Adipocyte differentiation-related protein promotes lipid accumulation in goat mammary epithelial cells

H. B. Shi,[†] K. Yu,[†] J. Luo,[‡] J. Li,^{*} H. B. Tian,^{*} J. J. Zhu,^{*} Y. T. Sun,^{*} D. W. Yao,^{*} H. F. Xu,^{*} H. P. Shi,^{*} and J. J. Looor[‡]

^{*}Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, P.R. China 712100

[†]College of Life Science, Zhejiang Sci-Tech University, Hangzhou, Zhejiang, P. R. China 310018

[‡]Mammalian NutriPhysioGenomics, Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana 61801

ABSTRACT

Milk fat originates from the secretion of cytosolic lipid droplets (CLD) synthesized within mammary epithelial cells. Adipocyte differentiation-related protein (ADRP; gene symbol *PLIN2*) is a CLD-binding protein that is crucial for synthesis of mature CLD. Our hypothesis was that ADRP regulates CLD production and metabolism in goat mammary epithelial cells (GMEC) and thus plays a role in determining milk fat content. To understand the role of ADRP in ruminant milk fat metabolism, ADRP (*PLIN2*) was overexpressed or knocked down in GMEC using an adenovirus system. Immunocytochemical staining revealed that ADRP localized to the surface of CLD. Supplementation with oleic acid (OA) enhanced its colocalization with CLD surface and enhanced lipid accumulation. Overexpression of ADRP increased lipid accumulation and the concentration of triacylglycerol in GMEC. In contrast, morphological examination revealed that knockdown of ADRP decreased lipid accumulation even when OA was supplemented. This response was confirmed by the reduction in mass of cellular TG when ADRP was knocked down. The fact that knockdown of ADRP did not completely eliminate lipid accumulation at a morphological level in GMEC without OA suggests that some other compensatory factors may also aid in the process of CLD formation. The ADRP reversed the decrease of CLD accumulation induced by adipose triglyceride lipase. This is highly suggestive of ADRP promoting triacylglycerol stability within CLD by preventing access to adipose triglyceride lipase. Collectively, these data provide direct in vitro evidence that ADRP plays a key role in CLD formation and stability in GMEC.

Key words: milk fat, lipid droplet, triacylglycerol, hydrolysis

INTRODUCTION

The lactating mammary gland is one of the most lipogenic organs of the female body in many species. The majority of the lipid produced during lactation is secreted into milk by a process of membrane envelopment of cytoplasmic lipid droplets (CLD; Hansen et al., 1984; Chong et al., 2011). The CLD are composed of a sterol esters and triacylglycerol (TG) core surrounded by a phospholipid monolayer and coated with specific proteins (Krahmer et al., 2009; Wilfling et al., 2014). Several proteins have been identified on the lipid droplet (LD; Reinhardt and Lippolis, 2006; Maller et al., 2010; Lu et al., 2014), including perilipin 1 (*PLIN1*), adipocyte differentiation-related protein (ADRP; also known as *PLIN2*), and tail-interacting 47-kDa protein (*TIP47*; also known as *PLIN3*), which are known as the PAT domain proteins (Brasaemle et al., 2004; Thiele and Spandl, 2008; Russell et al., 2011). Proteomic studies confirmed that ADRP is a major protein component of LD in the mammary gland (Wu et al., 2000; Lu et al., 2014), suggesting that it plays a role in the formation of LD during lactation.

Adipocyte differentiation-related protein is a 50-kDa protein initially described in adipocytes (Jiang and Serrero, 1992) and considered a marker of lipid accumulation. Ubiquitously expressed, ADRP participates in CLD formation in hepatocytes (Motomura et al., 2006), adipocytes (Brasaemle et al., 1997), mammary epithelial cells (Russell et al., 2011), fibroblasts (Imamura et al., 2002), and macrophages (Larigauderie et al., 2006). In adipocytes, ADRP is present at the surface of CLD and is the major protein during early stages of adipocyte differentiation. During adipocyte differentiation, even though ADRP mRNA remains elevated, its protein level is reduced dramatically after the onset of perilipin expression (Jiang and Serrero, 1992; Bickel et al., 2009).

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¹These authors contributed equally to this paper.

