Peroxisome proliferator-activated receptor delta regulates lipid droplet formation and transport in goat mammary epithelial cells

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ABSTRACT

Even though recent evidence in goat mammary epithelial cells (GMEC) suggest a role of peroxisome proliferator-activated receptor delta (PPARD) in regulating lipid homeostasis, its role is not fully understood. Our hypothesis was that PPARD regulates lipid transport processes in GMEC and, thus, plays a crucial role in regulating fat formation. The *PPARD* was overexpressed using an adenovirus system (Ad-PPARD) with recombinant green fluorescent protein (Ad-GFP) as the control. Results revealed that overexpression of *PPARD* markedly upregulated the mRNA abundance of *PPARD*. Compared with the control (Ad-GFP+dimethyl sulfoxide), overexpression of *PPARD* alone had no effect on mRNA expression of *CD36*, *SCD1*, *FABP4*, *ACSL1*, and *ADRP*. The cultures overexpressing *PPARD* with the PPARD ligand GW0742 (GW) upregulated the expression of *CD36*, *FABP3*, *FABP4*, *ACSL1*, and *ADRP*. Overexpression of *PPARD* in GMEC plus GW increased the concentration of 16:1 and 18:1-*trans* and was associated with upregulation of *SCD1*. Compared with the control (Ad-GFP+dimethyl sulfoxide), the decrease of triacylglycerol concentration coupled with upregulation of genes related to lipid droplet secretion (e.g., *ADRP* and *ACSL1*) induced by *PPARD* overexpression suggests a role in lipid droplet (LD) secretion. Luciferase assay revealed that GW increased the ADRP promoter activity in a dose-dependent manner. Knockdown of *PPARD* impaired the increase of ADRP promoter activity induced by GW, whereas GW enhanced the activity of ADRP promoter in GMEC overexpressing *PPARD*. Data with the *ADRP* 5'-flanking truncated luciferase reporter suggest a core region (−1,444 to −990 bp) response element

for the induction of GW. This core region contains a known PPARG response element (PPRE) at −1,003 to −990 bp. When the PPRE was mutated, the overexpression of *PPARD* had no effect on *ADRP* promoter activity. Collectively, these results reveal a novel role for PPARD in lipid homeostasis via promoting fatty acid transport and LD formation through a mechanism of direct binding to the promoter of key genes. Hence, PPARD activity may contribute to fatty acid transport and LD formation during lactation.

Key words: perilipin, promoter, peroxisome proliferator-activated receptor response element

INTRODUCTION

Transcription factors play important roles regulating fatty acid metabolism in ruminant mammary tissue (Harvatine et al., 2009; Oppi-Williams et al., 2013). Peroxisome proliferator-activated receptors (PPAR) are nuclear proteins that regulate the synthesis of lipid or lipid oxidation upon activation by endogenous or synthetic agonists (Bionaz et al., 2013). In the ruminant, the role of PPAR α (PPARA) (Bionaz et al., 2013) and PPAR γ (PPARG; Shi et al., 2013b, 2014b) in controlling lipid metabolism in mammary tissue has been well studied. Despite data indicating that its function seems to overlap with PPARA in terms of fatty acid (FA) oxidation (Giordano Attianese and Desvergne, 2015), compared with PPARG and PPARA, the biologic role of PPAR delta (PPARD) remains unclear. The finding in mice that PPARD activates an array of genes required for FA degradation and uncoupling suggested an opposite role of PPARD compared with PPARG (Wang et al., 2003). Thus, exploring and understanding the regulatory role of PPARD in lipid metabolism in mammary cells may contribute to improving efficiency and quality of ruminant milk production.

The lactating mammary gland is one of the most activate in terms of synthesis, utilization, and transport of FA. The FA transport process is related to the function

Received July 21, 2017.

Accepted November 27, 2017.

