2B), and resulted in a loss of 88.9% of ADRP mRNA and 35.3% of the ADRP protein, respectively (Fig. 2C). The knockdown of *ADRP* weakened the PPARG-induced lipid accumulation significantly (Fig. 2D), implying that some PPARG-regulated functional genes, maybe *ADRP*, could lead to lipid accumulation in GMEC.

CHARACTERIZATION OF THE *ADRP* GENE PROMOTER AND IDENTIFICATION OF A LUCIFERASE REPORTER CONSTRUCT IN CULTURED CELL

The transcriptional start site of goat *ADRP* promoter was identified according to the 5′UTR sequence obtained by 5′ RACE. An initiation codon ATG was located in exon 2, and a putative TATA box was located 35 nucleotides upstream of the transcriptional start site. Three putative Sp1 binding sites were underlined bordering the TATA box. On the basis of the results analyzed by PPRESearch program, a PPRE scored 93 points was located at −1003 to −990 (Fig. 3A).

Compartive alignment analysis of PPRE in *ADRP* promoter in humans, mice and goats, in our study, showed the element structure of PPRE was highly conserved in humans and mice, while the goats' *ADRP* promoter has another clearly distinct PPRE sequence (Fig. 3B). GMEC were transfected with pGL3-*ADRP* promoter constructs, which showed an approximately 60-fold higher levels of luciferase activity than the control group that transfected with pGL3-baisc (Fig. 3C).

To determine whether *ADRP* promoter could have a similar response to PPARG and its agonist from the tests above, PPARG adenovirus and ROSI were used to assay the change of luciferase activity (Fig. 3D). We found that adenoviral expression of PPARG raised the luciferase activity of pGL3-*ADRP* promoter constructs significantly, which was enhanced by ROSI and reached approximately to fourfold compared with the group treated with adenovirus expressing GFP only. Therefore, it could be concluded that PPARG, working as a specific transcription factor, promoted the activity of *ADRP* promoter in the presence of its agonist, which may be resulted from the direct interaction with a PPRE.

SITE-DIRECTED MUTAGENESIS AND DELETION ANALYSIS

To determine whether it was an indispensable part in the up-regulating effect of *ADRP* transcription induced by PPARG, the PPRE at −1003 to −990 was mutated or deleted by the site-directed mutagenesis and deletion, and sequencing was applied to assess the impregnability of the promoter except for PPRE. As shown in Figure 4, two types of mutants lost most or all of the effects of