²Corresponding authors: luojun@nwsuaf.edu.cn and jlloor@illinois.edu

Overexpression of the gene encoding ADRP in Swiss-3T3 cells increased the number and size of CLD without induction of other adipocyte-specific or lipogenic genes (Imamura et al., 2002), providing direct evidence that it can modulate CLD levels. However, the fact that ADRP-deficient mice displayed normal adipocyte and adipose tissue differentiation and function, but had reduced liver TG content with attenuation of hepatic steatosis induced by high-fat feeding (Chang et al., 2006), suggests that ADRP may not be a central factor modulating CLD in adipocytes.

Adipocyte differentiation-related protein plays roles in milk fat formation and secretion in mammary cells. At the mRNA and protein levels, ADRP in the lactating mammary gland increases over 30-fold compared with nonlactating mammary gland in mice (Russell et al., 2007), cows (Bionaz and Looor, 2008), and goats (Shi et al., 2015). Furthermore, the pattern of ADRP mRNA expression in the differentiating mammary gland correlates closely with the appearance, growth and accumulation of CLD in murine milk-secreting cells (Russell et al., 2007). Adipocyte differentiation-related protein deficiency in the mouse mammary gland did not prevent CLD formation; however, it disrupted the increase in CLD size (Russell et al., 2011).

Previous evidence implicates ADRP as a physiological regulator of milk lipid production. Beyond the role in lipid storage, ADRP is also hypothesized to participate in CLD secretion through formation of a tripartite complex with the transmembrane protein butyrophilin (BTN) and the ubiquitously-expressed cytoplasmic enzyme xanthine oxidoreductase (XOR) in mammary cells (McManaman et al., 2007; Chong et al., 2011). However, even though some evidence supports an important role of ADRP in milk fat formation, the mechanism of ADRP regulation of CLD metabolism in lactating mammary gland tissue is still unclear.

To date, most of the research about ADRP in lactating mammary gland was performed in rodents. Compared with rodents, less is known about how ADRP affects lipid accumulation in ruminants, despite the fact that ruminant animals produce the majority of milk and dairy products consumed by humans. To investigate the role of *ADRP* in lipid metabolism in mammary cells, the *ADRP* gene was knockdown or overexpressed in goat mammary epithelial cells (GMEC).

MATERIALS AND METHODS

Adenovirus Generation

Recombinant adenovirus with shRNA for the *ADRP* gene knockdown (**Ad-sh511**) was generated as reported previously (Kang et al., 2015). Recombinant adeno-

virus for overexpression of *ADRP* (**Ad-ADRP**) and adipose triglyceride lipase (*ATGL*; **Ad-ATGL**) were packed and amplified as described previously (Shi et al., 2013a, 2014; Li et al., 2015). Briefly, the cDNA of dairy goat *ADRP* (NCBI: HQ846827.1) and *ATGL* (NCBI: GQ918145) were subcloned into the pAdTrack-CMV plasmid vector (Invitrogen, Waltham, MA) between the *XhoI* and *KpnI* (New England Biolabs, Ipswich, MA) restriction sites to generate pAdTrack-CMV-ADRP and pAdTrack-CMV-ATGL vectors. These 2 vectors were inserted into an adenoviral vector (pAdEasy-1, Invitrogen) to generate adenoviral plasmids in BJ5183 cells. The adenoviral plasmids linearized by *Pac I* (New England Biolabs) were transfected into 293A cells to generate the adenovirus Ad-ADRP and Ad-ATGL. The adenovirus with green fluorescent protein (**Ad-GFP**) and small hairpin RNA (**shRNA**) negative control adenovirus (**Ad-NC**; noneffective control), gifts from Zhijie Chang (Tsinghua University, Beijing, China), were used as negative controls. In our study, all the virus vectors express green fluorescent protein.