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of several key proteins (Haunerland and Spener, 2004; Wang et al., 2015) such as fatty acid binding proteins (**FABP**). The integral membrane protein coded by *CD36* is believed to physically transport the FA across the plasma membrane (Pepino et al., 2014). A growing body of data suggests that FA uptake also requires concomitant FA activation to acyl-CoA (Mashek and Coleman, 2006). The protein long-chain FA acyl-CoA synthetase (**ACSL**) plays a central role in the FA uptake and also is associated with lipid droplet secretion (**LD**; Grevengoed et al., 2014). Once activated to acyl-CoA, FA have diverse cellular fates, but the 2 predominant routes of metabolism are degradation via oxidation or incorporation into complex lipids (e.g., triacylglycerol, **TAG**; Bionaz and Loor, 2008a). In the lactating mammary gland, the FA are mainly incorporated into lipid droplets and secreted out of the epithelial cells. Recent data underscored the role of adipocyte differentiation-related protein (**ADRP**) in promoting LD formation and secretion in dairy goat mammary epithelial cells (**GMEC**; Shi et al., 2015b). A growing body of data supports the view that the process of FA transport is regulated by multiple transcription factors, including PPAR (Bionaz et al., 2013; Shi et al., 2014b).

Data in GMEC demonstrating that PPARD activation by the synthetic ligand GW0742 (**GW**) upregulated the expression of genes containing *FABP4* and *ADRP* suggested a role of PPARD in promoting FA transport (Shi et al., 2017). Furthermore, the knock-down of PPARD selectively downregulated *ACSL1* and *ADRP* mRNA abundance in GMEC, underscoring a role for PPARD in lipid transport (Shi et al., 2017). It is hypothesized that overexpression of *PPARD* would upregulate the mRNA expression of genes related to FA transport and lipid droplet formation in ruminant mammary cells at least in part through binding the promoter region.

To investigate the role of PPARD in regulating FA transport and lipid droplet formation in ruminant mammary cells, in this study, goat *PPARD* was overexpressed in GMEC. The activity of the *ADRP* promoter was also measured to assess the effect of PPARD on *ADRP* transcription.

MATERIALS AND METHODS

Adenovirus Generation

The *PPARD* (GenBank no. XM_004018768.3) cDNA was cloned from GMEC, and then subcloned into the pAdTrack-CMV plasmid vector to generate pAdTrack-CMV-PPARD vector. The vector was inserted into an

adenoviral vector (pAdEasy-1) to generate adenoviral plasmids in BJ5183 cells. The adenoviral plasmids linearized by *PacI* (New England Biolabs, Ipswich, MA) were transfected into 293A cells to generate the adenoviral Ad-PPARD. The generation and proliferation of adenovirus (Ad-PPARD) was performed as previously described (Shi et al., 2013a). The 293A cell line for adenovirus generation was cultured in basal Dulbecco's modified Eagle medium (Gibco, Waltham, MA) containing 10% fetal bovine serum. A recombinant adenovirus of green fluorescent protein (Ad-GFP) was used as a control.

Cell Culture and Treatments

The GMEC were isolated from peak lactation Xining Saanen goats as described previously (Shi et al., 2014a). The GMEC were isolated from 3 individual goats. Details of cell culture are described elsewhere (Shi et al., 2013b, 2014a). Briefly, the collected mammary tissues were pooled together and cut into small pieces using scissors before plating on cell culture dishes. The GMEC were isolated from this mixture of tissue. To promote lactogenesis, GMEC were cultured in a lactogenic medium for 24 h before initial experiments. Lactogenic medium was composed of Dulbecco's modified Eagle medium/F12 (Hyclone, Beijing, China), insulin (5 mg/L, Sigma-Aldrich, St. Louis, MO), hydrocortisone (5 mg/L, Sigma-Aldrich), penicillin/streptomycin (10,000 units/L, Harbin Pharmaceutical Group, Harbin, China), prolactin (2 µg/mL, Sigma-Aldrich), and BSA (1 g/L, Sigma-Aldrich).

For the adenovirus infection, the GMEC cultured in 6-well plates were incubated with adenovirus medium Ad-GFP or Ad-PPARD, respectively. Treated GMEC were cultured with 1 µM GW [final concentration, diluted in dimethyl sulfoxide (**DMSO**), Sigma-Aldrich] or control (**DMSO**, Sigma-Aldrich) after 24 h of initial culture, and then harvested at 48 h (24 h later) for RNA extraction, cellular TAG assay, or lipid extraction. The adenovirus Ad-GFP was used as a control.

