Peroxisome proliferator-activated receptor delta facilitates lipid secretion and catabolism of fatty acids in dairy goat mammary epithelial cells

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ABSTRACT

In rodents, peroxisome proliferator-activated receptor delta (PPARD) is associated primarily with catabolism of fatty acids. However, the role of PPARD in regulating lipid metabolism in ruminant mammary gland remains unknown. In the present study, we assessed the mRNA abundance of *PPARD* at 3 stages of lactation in goat mammary tissue. Results revealed that *PPARD* had lower expression at peak lactation than in the nonlactating period. Luciferase assays revealed that GW0742 (GW), a specific PPARD ligand, enhanced the activity of the *PPARD* response element in goat mammary epithelial cells. Activation of *PPARD* by GW selectively upregulated the expression of genes related to fatty acid activation (*ACSL1*), lipid droplet formation (*PLIN2*), and transport (*FABP4*), and had no effect on genes involved in de novo fatty acid synthesis (*ACACA* and *FASN*), desaturation (*SCD*), hydrolysis and oxidation (*PNPLA2* and *CPT1A*), transport and uptake (*FABP3* and *CD36*), or triacylglycerol synthesis (*DGAT1* and *AGPAT6*) in goat mammary epithelial cells. In contrast, knockdown of *PPARD* using small interfering RNA dramatically decreased the expression of genes related to fatty acid activation (*ACSL1*) and lipid formation (*PLIN2*) and increased the expression of genes related to fatty acid transport (*FABP3*) and triacylglycerol synthesis (*AGPAT6* and *DGAT1*). The expression of genes related to fatty acid synthesis (*FASN*), hydrolysis (*PNPLA2*), and fatty acid oxidation (*CPT1A*) was downregulated significantly only after knockdown of *PPARD* in cells incubated with GW. We observed no significant change in fatty acid profiles. However, the total cellular triacylglycerol increased after knockdown of *PPARD* in goat mammary epithelial cells plus GW.

Collectively, these results highlight an important role for PPARD in the homeostasis of ruminant mammary cells by facilitating fatty acid activation and lipid droplet formation and secretion.

Key words: goat, peroxisome proliferator-activated receptor (PPAR), milk fat, gene expression

INTRODUCTION

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that belong to the nuclear receptor superfamily and include 3 closely related members: PPAR alpha (PPARA), gamma (PPARG), and delta (PPARD). Each member displays a tissue-selective expression pattern in ruminants, with PPARG and PPARA predominantly expressed in adipose tissue and liver, respectively, and PPARD broadly expressed in various tissues, including adipose, rumen, liver, and lung (Bionaz et al., 2013). In nonruminants, the role of PPARG in determining adipocyte differentiation and promoting lipid storage has been well established (Kawai and Rosen, 2010). Recent studies have also underscored a key role for PPARG in controlling lipid metabolism in goat mammary gland (Shi et al., 2013a; Kang et al., 2015). Similarly, a large body of evidence also supports the function of PPARA as a controller of the catabolism of fatty acids (Roberts et al., 2011; Pol et al., 2015). However, compared with PPARG and PPARA, knowledge about PPARD in the context of ruminant mammary lipid metabolism remains unknown.

In rodents, the primary role of PPARD is the control of catabolism of fatty acids in muscle (Barak et al., 2002). The expression of *PPARD* in skeletal muscle is increased upon fasting and upon exercise (Giordano Attianese and Desvergne, 2015), suggesting a role for PPARD in the adaptive response of skeletal muscle to increased demand for catabolism of fatty acids. Conversely, when *PPARD* is deleted in skeletal muscle, the muscle fibers exhibit lower oxidative activity, and body fat mass increases (Schuler et al., 2006).