Cell Culture and Treatment

The GMEC were isolated from peak lactation Xinong Saanen goats as described previously (Wang et al., 2010). The cells used in the experiments were isolated and pooled from at least 3 different individual goats. Details of cell culture were described recently (Lin et al., 2013; Shi et al., 2013b). Briefly, cells were incubated at 37°C in 5% CO₂ and air. Culture medium was changed every 24 h. Medium was composed of DMEM/F12 (Hyclone, Beijing, China) containing insulin (5 mg/L, Sigma-Aldrich, St. Louis, MO), hydrocortisone (5mg/L, Sigma-Aldrich), penicillin and streptomycin (10,000 U/L, Harbin Pharmaceutical Group, Harbin, China), epidermal growth factor (1 mg/L, Sigma-Aldrich), and fetal bovine serum (10%, Gibco, Grand Island, NY). The GMEC were transduced with adenovirus supernatant (Ad-ADRP, Ad-GFP, Ad-sh511, Ad-NC, or Ad-ATGL) until 70 to 80% confluence. Transfected GMEC were cultured with oleic acid (**OA**; O1008, Sigma-Aldrich) or ethyl alcohol (Sigma-Aldrich) after 24 h of the initial culture and then harvested at 48 h (24 h later) for RNA or protein extraction, quantification of total cellular triacylglycerol (**TG**) or oil red O staining. Oleic acid was diluted in ethyl alcohol and used at a 400 μ M working concentration.

RNA Extraction and Real-Time PCR

Total cellular RNA was extracted from cells using RNA prep pure cell kit (Tiangen Biotech, Beijing, China) and prepared for reverse transcription using

PrimeScript RT reagent kit (Takara, Shiga, Japan). Quantitative real-time PCR (**qPCR**) was performed with SYBR Premix Ex Taq II (Takara) on a CFX96 Real-time System (Bio-Rad, Hercules, CA). Relative mRNA expression levels of the target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primers of qPCR for *ADRP* and *GAPDH* were described previously (Shi et al., 2013b).

Western Blotting

Western blotting was performed as previously described (Kang et al., 2015). Briefly, GMEC incubated with Ad-GFP or Ad-ADRP were collected at 48 h. These samples were lysed with radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor mixture (Solarbio, Beijing, China). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. The blots were incubated with a polyclonal antibody against ADRP (1:200; sc-32888, Santa Cruz Biotechnology, Dallas, TX), and a monoclonal antibody against GAPDH (1:1,000; Cwbio-tech, Beijing, China). Secondary antibody used a goat anti-rabbit IgG with horseradish peroxidase or phycoerythrin (1:1,000; Transgen, Beijing, China). Signals were detected using a chemiluminescent ECL system (Pierce) and visualized by autoradiography with a cold CCD camera (Bio-Rad).

Oil Red O Staining

The GMEC cultured in 6-well plates (Nunc, Roskilde, Denmark) were incubated with adenovirus supernatant for 48 h. Oleic acid was then added for an additional 24 h before harvesting. Oil Red O staining was performed on GMEC according to a method described previously (Mehlem et al., 2013) with modifications. Briefly, the samples were washed twice with PBS and fixed with 4% formaldehyde for 30 min at room temperature. After 2 washes in PBS, cells were stained with ~1 mL of Oil Red O working solution (3.5 g/L, diluted in isopropyl alcohol) for 15 min and then examined microscopically. The images were monitored using a Leica fluorescence microscope (DMI4000B, Leica, Wetzlar, Germany).

Quantification of Total Cellular TG

Total cellular TG was extracted according to the protocol of the GPO-Trinder triglyceride assay kit (Ap-plygen Technologies, Beijing, China). Briefly, cells were collected and suspended in lysis buffer, following by sonication for 20 s. After centrifuging the cell suspension at $10,000 \times g$ for 10 min at 4°C, the supernatant

was removed for TG and protein analysis. The lysis buffer was used as blank control to correct the values of samples and standards. The concentration of TG 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 TG was normalized to the cellular protein concentration. Protein concentration of each well was determined using a BCA protein assay (Pierce) according to the manufacturer's instructions.

Immunocytochemical Staining

For the fluorescence experiments, GMEC were cultured on 6-well coverslip-bottomed plates (Nunc) and treated as described above. After harvesting, cells were fixed in 4% (vol/vol) paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature. After a brief rinse in PBS, cells were treated with Triton-X100 of 0.5% w/v for 25 min at room temperature and then blocked before staining with 1% skim milk powder in the presence of 0.1% wt/vol of Tween-20 (all from Sigma-Aldrich) for 2 h at room temperature. Primary antibody incubation was performed overnight at 4°C. Primary antibody for ADRP (rabbit polyclonal IgG, Santa Cruz Biotechnology) was diluted 1:200 in PBS with 1% dried bovine milk. After 3 washes with PBS with 0.1% wt/vol Tween-20, cells were incubated for 1 h with appropriate secondary antibodies (1:1,000; Cwbio-tech), phycoerythrin-conjugated goat anti-rabbit (1:1,000 dilution), or fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit (1:1,000 dilution).