Total RNA Extraction and Quantitative PCR

Total RNA was extracted using the RNA Prep pure cell Kit (Tiangen Biotech Co. Ltd., Beijing, China) and quantitative PCR (**qPCR**) was performed according to the manufacturer's instructions using SYBR Green (SYBR Premix Ex Taq II, Perfect Real Time, Takara Bio Inc., Dalian, China; Shi et al., 2014b). Genes studied included *PPARD*, *ACSL1*, *ADRP*, *CD36*, *FABP3*, *FABP4*, and *SCD1*. All the qPCR reactions were performed in a Bio-Rad CFX96 (Bio-Rad Laborato-

ries Inc., Hercules, CA) sequence detector, and data were normalized to the geometric mean of ubiquitously expressed transcript (*UXT*), mitochondrial ribosomal protein L39 (*MRPL39*), and ribosomal protein S9 (*RPS9*). The sequences of the primers for the genes are reported in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13543>).

Lipid Extraction and Analysis

Collected GMEC were washed 3 times with PBS and then scraped off the culture dish using a 2-mL aliquot of 2.5% (vol/vol) vitriol:methanol. Then, total lipid extraction and methylation was performed according to Shi et al. (2013a). Methylated lipid samples were analyzed using a GC-MS (Agilent Technologies, Santa Clara, CA) installed with an HP-5 column (35 m × 0.25 mm i.d. × 0.25 μm film thickness) following a published procedure (Yao et al., 2016). The relative proportion of each FA was determined as the ratio of the FA peak to the total peaks in each run. The data for each FA were analyzed as a proportion of the total FA.

Vector Construction

The goat *ADRP* promoter sequence was amplified from goat blood genomic DNA according to a previous publication (Kang et al., 2015). The PCR primers were designed to hybridize at −1,868, −1,444, −990, −788, and −38 bp to generate the corresponding deletion plasmid derivatives using the common downstream primer at +516 bp (deletion primers are provided in Supplemental Table S2; <https://doi.org/10.3168/jds.2017-13543>). The resulting amplicon was cloned into the pGL3-Basic vector (Promega, Madison, WI) by using *KpnI*/*BglII* (Takara Bio Inc.) enzyme sites.

According to the reported PPRE (−GGGGCAGGAGGAGA−, −1,003 to −990 bp), site-directed mutagenesis was performed to mutate the PPRE site in the ADRP promoter wild type (**ADRP-wild**, −1,868 to +516 bp) by altering 3 nucleotides within each site (“AGG” in original PPRE to “GTC”; Kang et al., 2015). The resulting amplicons were ligated to the pGL3-basic vector after digestion with *KpnI*/*BglII* (Takara Bio Inc.) to create a mutant construct (**ADRP-mut**). All the plasmids were confirmed by DNA sequencing.

RNA Interference

To knockdown the expression of *PPARD* (GenBank no.: XM_004018768.3), small interfering RNA (**siNC**, **siPPARD**) were designed and synthesized by Jima

Corp. (Shanghai, China). A scrambled siRNA was designed and used as a negative control. The siRNA sequence is 5′-GCUG GCCUCCAUGUCAAUTT-3′ (Shi et al., 2017).

Vector Transfection

For the luciferase assay, the GMEC cultured in 48-well plates at 80 to 90% confluent were co-transfected with 290 ng of the vector (*ADRP-wild*, *ADRP-mut*, or deletion-fragment of *ADRP* promoter) plus 10 ng of control vector (renilla luciferase) per well using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. For the transfection of *ADRP-wild* or *ADRP-mut*, transfected GMEC were incubated with adenovirus medium (Ad-GFP or Ad-PPARD), or incubated with siRNA (siNC or siPPARD) using Lipofectamine RNAiMAX (Invitrogen) after 12 h of initial culture. The transfected GMEC were treated with 1 μM GW (final concentration, diluted in DMSO, Sigma-Aldrich) or control (DMSO, with the same volume as GW solution) after 36 h of initial culture, and then harvested at 56 h for luciferase activity assays.

Based on the fact that a concentration of GW of 1 to 10 μM could markedly increase the activation of PPARE in GMEC (Shi et al., 2017), this compound was chosen to assess the response of *ADRP-wild* to PPARE activation. After 12 h of initial culture, the transfected GMEC were treated with 1 μM, 5 μM GW (final concentration, diluted in DMSO, Sigma-Aldrich), or control (0 μM, with the same volume as the GW solution) after 24 h of initial culture, and then harvested at 48 h for luciferase activity assays.