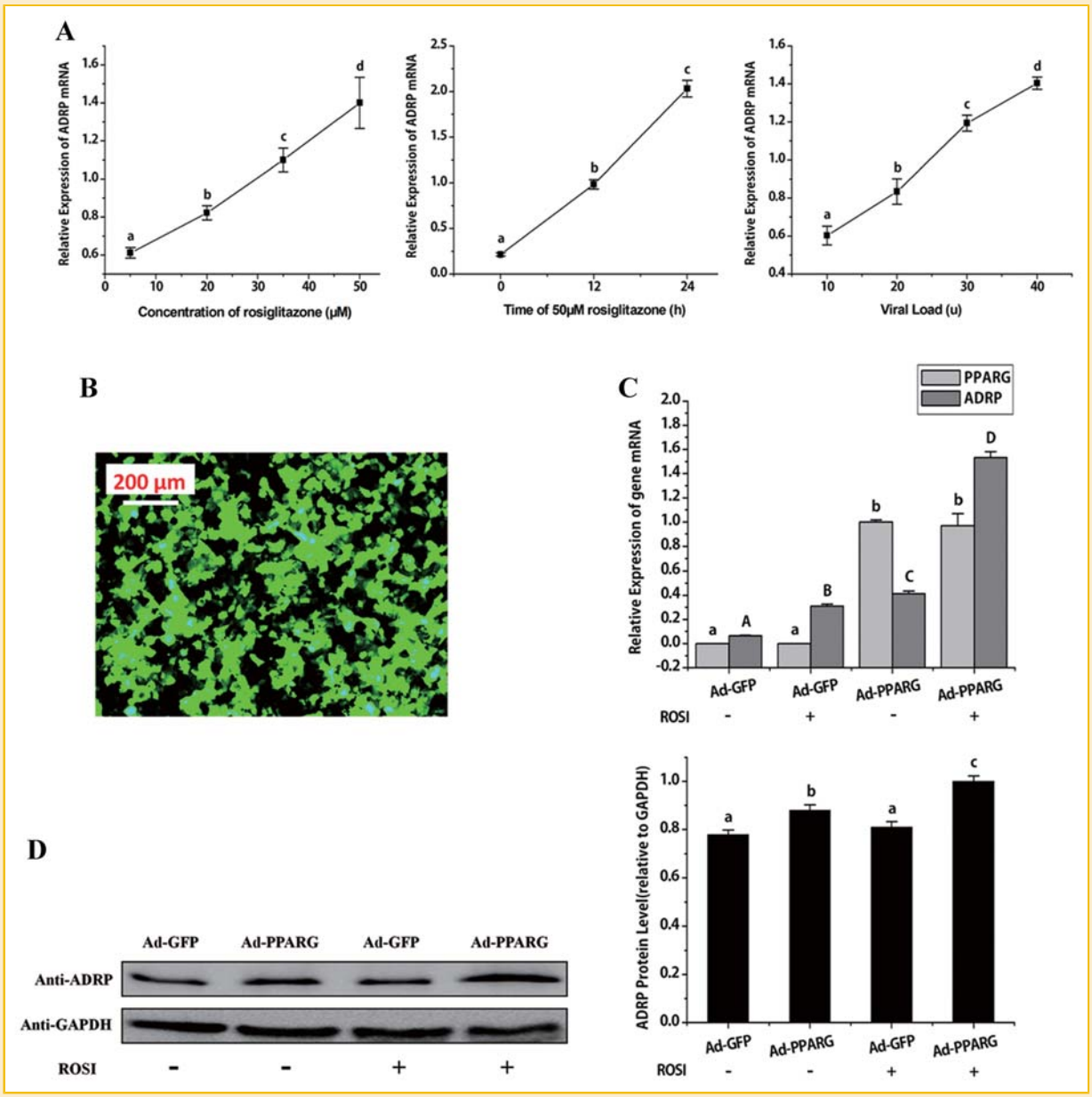

Fig. 1. PPARG and its agonist affected ADRP expression in goat mammary epithelial cells. A: Changes of ADRP mRNA expression at different concentrations and incubation times of ROSI treatment, and different levels of ligand (50 μM ROSI)-activated PPARG. Values of mRNA expression with different letters mean significantly different ($P < 0.05$) compared with other parallel treatments. The measurement of viral load "u" stands for unit of Ad-PPARG adenovirus (MOI = 200). B: Infection of GMEC with Ad-PPARG adenovirus after 48 h (MOI = 200) (×100). C: Effects of PPARG over-expression on ADRP mRNA expression with or without ROSI (50 μM). Bars with different letters indicate significant differences ($P < 0.05$) compared among cells treated and untreated with Ad-PPARG or ROSI. D: Effects of PPARG over-expression on ADRP protein level with or without ROSI (50 μM). Left panel: representative immunoblots; Right panel: corresponding mean gray values of ADRP protein which was expressed in relevance to GAPDH from three separate blots. Bars with different letters indicate significant differences ($P < 0.05$) compared among cells treated and untreated with Ad-PPARG or ROSI.

inducing luciferase activity compared with the wild *ADRP* promoter. The result suggested that the putative PPRE at −1003 to −990 could be a minimal key region involved in the PPARG induced augment of ADRP promoter activity in GMEC.