To visualize neutral lipids, 1 mg/mL of BODIPY 493/503 (Invitrogen) was added to cells during staining with the secondary antibody. To visualize the nucleus, after washing with PBS solution, the cells were incubated in 4',6-diamidino-2-phenylindole (**DAPI**; working solution, 1 µg/mL, Invitrogen) for 15 min at 37°C. The stained cells were visualized with a confocal laser microscope (A1R/A1, Nikon, Tokyo, Japan) using a 63× oil objective lens.

Data Analysis

Data for all measurements are from triplicate incubations, and all the experimental data are reported as means $\pm$ standard error of the mean. Data of qPCR were analyzed relative to the control using the $2^{-\Delta\Delta Ct}$ method, as described previously (Oppi-Williams et al., 2013), and differences among treatments determined via *t*-test (Shi et al., 2014). The data for total TG mass was normalized to the protein concentration before

statistical analysis. One-way ANOVA (SAS Institute Inc., Cary, NC) was used for statistical comparisons and for determining the difference of total TG mass among treatments. The statistical model included the fixed effect of treatment. Differences were considered significant at $P < 0.05$.

RESULTS

Expression of ADRP Protein in GMEC

The immunofluorescent staining was performed to detect the expression of ADRP in GMEC. As shown in Figure 1A, GMEC transfected with Ad-GFP expressed endogenous ADRP protein (stained red). Compared with the control group (Ad-GFP), Ad-ADRP significantly increased the expression of ADRP, which was confirmed by the qPCR and Western blotting (Figure 1B). Similar to our previous data from Western blotting (Kang et al., 2015; Supplementary Figure S1, <http://dx.doi.org/10.3168/jds.2015-9452>), Ad-sh511 decreased the expression of ADRP compared with the control Ad-NC (Figure 1A). Based on these results, Ad-sh511 and Ad-ADRP were used to block and overexpress expression of ADRP in GMEC.

ADRP Protein Co-Localizes with CLD in GMEC

In this experiment, CLD were stained with BODIPY 493/503 and ADRP was stained with a secondary antibody with FITC. As shown in Figure 2A, most CLD accumulated around the nucleus (stained by DAPI). The ADRP protein colocalized with the CLD. In response to OA supplementation, the formation of CLD was stimulated markedly and filled the cells. The Oil Red O staining (Figure 2B) and quantification of total cellular TG ($P < 0.001$) further confirmed that OA promoted the increase of lipid accumulation and TG mass (Figure 2C).

ADRP Enhances the Synthesis of CLD in GMEC

After overexpression of *ADRP*, Oil Red O staining revealed that the neutral lipid accumulation was enhanced compared with the control group (Ad-GFP; Figure 3A). The OA markedly increased the cellular lipid accumulation in the presence or absence of Ad-ADRP. Overexpression of *ADRP* in GMEC increased both the number and size of CLD, whereas both incubation with OA and Ad-ADRP had the strongest effect on the increase in number and size of CLD (Figure 3A). Consistent with the morphological changes, TG analysis confirmed that cells incubated with either *ADRP* or OA had higher TG concentrations compared with

the control (Ad-GFP group; Figure 3C). In addition, overexpression of *ADRP* further enhanced the concentration of TG induced by OA.

Compared with the control (Ad-NC), a significant morphological decrease in lipid accumulation was observed after knockdown of *ADRP* in cultures without OA supplementation (Figure 3A). Knockdown of *ADRP* decreased the lipid accumulation even when OA was supplemented (Figure 3A). Furthermore, the quantification of total cellular TG revealed that cells incubated with Ad-sh511 had lower TG concentration compared with the control (Ad-NC; $P = 0.010$). Despite the fact that OA enhanced the basal cellular lipid accumulation, knockdown of *ADRP* still decreased the concentration of TG ($P = 0.002$; Figure 3D).

Immunocytochemical staining was performed to evaluate the effect of *ADRP* on lipid accumulation in GMEC. We observed that overexpression of *ADRP* increased the accumulation of lipid (Figure 3B). However, despite of the treatment with OA, the CLD decreased to the lowest concentration after knockdown of *ADRP* (Figure 3B).