To assess the activity of deletion fragments of the *ADRP* promoter in response to PPARE activation, after 12 h of initial culture, the transfected GMEC were treated with 5 μM GW or DMSO after 24 h of initial culture, and then harvested at 48 h for luciferase activity assays.

Luciferase Analysis

After collection, the GMEC were washed 3 times with PBS and then incubated with passive lysis buffer (Promega) for 30 min at room temperature. The dual-Luciferase Reporter assay kit (Promega) was used to measure luciferase activity on a Fluoroskan Ascent apparatus (Thermo Fisher Scientific, Waltham, MA) according to the kit manufacturer's protocol (Shi et al., 2017). The relative luciferase activity was calculated as the ratio of firefly luciferase compared with renilla luciferase activity.

Cellular TAG Assay

Total cellular TAG was extracted according to the protocol of the GPO-Trinder triglyceride assay kit (Appligen Technologies, Beijing, China). The concentration of TAG was determined according to the manufacturer's instructions on a microtiter plate reader (BioTek, Winooski, VT). The concentrations were calculated using the equation obtained from linear regression of the standard curve. The quantification of total cellular TAG was normalized to the cellular protein concentration. Protein concentration of each well was determined using a BCA protein assay (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions (Shi et al., 2017).

Statistical Analysis

Treatments were replicated at least 3 times, and results are expressed as mean $\pm$ standard error of the mean. Data from qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to the control (Ad-GFP+DMSO). Statistical differences of mRNA expression and FA profile and activity of ADRP-wild treated with GW at different concentration were determined with a 1-way ANOVA using SPSS 19.0 (SPSS Inc./IBM Corp., Chicago, IL). Statistical analyses of each fragment of ADRP promoter activation were determined with a *t*-test (paired and 2-tailed; SPSS Inc./IBM Corp.). The activity of ADRP-wild or ADRP-mut were expressed within knockdown (siPPARD) or overexpression (Ad-PPARD) of PPARD, or control (Ad-GFP or siNC). This approach allowed us to assess directly the effect of different treatments for a given factor. Statistical differences were determined with a 1-way ANOVA using SPSS 19.0. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Overexpression of PPARD Increased the mRNA Abundance of Genes Related to Lipid Transport

To assess the effect of PPARD on the expression of genes related to lipid transport, *PPARD* was overexpressed in the GMEC using adenovirus (Ad-PPARD), with Ad-GFP as the control. As shown in Figure 1A, compared with the control (Ad-GFP+DMSO), incubation with the ligand (GW) had no effect on the expression of *PPARD* in the cells infected with Ad-GFP. Overexpression of *PPARD* without (Ad-PPARD+DMSO) or with (Ad-PPARD+GW) GW markedly upregulated the expression of *PPARD* compared with the control

(Ad-GFP+DMSO) and GW (Ad-PPARD+DMSO) treatments (Figure 1A). However, *PPARD* overexpression alone did not ($P > 0.05$) increase mRNA expression of *SCD1* and *CD36* compared with the control (Ad-GFP+DMSO). The addition of GW to cultures overexpressing *PPARD* (Ad-PPARD+GW) upregulated the expression of these 2 genes by ~ 3 - and ~ 17 -fold, respectively, relative to the control (Ad-GFP+DMSO; Figure 1B). Compared with the control (Ad-GFP+DMSO), the cells incubated with GW had a higher *CD36* mRNA expression, but not *SCD1* mRNA.

Compared with the control (Ad-GFP+DMSO), the addition of GW to cultures did not affect the mRNA abundance of *FABP3* ($P > 0.05$) but increased the expression of *FABP4* ($P < 0.05$; Figure 1C). Compared with the control (Ad-GFP+DMSO), the mRNA expression of *FABP3* and *FABP4* was upregulated ($P < 0.05$) when the transfected cells (Ad-PPARD+GW) were incubated with GW. Relative to the control (Ad-GFP+DMSO), the mRNA of *FABP3* and *FABP4* increased by ~ 1.8 - and 2-fold, respectively. Compared with the control (Ad-GFP+DMSO), the cells overexpressing *PPARD* (Ad-PPARD+DMSO) resulted in a decrease of *FABP3* mRNA but had no effect on *FABP4* mRNA expression.