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A biologic role for PPARD in the catabolism of fatty acids in white and brown adipose tissue is supported by evidence from *PPARD*-transgenic mice. In line with an increase in catabolism of fatty acids, transgenic mice expressing *PPARD* are resistant to lipid accumulation induced by high-fat diets and exhibit diminished lipidemia (Wang et al., 2003). However, the fact that mice carrying an adipose-specific deletion of *PPARD* did not have alterations in fat mass content was indicative of an indirect effect of PPARD in adipose tissue (Lee et al., 2006). Interestingly, the exploration of PPARD-mediated response in the liver suggested that PPARD also promotes liver lipogenesis (Liu et al., 2011).

Unlike in rodents, the function of PPARD in ruminants remains largely unknown. A similar expression of *PPARD* has been observed in various cow tissues, and *PPARD* was more abundant than *PPARG* in cow mammary gland (Bionaz et al., 2013). Contrasting roles for PPARG and PPARD in primary bovine mammary cells has been reported, where several PPARG ligands reduced the expression of *PPARD* (Lutzow et al., 2008). In contrast, the fact that activation of PPARD increased the activity of glyceraldehyde-3-phosphate dehydrogenase indicated a potential role for PPARD in adipogenesis in sheep (Soret et al., 1999). The opposite responses for catabolism of fatty acids and lipogenesis in these published experiments indicate that PPARD may play different roles in different tissues. Thus, it was hypothesized that activation or knockdown of *PPARD* would alter the mRNA expression of genes related to lipid metabolism.

To our knowledge, few available data directly support a role for PPARD in lipid metabolism in ruminants. To investigate the effect of PPARD on lipid metabolism in ruminant mammary gland in the present study, PPARD was activated by use of a chemical ligand or was knockdown via small interfering RNA (*siRNA*) in goat mammary epithelial cells (**GMEC**).

MATERIALS AND METHODS

mRNA Abundance of PPARD

The transcriptome data set from 3 lactating goat mammary tissue samples were deposited at the National Center for Biotechnology Information (GEO Series GSE87089). Details of these samples have been described previously (Shi et al., 2015). Briefly, we used 3- to 4-yr-old Xinong Saanen dairy goats from the experimental farm of Northwest Agricultural University, Shaanxi, China. Mammary tissue at peak lactation (3 goats, 100 d postpartum), late lactation (cessation of milking, 3 goats, 310 d postpartum), and non-lactation (3 goats, nonlactating and nonpregnant) was collected

by a veterinarian after slaughter. All goats were managed in a similar fashion, and were fed a mixed diet consisting of corn, soybean meal, bran, rapeseed meal, and a mineral-vitamin mixture. We used Bowtie 0.12.8 (<http://bowtie-bio.sourceforge.net/index.shtml>) to align reads to the goat genome (Dong et al., 2013). We used DESeq (a software for differential gene expression analysis based on negative binomial distribution of data; <http://www-huber.embl.de/users/anders/DESeq>) to analyze the abundance of gene expression. The single-end mapping method was used for read analysis; one read was compared with multiple genes. Reads per kilobase of exon model per million mapped reads (**RPKM**) were used for measuring gene expression of *PPARD*.

Cell Culture and Treatments

The GMEC were isolated from Xinong Saanen goats at peak lactation, as described previously (Wang et al., 2010; Shi et al., 2014). Details of cell culture were described recently (Lin et al., 2013; Shi et al., 2013b). To promote lactogenesis, GMEC were cultured in a lactogenic medium for 24 h before initial experiments. Cells were cultured in a 6-well culture plate and subcultured to 90% confluence; they were then treated with PPARD-specific ligand GW0742 (**GW**, Sigma-Aldrich, St. Louis, MO) at 1 μ M in lactogenesis medium. Then the cells were collected at 0, 12, and 24 h for total RNA extraction or triacylglycerol (**TAG**) assays. The lactogenesis medium was composed of DMEM/F12 (Hyclone, Beijing, China), insulin (5 mg/L, Sigma-Aldrich), hydrocortisone (5 mg/L, Sigma-Aldrich), penicillin/streptomycin (10k unit/L, Harbin Pharmaceutical Group, Harbin, China), prolactin (2 μ g/mL, Sigma-Aldrich) and BSA (1 g/L, Sigma-Aldrich).