ADRP Reduces Lipid Hydrolysis Induced by ATGL

To explore the role of *ADRP* on CLD stability and homeostasis, an Ad-ATGL adenovirus suspension was incubated with GMEC. The Oil Red O staining revealed that the OA treatment always enhanced the lipid accumulation and increased the total TG concentration of GMEC (Figure 4A, B, and C). Overexpression of *ATGL* decreased the ability of the lipid accumulation and total TG level in GMEC (Figure 4A and B). However, *ADRP* could overcome the effect of *ATGL*, and increased the lipid accumulation (Figure 4A) and TG concentration ($P = 0.039$; Figure 4C). In the presence of OA, *ADRP* still had the ability to promote TG and lipid accumulation which was inhibited by *ATGL* ($P = 0.019$). Such response was even more impressive given the high basal concentration of TG. In contrast, knockdown of *ADRP* further decreased the lipid accumulation and TG concentration in GMEC in the absence ($P = 0.029$) or presence of OA ($P = 0.04$).

DISCUSSION

During lactation, the mammary gland synthesizes and packages TG into CLD that are subsequently secreted into milk (McManaman, 2012). The ADRP protein, one member of the PAT family, was identified as an important element for stabilization of CLD in adipocytes and nonadipocyte cells (Sztalryd et al., 2006; Thiele and Spandl, 2008). Despite the fact that lactating mammary tissue is the most lipogenic organ

A

B

Figure 1. Adipocyte differentiation-related protein (ADRP) gene and protein expression in goat mammary epithelial cells (GMEC). Suspensions of adenovirus with shRNA (Ad-sh511), ADRP (Ad-ADRP), green fluorescent protein (Ad-GFP), or negative control (Ad-NC) were incubated with GMEC for 48 h. The cells were then collected for immunofluorescent staining, quantitative real-time PCR (qPCR) or Western blot. The cells were collected for immunofluorescent staining, qPCR, or Western blot. (A) ADRP protein levels were detected by immunofluorescent staining with anti-ADRP antibody/phycoerythrin (PE)-conjugated anti-mouse (red). The nuclear was stained using 4',6-diamidino-2-phenylindole (DAPI; blue). The GFP protein (green) is used as control, which is expressed by the adenovirus. Fluorescent images were captured at the same spot in each column (bars = 25 μ m). (B) Western blot and qPCR were used to detect the overexpression level of *ADRP* in GMEC incubated with Ad-ADRP. Values are means $\pm$ SEM for measurements of 3 wells. Difference was considered significant when $P < 0.05$. Color version available online.

Figure 2. Oleic acid enhances the localization of adipocyte differentiation-related protein (ADRP) on the lipid droplet and increases the concentration of triacylglycerol in goat mammary epithelial cells (GMEC). The GMEC were incubated with 400 μ M oleic acid (OA) for 24 h, and then were collected for immunofluorescent staining and quantification of total cellular triacylglycerol. (A) Immunofluorescent staining was performed to detect the co-localization of ADRP protein and lipid droplet in GMEC incubated with OA or not. The protein was detected by with anti-ADRP antibody/fluorescein isothiocyanate (FITC)-conjugated anti-mouse (green). The nuclear was stained using 4',6-diamidino-2-phenylindole (DAPI; blue). Lipid droplets were stained with BODIPY 493/503 (red; Invitrogen, Waltham, MA). Fluorescent images were captured at the same spot in each column (bars = 25 μ m). (B) Oil Red O staining detection for lipid accumulation after incubation with OA (OA+) compared with control group (OA-). Images were captured at the same spot in each column (50 $\times$ magnification). (C) Quantification of total triacylglycerol (TG) mass in OA treated GMEC. The concentration of TG was normalized to total protein. Values are means $\pm$ SEM for measurements of 3 wells. Difference in relative total TG concentration was considered significant when $P < 0.05$. Color version available online.