Compared with the control (Ad-GFP+DMSO), genes encoding proteins associated with FA activation (*ACSL1*) and lipid droplet secretion (*ADRP*) were upregulated ($P < 0.05$) in cultures incubated with GW (Figure 1D). Addition of GW to cells overexpressing *PPARD* enhanced the mRNA abundance of these 2 genes compared with the control (Ad-GFP+GW). Relative to the control (Ad-GFP+DMSO), the mRNA of *ACSL1* and *ADRP* in cells overexpressing *PPARD* and incubated with GW increased by ~ 3.8 - and ~ 35 -fold, respectively. Overexpressing *PPARD* alone, however, did not increase expression of these genes compared with the control (Ad-GFP+DMSO).

Overexpression of PPARD Altered the Cellular Fatty Acid Profile

The FA profile in GMEC was measured after *PPARD* overexpression. As shown in Table 1, compared with the control (Ad-GFP+DMSO), treatment with GW had no effect ($P > 0.05$) on the concentration of C14:0, C16:0, C16:1, C18:0, C18:1-*trans*, and C18:1-*cis*. However, the ratio of C16:1 ($P < 0.05$) and C18:1-*trans* ($P < 0.05$) in cells overexpressing *PPARD* and incubated with GW increased significantly at the expense of C16:0 ($P < 0.05$) and C18:0 ($P < 0.05$). Compared with the control (Ad-GFP+DMSO), overexpression of *PPARD* had no effect on the ratio of C14:0 and C18:1-*cis*.

Figure 1. Overexpression of peroxisome proliferator activated receptor delta (*PPARD*) affects the expression of genes related to lipid transport in goat mammary epithelial cells (GMEC). After a 24-h infection with Ad-GFP (control; adenovirus containing green fluorescent protein) or Ad-PPARD (adenovirus containing *PPARD*), the GMEC were incubated with dimethyl sulfoxide (DMSO) or the *PPARD* ligand GW0742 for 24 h. The total RNA was extracted to assess the gene expression changes. Panel A: the mRNA expression of *PPARD*. Panel B: the mRNA expression of *SCD1* and *CD36*. Panel C: the mRNA expression of *FABP3* and *FABP4*. Panel D: the mRNA expression of *ACSL1* and *ADRP*. Data are means $\pm$ SEM from 3 replicates. Different letters denote significant ($P < 0.05$) differences in expression.

Overexpression of *PPARD* Reduced the Cellular TAG Concentration

Compared with the control (Ad-GFP+DMSO), incubation with GW did not alter the cellular TAG profile in GMEC (Figure 2). Compared with the control (Ad-GFP+DMSO), the overexpression of *PPARD* in GMEC incubated with ($P < 0.05$) or without ($P < 0.05$) GW reduced the total cellular TAG. However, no significant change occurred in TAG in cells overexpressing *PPARD* compared with the control (Ad-GFP+GW; Figure 2).

Activation of *PPARD* by GW Enhanced the Promoter Activity of *ADRP*

To investigate the role of *PPARD* in promoting lipid droplet formation, the activity of *ADRP* promoter fragments was measured. As shown in Figure 3A, GW increased the activity of the *ADRP* promoter in a dose-dependent manner. The cells incubated with GW at 5 μ M had the highest relative luciferase level. Treatment with GW at 1 μ M also increased the relative luciferase level compared with the control (GW at 0 μ M).

A series of 5'-flanking deletion constructs (−1,868/+516, −1,444/+516, −990/+516, −788/+516, and −38/+516 bp) of the *ADRP* promoter were generated and transfected into GMEC. Results in Figure 4B revealed no reduction in the activity of the *ADRP* promoter due to deletion fragments of −1,868/+516, −1,444/+516, −990/+516, and −788/+516 bp, whereas a dramatic decrease in *ADRP* promoter activity was observed when deleting up to −38/+516 bp (Figure 3B). Compared with each control (cells incubated with DMSO), the *PPARD* agonist GW significantly increased the activation of −1,868/+516 bp ($P < 0.05$) and −1,444/+516 bp ($P < 0.05$) fragments, but failed to do so with −990/+516, −788/+516, and −38/+516 bp fragments ($P > 0.05$).