RNA Interference

To determine *PPARD* mRNA interference, cells cultured in 6-well plates were transfected with 100 nM siRNA and 7.5 μ L of transfection reagent (Lipofectamine RNAiMAX; Thermo Fisher Scientific Inc., Waltham, MA) in lactogenesis medium without antibiotic. The transfection was performed according to the manufacturer's instructions (http://tools.thermofisher.com/content/sfs/manuals/Lipofectamine_RNAiMAX_Reagent_protocol.pdf). Transfected GMEC were cultured with 1 μ M GW (Sigma-Aldrich) or control (dimethyl sulfoxide, **DMSO**; Sigma-Aldrich) after 24 h of initial culture, and then harvested at 48 h (24 h later) for RNA extraction, TAG assays, and fatty acid extraction. The sequences of the siRNA oligonucleotides for *PPARD* (accession XM_004018768.3) were 5'-GCUG-

GCCUCCAUGUCAAUTT-3' (designed and synthesized by Jima Biotechnology Co. Ltd., Shanghai, China). Scrambled siRNA was a functional nontargeting siRNA used as a negative control (siNC). In total, we included 4 treatments (siNC + DMSO, siPPARD + DMSO, siNC + GW, siPPARD + GW) in the RNA interference experiment.

RNA Extraction and Quantitative Real-Time PCR

The procedures for total RNA extraction, purification, and quantitative PCR have been recently described (Lin et al., 2013). Total RNA from GMEC was extracted using the RNeasy Pure Kit (Qiagen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol (<http://www.qiagen.com/asset/imsupload/up0203129001467351047.pdf>). During RNA extraction, genomic DNA contamination was removed using DNase provided with the kit. We synthesized cDNA from 0.5 µg RNA using the PrimeScript RT kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. We performed quantitative real-time PCR (qPCR) according to the manufacturer's instructions using SYBR Green (SYBR Premix Ex Taq II, Perfect Real Time; Takara Bio Inc.; http://www.clontech.com/CN/Products/Real-Time_qPCR_and_Reverse_Transcription/Real-Time_qPCR_Probe_Detection/Pre-mix_Ex_Taq?site=10022:22372:US). A melting curve was included in each qPCR reaction, and a positive control was included in each plate.

Genes studied included lipoprotein lipase (*LPL*), CD36 molecule (thrombospondin receptor, *CD36*), carnitine palmitoyltransferase 1A (*CPT1A*), acyl-coA synthetase long-chain family member 1 (*ACSL1*), fatty acid binding protein 3 (*FABP3*), *FABP4*, fatty acid synthase (*FASN*), acetyl-Coenzyme A carboxylase α (*ACACA*), stearoyl-CoA desaturase 1 (*SCD1*), 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6*), diacylglycerolacyltransferase 1 (*DGAT1*), patatin-like phospholipase domain containing 2 (*PNPLA2*), perilipin2 (*PLIN2*), and *PPARD*. All qPCR reactions were performed in a Bio-Rad CFX96 (Bio-Rad Laboratories Inc., Hercules, CA) sequence detector, and data were normalized to ubiquitously expressed transcript (*UXT*), mitochondrial ribosomal protein L39 (*MRPL39*), and ribosomal protein S9 (*RPS9*). The sequences of the primers for the genes are described in Supplementary Table S1 (<https://doi.org/10.3168/jds.2016-11647>).

Fatty Acid 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, we performed

total lipid extraction and methylation according to Shi et al. (2013a). Methylated lipid samples were analyzed using a gas chromatography-mass spectrometer (Agilent Technologies, Santa Clara, CA) installed with an HP-5 column (35 m $\times$ 0.25 mm i.d. $\times$ 0.25 µm film thickness) following a published procedure (Yao et al., 2016). The relative proportion of each fatty acid was determined as the ratio of the fatty acid peak to the total peaks in each run. The data for each fatty acid were analyzed as a proportion of the total fatty acids.