PPARG DIRECTLY BONDED TO PPRE ON *ADRP* PROMOTER

To verify that PPARG can bind to PPRE on goat *ADRP* promoter, EMSA was performed with the specific oligonucleotide probes containing PPRE as described above. A complete set of three reactions were performed which is shown in Figure 5A. An apparent complex was formed when the biotin-labeled probes were incubated with the nuclear extracts from the normal GMEC, and the complex-forming ability was almost abolished by an addition of 200-fold unlabeled competitors. In order to identify the complex, with or without the incubation of ROSI, the nuclear extracts from the treatment including null, Ad-GFP and Ad-

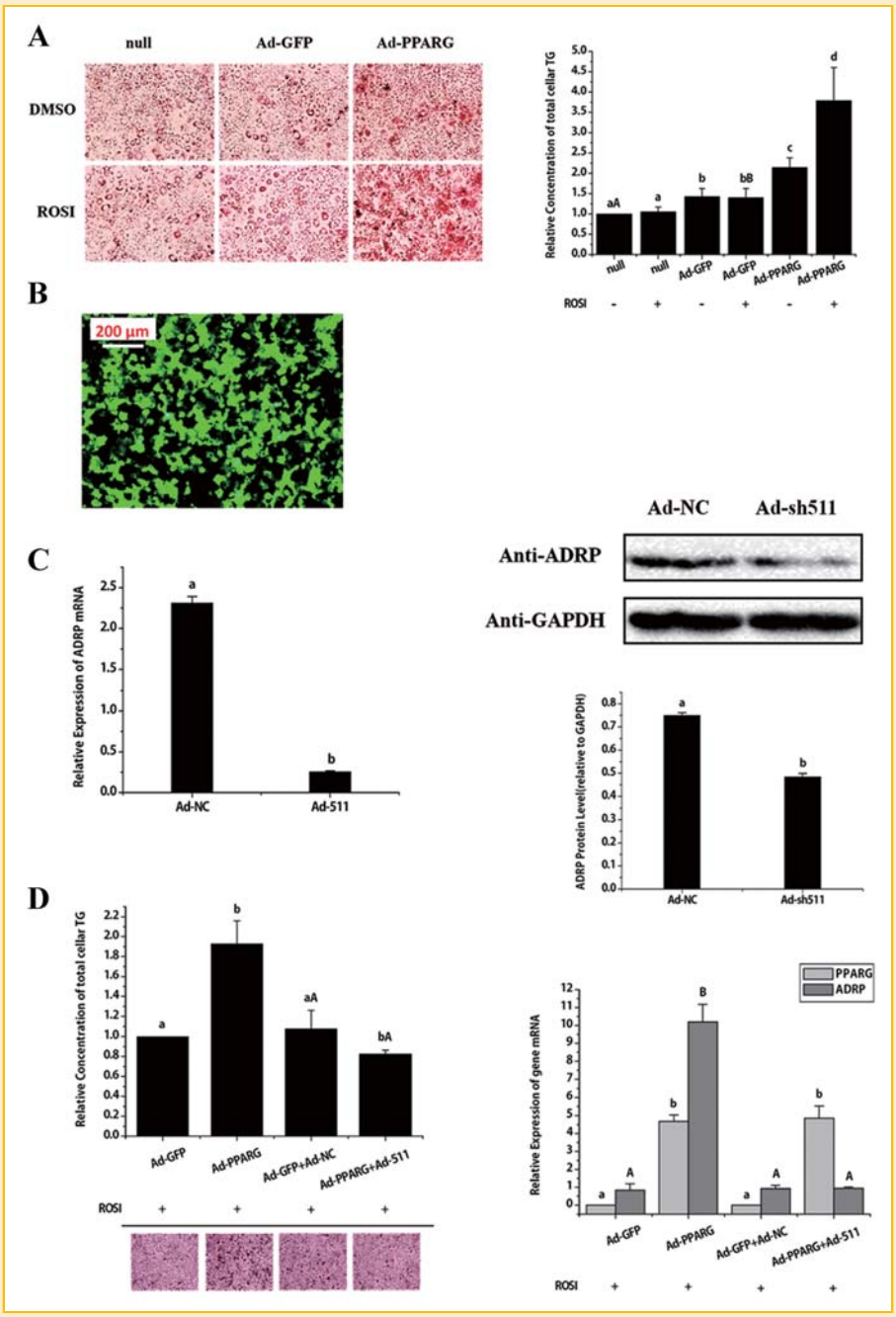

Fig. 2. Lipid accumulation induced by PPARG over-expression in GMEC. A: Effects of PPARG over-expression on lipid accumulation. Oil Red O staining of the cells was performed after the treatment of Ad-PPARG and ROSI (50 μM). Quantification of total TG in cells was measured at 550 nm. Bars with different letters indicate significant differences ($P < 0.05$) compared among cells treated and untreated with Ad-PPARG or ROSI. B: Infection of Ad-sh511 adenovirus in GMEC after 48 h (MOI = 200) (×100). C: Detection of ADRP knockdown efficiency. Corresponding mean gray values of ADRP protein level was from three separate blots. Values of ADRP mRNA and protein level with different letters indicate highly significant differences ($P < 0.01$). D: Effects of ADRP knockdown on PPARG-induced lipid accumulation and the changes of PPARG and ADRP mRNA levels after the treatments. The titer of adenovirus used was halfed to allow for the cells to receive two different types of adenovirus. Relative TG concentration was corresponding with Oil Red O staining effects. Bars with different letters indicate significant differences ($P < 0.05$) compared among cells treated with Ad-PPARG, Ad-sh511 and null adenovirus.

PPARG were prepared. Over-expression of *PPARG* facilitated the complex-forming ability, which could be enhanced by ROSI (Fig. 5B). Collectively, these results further supported our speculation that PPARG protein can directly interact with PPRE on goat *ADRP* promoter.

DISCUSSION

PPARG has been reported to play a key role in the regulation of adipocyte differentiation in an early study [Tontonoz et al., 1994], and found to modulate lipid metabolism and storage in different types of