PPARD Promoted the Activity of *ADRP* Through Binding to the *PPRE*

To further investigate the mechanism whereby *PPARD* enhances *ADRP* promoter activation, *PPARD* was knocked down using siRNA (siPPARD) or overexpressed in GMEC that were transfected with the *ADRP* promoter. In the cells incubated with siNC, treatment with GW increased ($P < 0.05$) the activity of *ADRP*-wild compared with the control (cells treated with DMSO). However, knockdown of *PPARD* impaired the induction of GW on *ADRP*-wild activity ($P > 0.05$; Figure 4A). In the cells incubated with Ad-GFP, GW increased ($P < 0.05$) the activity of *ADRP*-wild compared with the control (Ad-GFP+DMSO). In

Figure 2. Changes in total cellular triacylglycerol in goat mammary epithelial cells (GMEC) in response to overexpression of peroxisome proliferator-activated receptor delta (*PPARD*; Ad-PPARD+DMSO), incubation with the *PPARD* ligand GW0742 (Ad-GFP+GW0742) or without GW0742 (Ad-GFP+DMSO), or overexpression of *PPARD* plus GW0742 (Ad-PPARD+GW0742). Data are means $\pm$ SEM from 3 individual cultures. Different letters denote significant ($P < 0.05$) differences. DMSO = dimethyl sulfoxide.

contrast, in the cells transfected with Ad-PPARD, GW significantly upregulated the activity of ADRP-wild compared with the control (Ad-PPARD+DMSO; Figure 4B). In cells transfected with ADRP-mut, addition of GW had no effect on the relative luciferase activity regardless of the overexpression of *PPARD* ($P > 0.05$; Figure 4C).

DISCUSSION

In rodents, the role of *PPARD* is primarily associated with FA oxidation (Schuler et al., 2016). Recent data in GMEC support a role of *PPARD* in facilitating lipid secretion and catabolism of FA (Shi et al., 2017). However, the exact mechanism for the *PPARD* effect in mammary cells has not been fully resolved. In the pres-

ent study, the role of *PPARD* on FA transport was assessed using a combination of activation with GW and alteration of *PPARD* expression. The novel findings underscore an important role of *PPARD* in regulating FA transport directly via binding to the promoter of downstream genes (e.g., *ADRP*).

The gene encoding the FA translocase CD36 is a target of *PPAR* in rodents (Zhou et al., 2008) and was markedly upregulated by overexpression of *PPARG* in GMEC (Shi et al., 2014b). The finding that the *CD36* expression was increased in the cells overexpressing *PPARD* following incubation with GW is highly suggestive that *CD36* is a target of *PPAR* in GMEC.

Fatty acid binding proteins are thought to be the main intracellular FA transporters in ruminant cells (Bionaz and Loor, 2008a). The increase of *FABP4* in cultures incubated with both GW or overexpressing *PPARD* plus GW is consistent with the fact that *FABP4* is a target of *PPAR* (Shin et al., 2009). The lack of effect of GW on the expression of *FABP3* agrees with previous data in GMEC (Shi et al., 2017), and suggests it is not a target of *PPAR*. However, the fact that overexpression of *PPARD* plus GW increased the mRNA level of *FABP3* seems to suggest an alternate mechanisms whereby activation of *PPAR* may induce activation of another transcription regulator controlling the expression of *FABP3*. This idea is supported by the fact that *FABP3* has the highest expression level among *FABP* in the lactating ruminant mammary gland (Bionaz and Loor, 2008b).

Esterification with CoA catalyzed by *ACSL* is essential for the participation of long-chain FA in metabolic pathways or cellular transport. The high expression of *ACSL1* suggests an important role for this isotype in the lactating dairy goat mammary gland (Shi et al., 2015a). In this study, the significant increase of *ACSL1* mRNA after overexpression of *PPARD* is consistent with the fact that knockdown of *PPARD* decreased *ACSL1* expression (Shi et al., 2017). Because esterification with CoA enhances FA transport (Mashek and

Table 1. Effect of overexpression of peroxisome proliferator-activated receptor delta (*PPARD*) on the fatty acid composition (%; mean $\pm$ SEM) in goat mammary epithelial cells¹