Cellular TAG Assays

Total cellular TAG was extracted according to the protocol of the GPO-Trinder triglyceride assay kit (Applygen Technologies, Beijing, China; <http://www.applygen.com/a/meixueyushenghuaceding/276.html>). The concentration of TAG was determined according to the manufacturer's instructions on a microtiter plate reader (BioTek, Winooski, VT). We calculated the concentrations using the equation obtained from linear regression of the standard curve. The quantification of total cellular TAG was normalized to the cellular protein concentration. We determined the protein concentration for each well using a bicinchoninic protein assay (Pierce; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Luciferase Activity Analysis

The GMEC at 80% confluence in 48-well plates were transfected with 0.2 µg of *PPARD* response element (PPRE) $\times$ 3-Luc reporter plasmid along with a Renilla vector (pRL-TK) as a control at a ratio of 25:1 using the Lipofectamine 2000 (Thermo Fisher Scientific Inc.). After a 24-h recovery period in lactogenesis medium, one group of cells was treated with GW at final concentrations of 0, 0.1, 1, 5, and 10 µM and harvested after 48 h. The other group was treated with GW at 1 µM (final concentration), and cells were harvested after 0, 12, and 24 h, respectively. Lysates were made using reporter lysis buffer (Dual-Luciferase Reporter Assay System, Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity in the cell extract was determined using luciferase assay buffer and luciferase assay substrate according to the manufacturer's protocol.

Statistical Analysis

Treatments were replicated at least 3 times in culture wells, and results were expressed as mean $\pm$ standard error of the mean. Data for qPCR were analyzed us-

ing the $2^{-\Delta\Delta Ct}$ method. Data for qPCR in cells treated only with GW were analyzed relative to the control (0 h) using one-way ANOVA. All data for qPCR were normalized to 3 internal control genes (*UXT*, *MRPL39*, and *RPS9*). The significance of relative luciferase activity across different concentrations and times was determined by one-way ANOVA. The significance of RNA expression after *PPARD* knockdown was determined by one-way ANOVA. Significance was declared at $P < 0.05$.

RESULTS

PPARD Expression in Dairy Goat Mammary Gland Tissue

To assess the expression of *PPARD* in goat mammary gland tissue, we evaluated RNA sequencing data from 3 stages of lactation. As shown in Figure 1, the nonlactating mammary gland had the highest RPKM value for *PPARD*, followed by the late-lactation samples. Compared with the nonlactating period, we detected a lower expression of *PPARD* at peak lactation ($P < 0.05$).

A *PPARD* Ligand Enhanced Activity of the PPAR Response Element in GMEC

As shown in Figure 2, treatments with GW caused a dramatic activation of *PPARD* in GMEC. The luciferase levels between each treatment group and the control group (treatment with 0 μM GW) increased significantly ($P < 0.05$). Activation of *PPARD* by GW reached a peak at the 5 μM dose (~ 4 -fold; Figure 2A).

Figure 1. The mRNA abundance of peroxisome proliferator-activated receptor delta (*PPARD*) at 3 stages of lactation in goat mammary tissues. The transcriptome data set is from mammary tissue harvested at peak lactation (3 goats, 100 d postpartum), in late lactation (cessation of milking, 3 goats, 310 d postpartum), and non-lactation (3 goats, nonlactating and nonpregnant) by a veterinarian after slaughter. The DESeq software <http://www-huber.embl.de/users/anders/DESeq> was used to analyze abundance of gene expression. Reads per kilobase of exon model per million mapped reads (RPKM) was used to assess gene expression. Values are means $\pm$ SEM for 3 individual goats. The different letters (a, b) denote significant ($P < 0.05$) differences.