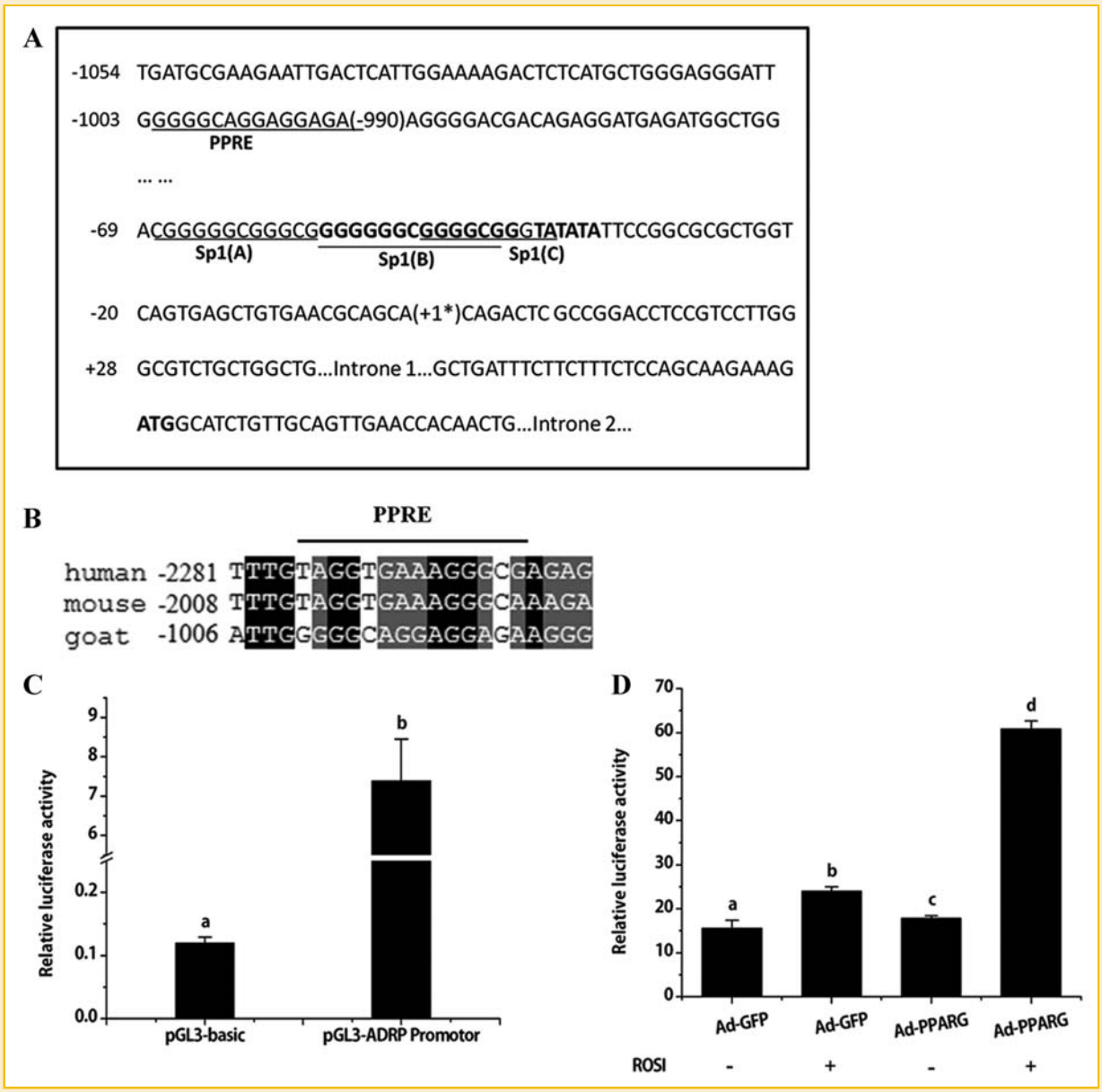

Fig. 3. PPARG up-regulated the activity of goat ADRP promoter in response to the PPARG agonist (50 μM). A: Characteristics of the goat ADRP promoter. +1* indicates the transcriptional start site. Initiation codon and TATA box are highlighted in bold. Putative PPRE and Sp1 binding sites are underlined. B: Alignment of the segments of ADRP gene promoter containing PPRE from humans, mice and goats. The promoter sequence of ADRP gene from humans and mice were reported by Fan (2009). Nucleotides with black, gray background indicate matching fully (with the exactly same single nucleotide) and preferably (all with purines or all with pyrimidines), respectively. Otherwise, mismatching is shown as no background. C: Identification of the luciferase activity of pGL3-ADRP promoter constructs in GMEC. Luciferase activity with different letters indicates a highly significant difference ($P < 0.01$). D: Effects of PPARG over-expression and its agonist on transcriptional activity of ADRP promoter. Bars with different letters indicate significant differences ($P < 0.05$) compared among cells treated and untreated with Ad-PPARG or ROSI (50 μM).

cells and various conditions [Chawla et al., 2001; Bogacka et al., 2004; Szatmari and Nagy, 2008; Almeida et al., 2012]. Some specific ligands for PPARG were elaborated to up-regulate *ADRP* expression [Buechler et al., 2001; Bildirici et al., 2003; Schadinger et al., 2005; Suzuki et al., 2010]. Moreover, *ADRP* has been reported to be regulated by PPARG in a few types of cells [Gupta et al., 2001; Schadinger et al., 2005]. However, whether PPARG directly regulates *ADRP* in ruminants is still unknown. As found in this paper, the mRNA level of *ADRP* gene was dramatically up-regulated by treatments with ROSI, Ad-PPARG and their combination. Intriguingly, the ADRP protein level did not increase as markedly as its mRNA level, maybe due to the limited surface area and volume of LDs. ADRP protein would be degraded by proteasomes after translation unless interacting with LDs [Xu et al., 2005]. In addition, although it was hard to notice the difference in Figure 1C, the mRNA level of PPARG was actually lifted by about 33.3% with ROSI treatment. Our result is similar to the result