Figure 3. Adipocyte differentiation-related protein (ADRP) gene overexpression enhances the synthesis of lipid droplets in goat mammary epithelial cells (GMEC). The GMEC were incubated with adenovirus suspension [with shRNA (Ad-sh511), ADRP (Ad-ADRP), green fluorescent protein (Ad-GFP), or negative control (Ad-NC)] for 48 h with or without 400 μ M oleic acid (OA) and then the cells were collected for fluorescence staining, Oil Red O staining and quantification of total triacylglycerol mass. (A) Fluorescence detection of alteration of lipid accumulation via knockdown or overexpression of *ADRP* in GMEC. The cells for fluorescence detection of lipid accumulation under treatment with Ad-GFP and Ad-ADRP were incubated without OA, and cells treated with Ad-NC and Ad-sh511 were incubated with OA. The nucleus was stained using 4',6-diamidino-2-phenylindole (DAPI; blue). Lipid droplets were stained with BODIPY 493/503 (red; Invitrogen, Waltham, MA). Fluorescent images were captured at the same spot in each column (bars = 25 μ m). (B) Oil Red O staining detection for lipid accumulation after knockdown (Ad-sh511) or overexpression of *ADRP* (Ad-ADRP) compared with control groups (Ad-NC or Ad-GFP). Images were captured at the same spot in each column (50 $\times$ magnification). (C) Relative total triacylglycerol (TG) mass in GMEC after overexpression of *ADRP*. (D) Relative total TG mass in GMEC after knockdown of *ADRP*. Values are means $\pm$ SEM for measurements of 3 wells. The different letters (a–d) denote significant ($P < 0.05$) differences in relative total TG concentration. Difference in fold change was considered significant when $P < 0.05$. Color version available online.

Figure 4. Adipocyte differentiation-related protein (ADRP) gene overexpression reduces lipid hydrolysis induced by adipose triglyceride lipase (ATGL) gene overexpression. Goat mammary epithelial cells (GMEC) were incubated with 1 or 2 adenovirus suspension [with ADRP (Ad-ADRP), green fluorescent protein (Ad-GFP), adipose triglyceride lipase (Ad-ATGL), negative control (Ad-NC), and shRNA (Ad-sh511)] for 48 h without or with oleic acid (OA) at 400 μ M and then the cells were collected for Oil Red O staining and quantification of total triacylglycerol (TG) mass. (A) Oil Red O staining detection for lipid accumulation after treatment with Ad-sh511, Ad-ADRP, or Ad-ATGL compared with control groups (Ad-NC or Ad-GFP). Images were captured at the same spot in each column (50 $\times$ magnification). (B) Relative total TG mass in GMEC after overexpression of *ADRP* and *ATGL* along with or without OA. (C) Relative total TG mass in GMEC after knockdown of *ADRP* and overexpression of *ATGL* along with or without OA. Values are means $\pm$ SEM for measurements of 3 wells. Difference in fold change was considered significant when $P < 0.05$. Color version available online.

of the body, its role in lactating ruminant mammary tissue is poorly understood. The ability of *ADRP* to enhance the synthesis of CLD in GMEC suggests that it plays an important role in regulating CLD formation in mammary cells.

Using proteomics, the overall protein composition of CLD isolated from milk-secreting cells of mice (Wu et al., 2000) and the milk fat layer of cows (Lu et al., 2014) confirmed that ADRP is one of the most abundant proteins on the milk CLD. The fact that ADRP colocalized with CLD in GMEC agrees with previous cell culture experiments (Imamura et al., 2002; McManaman et al., 2007; Krahmer et al., 2009).

In nonruminant hepatocytes, long-chain FA (e.g., OA) stimulate lipid accumulation in parallel with the expression of *ADRP* (Fan et al., 2009). Data from the present study confirmed this effect in GMEC. The greater colocalization of ADRP with CLD in response to exogenous OA is suggestive of a role for this protein in binding and transporting intracellular NEFA, hence leading to greater TG and CLD synthesis (Gao and Serrero, 1999).

In ruminants, the mRNA expression of *ADRP* in mammary tissue closely followed the pattern of milk synthesis and increased remarkably through ~100 d postpartum (Bionaz and Looor, 2008). The marked increase in the lipid accumulation detected with both Oil Red O staining and immunocytochemical staining confirms previous suggestions that overexpression of *ADRP* could stimulate CLD formation in fibroblasts (Imamura et al., 2002; Larigauderie et al., 2004). The fact that incubation of cells overexpressing *ADRP* with OA increased lipid accumulation suggests that ADRP facilitates transport of NEFA within GMEC, which agrees with a previous hypothesis in Cos-7 cell line (Gao and Serrero, 1999; Ohsaki et al., 2008). Mechanistically, the binding of NEFA by ADRP likely contributes to the rapid appearance of FA at the surface of CLD. Whether such a response also is associated with enhanced TG synthesis and subsequent CLD formation (Serrero et al., 2000) is yet to be established.