Fatty acid	Ad-GFP+DMSO	Ad-GFP+GW0742	Ad-PPARD+GW0742
C14:0	0.89 $\pm$ 0.17	1.06 $\pm$ 0.1	0.97 $\pm$ 0.07
C16:1	1.06 $\pm$ 0.01 ^a	1.17 $\pm$ 0.04 ^{ab}	1.45 $\pm$ 0.34 ^b
C16:0	22.18 $\pm$ 0.44 ^b	22.87 $\pm$ 1.26 ^{ab}	21.64 $\pm$ 0.19 ^a
C18:1- <i>trans</i>	26.13 $\pm$ 0.55 ^a	27.22 $\pm$ 1.46 ^{ab}	27.71 $\pm$ 0.2 ^b
C18:1- <i>cis</i>	4.88 $\pm$ 0.17	4.8 $\pm$ 0.18	5.08 $\pm$ 0.22
C18:0	13.52 $\pm$ 0.4 ^b	13.53 $\pm$ 1.10 ^{ab}	12.27 $\pm$ 0.00 ^a

^{a,b}Values within a row with different superscripts differ significantly ($P < 0.05$).

¹Ad-GFP = recombinant adenovirus of green fluorescent protein as control; Ad-PPARD = recombinant adenovirus containing peroxisome proliferator-activated receptor delta. DMSO = dimethyl sulfoxide; GW0742 = synthetic ligand.

Coleman, 2006), an upregulation of *ACSL1* in GMEC was hypothesized to enhance FA transport and efficiency of FA metabolism for TAG synthesis (Shi et al., 2017). This idea is supported by observations in dairy cow mammary epithelial cells demonstrating that *ACSL1* activation enhanced TAG synthesis (Lian et al., 2016), and in mouse liver where the loss of *ACSL1* decreased TAG synthesis (Li et al., 2009). These data suggest *PPARD* may promote the synthesis of TAG via controlling the activation of *ACSL1*.

Figure 3. The compound GW0742 (GW) activated the peroxisome proliferator-activated receptor delta (*PPARD*) promoter activity in goat mammary epithelial cells (GMEC). Panel A: Cells were co-transfected with adipocyte differentiation-related protein (*ADRP*) promoter and Renilla luciferase vectors. After transfection for 24 h, cells were treated with GW at 0, 1, and 5 μ M for 24 h, respectively. The relative luciferase activity is expressed relative to the control (0 μ M) and normalized with renilla luciferase activity, respectively. Panel B: After 24 h co-transfection with each 5'-flanking deletion construct (-1,868/+516 bp, -1,444/+516 bp, -990/+516 bp, -788/+516 bp, or -38/+516 bp) and pRL-TK vectors, cells were treated with GW at 5 μ M for 24 h. The relative luciferase activity of each fragment is expressed relative to the control (dimethyl sulfoxide; DMSO) and normalized with renilla luciferase activity. The data represent mean $\pm$ SEM from replicates. Different letters denote significant ($P < 0.05$) differences in relative luciferase activity in panel A. In panel B, asterisks denote significant ($P < 0.05$) differences.

Figure 4. Overexpression of peroxisome proliferator-activated receptor delta (*PPARD*) or knockdown of *PPARD* altered the activity of adipocyte differentiation-related protein (*ADRP*) promoter in goat mammary epithelial cells (GMEC) containing a wild type construct (*ADRP-wild*) or a mutation of the *PPARG* response element (*ADRP-mut*) and incubated with the *PPARD* agonist GW0742 (GW) or dimethyl sulfoxide (DMSO). A vector containing renilla luciferase was used as control and co-transfected with each *ADRP* promoter in GMEC. Panels A and B: relative luciferase activation of *ADRP-wild*; panel C: relative luciferase activation of *ADRP-mut*. Values are means $\pm$ SEM from 3 individual cultures. Asterisks denote significant ($P < 0.05$) differences. GFP = green fluorescent protein; siNC = small RNA as negative control; siPPARD = small RNA interfering *PPARD*.

The finding that overexpression of *PPARD* resulted in a decrease of TAG concentration is noteworthy. It is possible that the mechanism resulting in decrease of TAG is mainly caused by changes in β -oxidation. Previous work with nonruminants has clearly identified PPARG as an activator of genes required for FA oxidation (Wang et al., 2003; Gan et al., 2011). Our recent work also provided evidence in GMEC that activation of PPARG upregulated genes related to β -oxidation of FA (Shi et al., 2017). The decrease of TAG is consistent with the finding in mice that activation of PPARG by chemical agonists causes lipid depletion (Wang et al., 2003). A limitation of the present study is that the secreted TAG in the culture medium could not be assessed due to the extremely low concentration in the serum-free in vitro culture (Li et al., 2014). Further in vivo and vitro experiments will have to be conducted to verify the mechanism of TAG metabolism in response to PPARG activation. Despite this limitation, the data still support an important role for PPARG in lipid homeostasis in the lactating ruminant mammary gland.