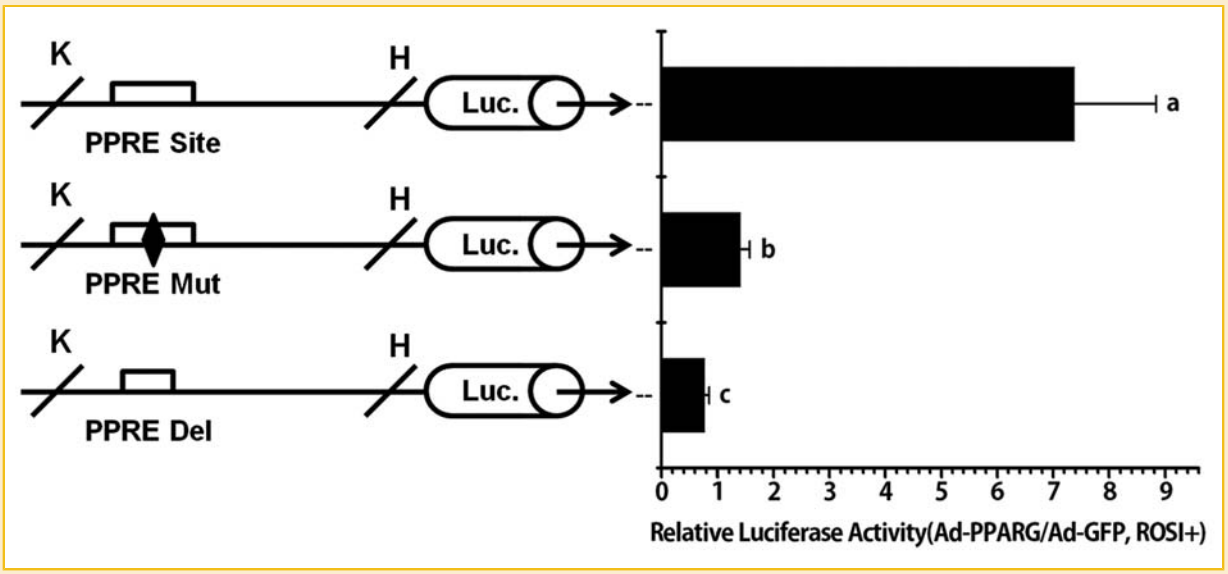

Fig. 4. A PPRE mediates the up-regulation of ADRP promoter activity induced by PPARG and its agonist (50 μM). The line from K (restriction site Kpn I) to H (restriction site Hind III) stands for the ADRP promoter equence. The putative PPRE is marked on the schematic diagram. The PPRE filled with black rhombus and shortened indicates the nested mutation and nested deletion, respectively. In the histogram for Ad- PPARG/Ad-GFP, the value of X-axis represents the uplifted relative luciferase activity after an infection with PPARG adenovirus compared with null adenovirus. Bars with different letters indicate significant differences ($P < 0.05$) among cells transfected with the reporter that was constructed with mutated, partly deleted or normal PPRE.

reported by Kadegowda et al. (2009) on bovine mammary epithelial cells. However, the slight fluctuation of *PPARG* mRNA level resulted from ROSI could cause a significant change in the transcriptional activity of *ADRP* (Figs. 1A and 3D). Furthermore, the over-expression of PPARG indeed induced lipid accumulation in GMEC in this research, consistent with its cellular efficiency in hepatocytes reported previously by Schadinger et al. (2005). Our findings and these reports prompt us to colligate the effect of *ADRP* gene in mammary gland. *ADRP* gene has been shown to be particularly involved in lipid accumulation in GMEC in our recent studies (data unpublished). It may also function as a lipid storage marker not only in mammary gland (as per this study), but also in many other tissues and cell types [Larigauderie et al., 2004; Magnusson et al., 2006; Motomura et al., 2006; Mak et al., 2008; Shaw et al., 2009; Shepherd et al., 2012]. Our data also showed that knockdown of *ADRP* observably decrease the content of TG induced by PPARG, which opens a possibility that PPARG conducts lipid accumulation via the control of *ADRP*. Furthermore, in this experiment the incubation with ROSI without over-expression of PPARG did not induce the increase of TG content in GMEC (Fig. 2B), although those cells remained expressing PPARG in a background level. This phenomenon is similar to the earlier study on troglitazone, and the work on PPARG′ s ligand in hepatocytes by Schadinger et al. (2005). All these findings worth pondering that ROSI do not actually boost the ADRP protein in GMEC.