Figure 2. The peroxisome proliferator-activated receptor delta (*PPARD*)-specific ligand GW0742 (GW) ligand activated the *PPARD* response element (PPRE) effectively in goat mammary epithelial cells. Cells were transfected with pGL3-PPRE $\times$ 3 and pRL-TK vectors. (A) After transfection, cells were treated with GW at 0, 0.1, 1, 5, and 10 μM for 24 h, respectively. (B) Transfected cells at a 1- μM dose were collected at 0, 6, 12, and 24 h for luciferase assays. Luciferase and Renilla luciferase assays were performed in triplicate. Relative luciferase activity was expressed relative to control (0 μM) or 0 h and normalized with Renilla luciferase activity, respectively. Values are mean $\pm$ SEM from 3 independent experiments. The different letters (a to d) denote significant ($P < 0.05$) differences in relative luciferase activity.

The relative luciferase activity increased ~ 3 -fold at 24 h in GMEC treated with 1 μM GW.

Activation of *PPARD* by GW Affects Expression of Genes Related to Fatty Acid Metabolism in GMEC

Compared with the control group (cells treated with GW at 0 h), GW had no significant effect on the mRNA expression of *PPARD* (Figure 3A). Among the genes related to de novo synthesis and desaturation, activation of *PPARD* by GW had no effect on *ACACA*, *FASN*, or *SCD1* (Figure 3B). We observed no effect on *PNPLA2*, *CPT1A*, *DGAT1*, or *AGPAT6* after activation of *PPARD* by GW (Figures 3C and E). In contrast,

activation of PPARD by GW led to significant upregulation of *ACSL1* at 12 and 24 h (Figure 3C). Among the genes related to fatty acid transport and uptake, *FABP4* was upregulated ($P < 0.05$) by treatment with GW (Figure 3D). We observed a dramatic upregulation of *PLIN2* in cells incubated with GW at 12 and 24 h (~10- and ~14-fold, respectively, $P < 0.01$).

Knockdown of PPARD Affects the Expression of Genes Related to Fatty Acid Metabolism in GMEC

As shown in Figure 4A, treatment of GMEC with siRNA reduced *PPARD* mRNA abundance by 85%. Compared with the control group (siNC + DMSO), knockdown of *PPARD* (siPPARD + DMSO) did not significantly alter the mRNA abundance of *ACACA*, *FASN*, *SCD1*, *PNPLA2*, *CPT1A*, *CD36*, or *FABP4*; however, it did increase *FABP3* ($P = 0.023$), *AGPAT6* ($P = 0.04$), and *DGAT1* ($P = 0.023$; Figures 4D and E). Knockdown of *PPARD* in cells cultured with GW downregulated the mRNA abundance of *FASN* ($P = 0.035$), *PNPLA2* ($P = 0.022$), and *CPT1A* ($P = 0.047$; Figures 4B and C). It is noteworthy that the pattern of expression of *PLIN2* and *ACSL1* was strongly consistent with the activation and knockdown of *PPARD* (Figures 4C and E). Treatment of GMEC with *PPARD* siRNA significantly downregulated both *ACSL1* and *PLIN2*, regardless of GW.

PPARD Alters Cellular TAG but Fails to Change Fatty Acid Composition in GMEC

Compared with DMSO, incubation with GW for 24 h did not alter the cellular TAG profile in GMEC (Figure 5). We also observed no significant change in TAG in cells treated with *PPARD* siRNA (siPPARD + DMSO) compared with the control group (siNC + DMSO). However, knockdown of *PPARD* in GMEC incubated with GW increased the total cellular TAG ($P < 0.05$; Figure 5). The results of fatty acid analysis illustrated that knockdown or activation of *PPARD* did not affect the ratio of C14, C16:0, C16:1, C18:0, C18:1n-7, or C18:1n-9 in GMEC (Figure 6).