Consistent with the mRNA expression of *ADRP* induced by GW, GW treatment increased the activity of the *ADRP* promoter in a dose-dependent manner. The finding in cultures treated with GW that knockdown of *PPARD* impaired the activity of ADRP-wild and that overexpression of *PPARD* enhanced the activity of ADRP-wild strongly suggest that *ADRP* is a target of PPARG. By transiently transfecting the *ADRP* 5'-flanking truncated luciferase reporter vector into GMEC, we identified a core region at -1,444 to -990 bp that is responsive to PPARG activation (Figure 4B). The core region is consistent with the previous finding that the PPRE spanning at -1,003 to -990 bp on the *ADRP* promoter was responsive to the activation of PPARG (Kang et al., 2015). Furthermore, the data that a PPRE mutation impaired the activity of *ADRP* promoter induced by GW and overexpression of *PPARD* indicate that PPARG regulates *ADRP* expression by directly binding to its promoter through a similar regulatory mechanism as PPARG (Kang et al., 2015).

The upregulation of *SCD1* in the cells overexpressing *PPARD* and cultured with GW is consistent with observations in mouse liver, where adenovirus-mediated restricted PPARG activation in the liver and increased the mRNA level of *SCD1* (Liu et al., 2011). The increase of MUFA concentrations may have been due to the upregulation of *SCD1* after treatment with Ad-PPARG and GW. Overexpression of *PPARD* plus GW increased the ratio of C16:1 and C18:1 at the expense of C16:0 and C18:0 and led to a similar pattern when *PPARG* was overexpressed and incubated with rosiglitazone in GMEC (Shi et al., 2013a). This suggests

that both PPARG and PPARG may have overlapping functions in regulating FA desaturation. The similar change in FA profiles elicited by PPARG and PPARG also suggest these 2 isoforms may share a functional PPRE in the *SCD1* promoter (Yao et al., 2017). However, the fact that GW did not induce *SCD1* expression strongly suggests a different PPRE binding model in the promoter for these 2 isoforms (at least for *SCD1*). This phenomenon may partly result from differences in the co-activator proteins that work with PPAR (Liu et al., 2011).

Previous data indicated that *ADRP*, *SCD1*, and *FABP4* are targets of PPARG (Bionaz et al., 2013); thus, the induction of these genes by *PPARD* suggests a functional overlap between PPARG and PPARG. This idea agrees with the fact in rodents that PPAR are activated by overlapping groups of FA derivatives and share many target genes (Poulsen et al., 2012). However, the different regulatory mechanism on *SCD1* also suggest a distinct function for each isoform. The distinct function for PPARG is partly related to the integration of the PPARG subtypes into transcriptional complexes and signaling networks (Wang, 2010; Poulsen et al., 2012).

Collectively, the data reveal a novel role of PPARG in regulating lipid transport and promoting LD formation in GMEC. This role could be helpful for manipulating milk lipid synthesis in the lactating ruminant mammary gland. Our data also indicate that the mechanism of action of PPARG is through regulating downstream gene expression via binding to the promoter (at least on *ADRP*).

CONCLUSIONS

The combination of gene overexpression and promoter analysis underscore a novel role for PPARG in lipid homeostasis via promoting lipid transport. This function involves the cellular FA trafficking and LD formation. There appears to be a functional overlap in GMEC among the PPAR family. More controlled molecular experiments will have to be performed to address questions about the functional overlap among PPAR. Together, results reveal a crucial role for PPARG in the regulation of milk fat metabolism.

ACKNOWLEDGMENTS

This research was jointly supported by a National Natural Science Foundation of China (31702090, Beijing, China), Open Fund of Zhejiang Provincial Top Key Discipline of Biology (Hangzhou, China), and a Special Fund for Agro-Scientific Research in the Public

Interest of Zhejiang Province (2016C32061, Hangzhou, China).

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