To explore whether PPARG could regulate the expression of *ADRP* gene directly in GMEC, we cloned goat *ADRP* promoter including a potential PPRE in the promoter. The site-directed mutagenesis and deletion analysis for the PPRE in the ADRP promoter demonstrated that the PPRE was a fateful element for lifting transcriptional activity induced by PPARG and its specific agonist. Interestingly, through the sequence alignment of *ADRP* promoters (Fig. 3B), the PPRE in goat *ADRP* promoter was quite discrepant with these reported for humans and mice [Fan et al., 2009]. The differences among the species may be caused by the gene regulation patterns from PPARs which are not ubiquitously conserved [Targett-Adams et al., 2005]. However, a PPRE in murine *FATP* gene [Frohnert, 1999] has been found to be quite analogous to our one. Similar to *FATP*, *ADRP* gene is also recognized to play a role in LCFA uptake as a fatty acid binding protein [Gao and Serrero, 1999; Gao et al., 2000; Serrero et al., 2000]. It is fascinating to propose that a specific PPRE in one gene may strongly link to its specific function through the regulation of PPARG in particular types of cells and conditions. To further verify this proposed molecular mechanism by which PPARG directly interacts with the putative PPRE, the differently prepared nuclear extracts were applied in the EMSA in this study. The significantly enhanced complex-forming ability was displayed after the over-expression of *PPARG* or even infected with more adenovirus. Therefore, we confirmed that PPARG could work as a special transcription factor and directly regulate *ADRP* gene which is then responsible for cellular lipid accumulation in GMEC.

Recent studies by Schadinger et al. (2005) showed that PPARG can mediate lipid accumulation through the induction of expression of some genes related to de novo lipid synthesis like SREBP-1 and FAS. In addition, Cidea, a gene that promotes LD enlargement through the LD- LD lipid transference, is also reported by Viswakarma et al. (2007) to be regulated by PPARG. Besides, PPARG can mediate TG content by regulating Gyk which is required for the recycling of glycerol and fatty acids for formation of TG [Guan et al., 2002]. Here, we put forward another pathway that some genes like *ADRP*, which are responsible for lipid