DISCUSSION

In ruminant mammary gland, recent data have strongly underscored the role of PPARG in lipogenesis and TAG secretion via control of gene expression (Shi et al., 2013a; Kang et al., 2015). The fact that the relative mRNA abundance of *PPARD* is higher than that of *PPARG* in bovine mammary tissue (Bionaz et al., 2013) led us to hypothesize that PPARD also plays an important role in mammary gland lipid metabolism.

The present study is novel in that we studied the selective regulation of gene expression and cellular TAG content by PPARD in GMEC using a combination of activation with GW and knockdown via siRNA. The findings from the present study underscore an important role for PPARD in regulating the transcription of *PLIN2* and *ACSL1*, which are related to TAG synthesis and lipid droplet formation, 2 key processes required for milk fat secretion.

The markedly lower expression of *PPARD* at peak lactation compared with the dry period indicated an opposite role for PPARD compared with PPARG during lactation. Together with the fact that *PPARG* expression in lactating goat mammary tissue is higher than in the dry period (Shi et al., 2013a), the present data agree with the observation that several PPARG ligands decreased the expression of *PPARD* in primary bovine mammary cells (Lutzow et al., 2008). The present data also support a previous hypothesis that PPARD may perform a role opposite to PPARG (Bionaz et al., 2013). Clearly, additional experiments are needed to better define the mechanisms of PPARD biology in the ruminant mammary gland.

Once activated by a ligand, PPARD forms a heterodimer with retinoid X receptor and binds to PPRE in the promoter region of its target genes (Tan et al., 2005). The marked increase in luciferase levels upon incubation with GW indicated that PPARD activation was ligand-dependent. The higher level of luciferase induced by GW in this study compared with that induced by rosiglitazone (Shi et al., 2013b) indicates that PPARD is more readily activated than PPARG in GMEC. Such a response may be partly due to the higher expression of PPARD demonstrated in bovine mammary tissue (Bionaz et al., 2013). The higher activation of PPARD in mammary tissue strongly underscores its importance in ruminant mammary tissue.

The proteins encoded by *ACACA*, *FASN*, and *SCD* play an important role in de novo fatty acid synthesis in the mammary gland. The lack of change in expression of *ACACA*, *FASN*, and *SCD1* with GW agree with data from *PPARD*-transgenic mice (Wang et al., 2003). The downregulation of *FASN* after knockdown of *PPARD* in GMEC incubated with GW indicated that PPARD elicits some control over fatty acid synthesis, at least in GMEC. From a mechanistic standpoint, the alteration of *FASN* upon PPARD activation may be functionally related to the unique characteristics of ruminant mammary cells. This hypothesis is supported by the marked increase in rates of fatty acid synthesis in lactating ruminant mammary gland, which is required for milk fat synthesis and secretion (Mellenberger et al., 1973).

The lack of change in C16:1 and C18:1 percentage in this study agrees with the lack of change in *SCD1* with

A

B

C

D

E

Figure 3. Activation of peroxisome proliferator-activated receptor delta (PPARD) by GW0742 (GW) alters the expression of genes coding for proteins related to lipid metabolism in goat mammary epithelial cells. Cells were incubated with GW at 1 μ M and harvested after 0, 12, and 24 h to assess gene expression. Panels show the mRNA expression of (A) *PPARD*; (B) genes related to de novo synthesis (*ACACA* and *FASN*) and desaturation (*SCD1*); (C) genes related to triacylglycerol hydrolysis (*PNPLA2* and *CPT1A*) and fatty acid activation (*ACSL1*); (D) genes related to fatty acid transport (*FABP3* and *FABP4*) and uptake (*CD36*); and (E) genes related to triacylglycerol synthesis (*DGAT1* and *AGPAT6*) and lipid droplet formation (*PLIN2*). Values are means $\pm$ SEM from 3 individual cultures. The different letters denote significant ($P < 0.05$) differences.