Knocking out *ADRP* in fibroblasts did not cause a significant change in lipid accumulation due to the compensatory upregulation of TIP47 protein (Sztalryd et al., 2006), which plays a pivotal role in CLD formation and biogenesis (Bickel et al., 2009; Nose et al., 2013; Patel et al., 2014). The decrease of TG concentration after downregulation of *ADRP* in our study is consistent with the fact that knocking down both *ADRP* and *TIP47* decreased CLD formation and increased the ability of TG lipolysis in fibroblasts (Sztalryd et al., 2006). The fact that knockdown of *ADRP* could not completely eliminate lipid accumulation at a morphological level in the present study is highly

suggestive that some unidentified factor (e.g., TIP47, PLIN1, or PLIN4) compensates for ADRP activity in lipid formation. However, the lower TG concentration after knockdown of *ADRP* indicated that compensatory factors could not completely rescue the effect of *ADRP* knockdown. Because *TIP47* and *PLIN1* have a low expression in goat mammary gland (data not shown), it is speculated that *ADRP* is most important in CLD formation in goat mammary gland. Further work should be performed to address whether and how other PAT family members work in concert with ADRP to control CLD formation in goat mammary cells.

The present study did not address the exact mechanism whereby ADRP gene and protein promotes the increase of CLD clustering and fusion. The ADRP protein may serve as a factor maintaining the stability of CLD as, for example, PLIN1 in adipocytes (Brasaemle et al., 2000). Previous evidence that ADRP deficiency did not prevent CLD formation but disrupted the increase in CLD size in differentiating mouse mammary epithelial cells (Russell et al., 2011) was highly suggestive that ADRP is a unique factor altering the diameter of CLD. Recently, several protein factors including the cell death-inducing DFF45-like effector family (Yang et al., 2012; Wilfling et al., 2014) and atlastin GTPases (Klemm et al., 2013) were identified as promoting the enlargement of CLD. More experiments are needed to explore the interaction among these proteins in the process of CLD formation and enlargement in ruminant mammary gland.

Adipose triglyceride lipase catalyzes the initial step in the hydrolysis of TG in adipose and other tissues (Zechner et al., 2009). Absence of *ATGL* in mice increases adipose tissue mass and promotes lipid storage in multiple tissues (Haemmerle et al., 2006). In adipocytes, PLIN1 helps stabilize CLD by forming a barrier that reduces the access of ATGL to TG within CLD, thus inhibiting hydrolysis (Brasaemle et al., 2000). Recent data revealed that ATGL hydrolyzes significant amounts of TG in GMEC (Li et al., 2015), which is consistent with the fact that ATGL altered CLD morphology and made it difficult for CLD to accumulate in the present study. The fact that overexpression of *ADRP* rescued the decrease in TG concentration and CLD accumulation induced by ATGL agrees with a previous hypothesis that ADRP inhibits TG hydrolysis via preventing ATGL from binding to the CLD surface in HEK 293 cells (Listenberger et al., 2007) and mouse mammary tissue (Russell et al., 2011). These results underscore the importance of ADRP gene and protein in regulating size of CLD and suggest that the potential mechanism by which ADRP mediates CLD enlargement is partly due to preventing ATGL-dependent lipolysis in GMEC.

A limitation of the present study is that because of the use of a two-dimensional cell culture approach; the cells lacked polarity, which is critical for lipid assembly and secretion (Jin and Amberg, 2000). Thus, the absence of cell polarity may partly explain why the lipid droplets were mainly located paranuclearly (Figures 1A and 2A). Despite this limitation, the data still support a role for ADRP gene and protein in ruminant mammary gland. Further in vivo experiments utilizing, for example, mammary tissue at different stages of lactation will have to be conducted to validate the importance of ADRP in the mammary gland.

In summary, our data directly demonstrated that *ADRP* is a regulator of TG accumulation in the GMEC. The localization of ADRP on CLD surface underscores the important role of ADRP in CLD metabolism and CLD secretion in GMEC. Overexpression of *ADRP* increases the lipid accumulation, whereas knockdown of *ADRP* prevented the lipid accumulation. Furthermore, the fact that ADRP rescued the CLD hydrolysis induced by *ATGL* is highly suggestive of ADRP playing a role in stabilizing CLD in GMEC.

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