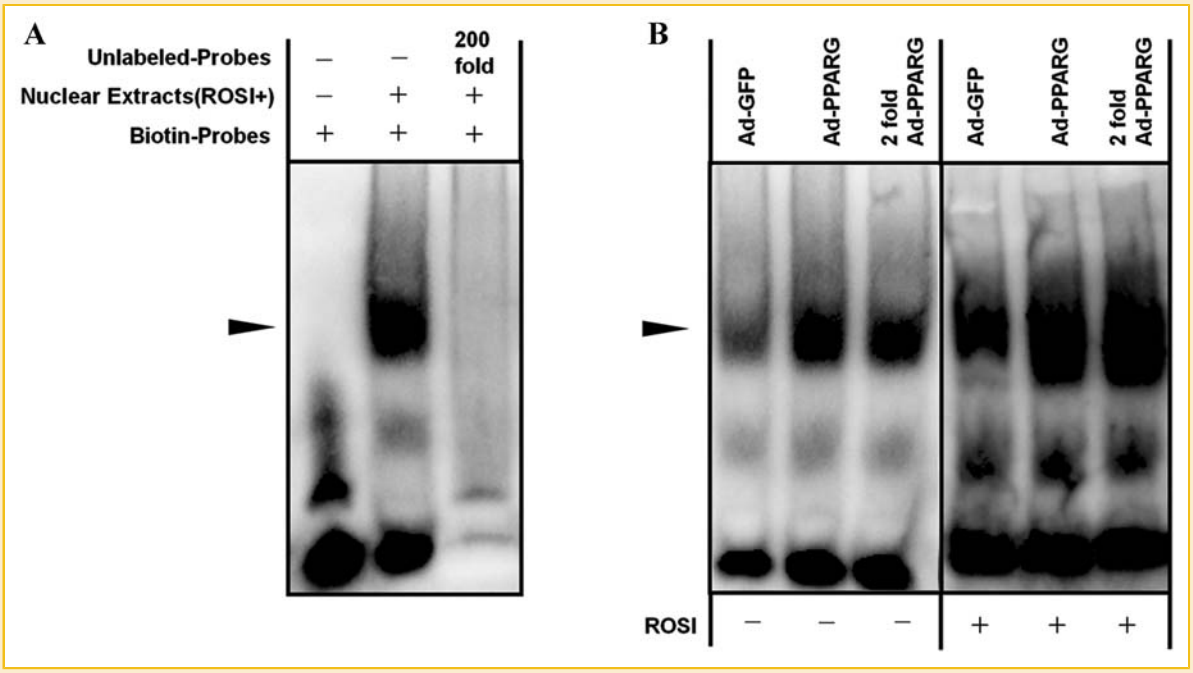

Fig. 5. The direct bonding of PPARG protein to the PPRE in vitro as revealed by electrophoretic mobility shift assay. A: A complete set of three reactions were performed using the nuclear extracts prepared from normal GMEC treated with ROSI (50 μM). 200-fold of unlabeled probes were used as specific competitors to demonstrate the signal shift was resulted from the specific protein: DNA interaction. B: Nuclear extracts obtained from cells infected with PPARG adenovirus with or without ROSI (50 μM) were used to certify the complex formed from PPARG protein bonding to the PPRE in ADRP promoter. 2-fold Ad-PPARG means the titer of adenovirus was doubled than the AD-PPARG group. Arrowheads indicate the specific complexes.

storage, stabilization of lipid droplets and even uptake of LCFA, also can be directly regulated by PPARG. This pathway may also respond to the signals, such as lipid loading, which can activate PPARG and then dictate the cell to accumulate lipid. To induce lipid loading in GMEC, the cell culture medium was supplemented with 200 μM oleic acid complexed to bovine serum albumin. We determined the time course of the mRNA level of *PPARG* and *ADRP* up to 24 h, and found that the expression of the two genes showed a quadratic pattern: earlier increases followed with later decreases (data not shown), with *PPARG* mRNA peaked nearby 3 h and *ADRP* mRNA peaked around 12 h. Thus, rising transcriptional activity of *PPARG* responded to stimulation of lipid loading and then induced the increase of ADRP which could eventually result in cellular lipid accumulation.

Perilipin that is directly regulated by PPARG executes a function to protect lipid droplets from hydrolyzation, leading to lipid accumulation in adipocytes [Rosen et al., 1999]. It is worth addressing here that perilipin is expressed at an extremely low level in mammary gland. Consequently, ADRP, another important member in the PAT family, may substitute the duty of perilipin, and be regulated by PPARG analogously. Furthermore, research should focus not only on the ability of ADRP in leading and sustaining lipid accumulation, but also on its effects and vital roles in milk lipid formation and secretion. Therefore, further research is required to understand practical functions of PPARG in mammary gland.

In conclusion, we have demonstrated that PPARG could directly regulate the expression of *ADRP* gene, which may induce lipid accumulation in GMEC. Compared with *ADRP* genes of humans and mice, a different type of PPRE in goat *ADRP* gene was discovered, and working as an indispensable cis-acting element in the regulatory relationship between PPARG and *ADRP* genes. Further study is needed to explore whether there are other crucial pathways that are regulated by PPARG in modulating lipid metabolism in mammary gland of the ruminants.

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