Figure 4. Changes in gene expression in goat mammary epithelial cells in response to knockdown of peroxisome proliferator-activated receptor delta [*PPARD*; siPPARD + DMSO (dimethyl sulfoxide)], incubation with *PPARD*-specific ligand GW0742 (GW; siNC + GW), or knockdown of *PPARD* plus GW (siPPARD + GW). Panels show the mRNA expression of (A) *PPARD*; (B) genes related to de novo synthesis (*ACACA* and *FASN*) and desaturation (*SCD1*); (C) genes related to triacylglycerol hydrolysis (*PNPLA2* and *CPT1A*) and fatty acid activation (*ACSL1*); (D) genes related to fatty acid transport (*FABP3* and *FABP4*) and uptake (*CD36*); and (E) genes related to triacylglycerol synthesis (*DGAT1* and *AGPAT6*) and lipid droplet formation (*PLIN2*). Values are means $\pm$ SEM from 3 individual cultures. Two comparisons (siPPARD + DMSO vs. siNC + DMSO and siPPARD + GW vs. siNC + GW) were performed. The different letters (a to c) denote significant ($P < 0.05$) differences.

Figure 5. Changes in total cellular triacylglycerol in goat mammary epithelial cells in response to knockdown of peroxisome proliferator-activated receptor delta [*PPARD*; siPPARD + DMSO (dimethyl sulfoxide)], incubation with PPARD-specific ligand GW0742 (GW; siNC + GW), incubation control and GW (siNC + DMSO), or knockdown of *PPARD* plus GW (siPPARD + GW). Data are means $\pm$ SEM from 3 individual cultures. The different letters (a, b) denote significant ($P < 0.05$) differences.

both GW treatment and knockdown of *PPARD*. The fact that *SCD1* is a verified target of PPARG in GMEC (Shi et al., 2013a) indicates that target gene regulation by PPARG is mediated via a different PPARE (Tan et al., 2005).

Previous work with nonruminants has clearly identified PPARG as an activator of genes required for oxidation in brown adipocyte tissue. The decrease in expression of *PNPLA2* and *CTP1A* after *PPARD* knockdown and GW supplementation indicates that PPARG is not required for the basal transcription of

genes associated with the catabolism of fatty acids in ruminant mammary gland. A similar transcriptional regulatory mechanism in bovine mammary cells has also been reported for other transcription factors, such as liver X receptor α (Oppi-Williams et al., 2013).

The dynamic activation of PPARG seems to participate in balancing mammary cell homeostasis to avoid excessive lipogenesis, or to control it. In the physiological context of milk fat synthesis in lactating mammary gland, this mechanism is not far-fetched, given that the lactating mammary cell must synthesize TAG and export it to ensure that intracellular TAG does not accumulate. Several knockout studies in mouse have demonstrated that excessive intracellular TAG accumulation could be toxic to mammary epithelial cells and elicit a negative effect on lactation (Ogg et al., 2004; Beigneux et al., 2006).

The putative function of PPARG to prevent excessive lipogenesis also is supported by the response of *PLIN2* in cultures incubated with GW or with the PPARG knockdown using siRNA. The *PLIN2* protein is essential for the formation and secretion of lipid droplets during lactation (Chong et al., 2011; Russell et al., 2011). The dramatic changes in *PLIN2* in response to GW (present study) or the overexpression of *PPARG* in adipose tissue (Wang et al., 2003) agree with the notion that *PLIN2* is a target of PPARG. Our recent data demonstrated that PPARG directly regulates *PLIN2*

Figure 6. Knockdown of peroxisome proliferator-activated receptor delta (*PPARD*) did not alter the fatty acid (FA) composition of goat mammary epithelial cells. Panels A, B, C, D, E, and F depict the percentage of C14:0, C16:0, C16:1, C18:0, C18:1n-7, and C18:1n-9, respectively, in goat mammary epithelial cells in response to knockdown of *PPARD* [siPPARD + DMSO (dimethyl sulfoxide)], incubation with PPARG-specific ligand GW0742 alone (siNC + GW), incubation with control and DMSO (siNC+DMSO), or knockdown of *PPARD* plus GW (siPPARD + GW). Data are means $\pm$ SEM for 3 individual experiments. No significant change were observed among the comparisons using one-way ANOVA.

by binding to the PPARE site on the promoter (Kang et al., 2015). Whether the 2 transcription factors share the same PPARE is still unknown. However, the lack of significant change in cellular TAG in response to GW or knockdown of *PPARD* indicates a potential balance or concerted action between PPARG and PPARD on *PLIN2* to regulate the synthesis of lipid droplets in mammary cells.

Although *PLIN2* certainly is not the only protein that could alter lipid droplet formation, it does determine lipid droplet formation in mammary cells (Shi, et al., 2015). However, other proteins such as tail-interacting 47-kDa protein and cell death-inducing DFF45-like effector family also play a role. The observed regulation of *PLIN2* by PPARD in the present study also helps explain the fact that *PLIN2* expression remained high even after knockdown of *PPARG* in GMEC (Shi et al., 2013b). Collectively, the findings in the present study underscore a central role for PPARD in the regulation of milk lipid droplet secretion.

The induction of *FABP4* by GW in the present study is consistent with the fact that there are 2 PPAREs in the promoter region of bovine *FABP4* (Shin et al., 2009). The CD36 protein is an essential plasma membrane transporter that allows for fatty acid uptake by mammary cells (Bionaz and Loor, 2008). Despite the fact that there are PPARE sites on its promoter, the lack of change in *CD36* expression with supplementation of GW and knockdown of *PPARD* indicates that it is not a target gene of PPARD in ruminant mammary cells (Zhou et al., 2008).

Long-chain fatty acids are esterified with CoA in the inner face of the plasma membrane before participating in metabolic pathways. The *ACSL1* gene is predominant among ACSL isoforms in bovine mammary tissue (Bionaz and Loor, 2008) and is considered a key enzyme in activating fatty acids destined for TAG synthesis and β -oxidation (Li et al., 2009). Recent data have also demonstrated that *ACSL1* could enhance fatty acid synthesis in bovine mammary cells (Lian et al., 2016). In the present study, the alteration of *ACSL1* mRNA abundance response to activation and knockdown of PPARG strongly suggest that *ACSL1* is a target of PPARD in ruminant mammary cells. The fact that activation or knockdown of PPARD did not change the total cellular TAG is consistent with observations in mouse liver, where the content of TAG was unchanged in liver of *Acs11*^{-/-} mice after feeding either low- or high-fat diets (Li et al., 2009). The higher cellular TAG after knockdown of PPARD plus GW compared with control (siNC-GW) might have been due to a decrease in TAG hydrolysis, which was partly confirmed by the downregulation of *PNPLA2* and *CPT1A* upon PPARD knockdown and GW supplementation. In contrast, the

upregulation of *AGPAT6* and *DGAT1* upon the knockdown of PPARD is consistent with the fact that knock-out of *ACSL1* diminished the incorporation of [¹⁴C] oleate into TAG (Li et al., 2009). Thus, the data indicate that PPARD suppresses cellular TAG biosynthesis.

Collectively, our data highlight a role for PPARD in facilitating the formation and secretion of lipid droplets in goat mammary cells. This role seems to extend to the suppression of biosynthesis of cellular TAG. The induction of *PNPLA2* and *CPT1A* supports a role for PPARD in the catabolism of fatty acids. The mechanism leading to this function likely involves the regulation of *ACSL1* and *PLIN2*.

CONCLUSIONS

In the present study, the combination of ligand activation and gene knockdown using siRNA underscore a novel role for PPARD in homeostasis in ruminant mammary cells by facilitating the catabolism of fatty acids, as well as lipid droplet formation and secretion. More controlled molecular experiments will have to be performed to address questions about the precise signaling pathway of PPARD. Together, results from the present study indicate a crucial role for PPARD in the regulation of milk fat synthesis.

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