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RESEARCH ARTICLE

The Isolation of Pre-Adipocytes from Dairy Cow Adipose Tissue and the Development of Pre-Adipocytes into Mature Adipocytes

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A B S T R A C T

Serious lipid mobilization in adipose tissue of dairy cow is the pathological basis of ketosis and fatty liver. Many Studies demonstrated that pre-adipocytes are unstable and can differentiate into multiple cells *in vitro*. The aim of this study was to investigate the differentiation process of dairy cow pre-adipocytes and to establish a stable pre-adipocyte induction method. Pre-adipocytes were isolated from dairy cow adipose tissue and were cultured for 15 days. The originally rounded cells converted to a spindle fibroblast-like morphology and accumulated lipids during culturing. The lipid droplets and TG content in the adipocytes gradually increased from 4 to 15 days in culture. The adipocytes reached maximal proliferation after 11 days in culture. Additionally, the expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) and sterol regulatory element binding protein 1c (SREBP-1c) increased during the initial 4 days of differentiation and decreased thereafter. However, the level of SREBP-1c increased after 10 days of differentiation. The PPARγ2 and SREBP-1c proteins levels were significantly higher on day 13 than on day 0. PPARγ2 and SREBP-1c were primarily localized to the cytoplasm on day 0 and to the cytoplasm and nucleus on day 13. In conclusion, a stable dairy cow pre-adipocyte culture was established, and SREBP-1c and PPARγ2 were increased during (demonstrated to be involved in) pre-adipocyte differentiation. Isolated adipocytes can be used as a cellular model to elucidate the process of lipogenesis in dairy cows and to further investigate metabolic diseases.

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INTRODUCTION

Adipose tissue is a complex, essential and highly active metabolic tissue. This tissue type has attracted extensive attention because it was established as an endocrine organ when leptin was identified (Kershaw and Flier, 2004). Adipose tissue secretes several cytokines, such as interleukin, adiponectin, resistin and so on. Most perinatal cows experience negative energy balance (NEB) induced by decreased dry matter intake and increased energy demands to support milk production (Wathes *et al.*, 2011; Chen *et al.*, 2013). This NEB initiates lipid mobilization and a subsequent increase in the concentration of blood non-esterified fatty acids (NEFA) (Li *et al.*, 2014) . A high concentration of blood NEFA plays a pivotal role in the pathogenesis of ketosis and fatty liver syndrome in dairy cows(Sumithran *et al.*, 2013). Adipose tissue is the principal organ for the metabolism of NEFA, and physiological, metabolic and endocrine adaptations occur in adipose tissue to support lipid mobilization during transition periods. The pathological basis of ketosis and fatty liver is NEB-mediated lipid metabolism disorder. Therefore, it is necessary to clarify the mechanism underlying lipid deposition and mobilization in dairy cow adipose tissue.

Adipocyte differentiation was the process of lipid deposition. Peroxisome proliferator-activated receptor-γ (PPARγ) and sterol regulatory element-binding protein 1c (SREBP-1c) have been identified as adipogenic regulatory factors involved in adipocyte differentiation and lipid

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metabolism (White and Stephens, 2010; Zhong *et al.*, 2014). For PPARγ, the activation of different promoters generates variety mRNA isoforms (PPARγ1-4) but only two receptor proteins (PPARγ1 and PPARγ2) because protein that yield by PPARγ3 and PPARγ4 mRNA variants is identical with PPARγ1 (Ling *et al.*, 2003; Gurnell, 2005). PPARγ1 is widely expressed, including in adipose tissue, intestines, liver, kidneys, macrophages and so on, whereas PPARγ2 is almost exclusively expressed in adipose tissue, except for that in the liver (Gurnell, 2005). SREBP-1c regulates the transcription of many genes that are important for cholesterol and fatty acid metabolism. In contrast, several recent studies of adipocytes in rats, mice and the 3T3-L1 cell line have shown that the expression levels of SREBP-1c do not coincide with changes in lipogenic gene expression, indicating that SREBP-1c does not contribute to the regulation of adipogenesis in adipocytes (Bertile and Raclot, 2004; Sekiya *et al*., 2007). However, currently, few studies have been performed on the effect of SREBP-1c on the differentiation and adipogenic gene expression of adipocytes in dairy cows.

NEB-induced fat mobilization is the pathological basis of energy metabolism diseases, such as ketosis and fatty liver, in dairy cows. Therefore, exploring adipocyte differentiation and lipid mechanism will provide valuable information to demonstrate the mechanism by which NEB factors induce fat mobilization. In this study, pre-adipocytes were isolated from the fat tissue of dairy cows. Based on the morphological changes and the increases in lipid droplets and triglyceride (TG) levels in cells, we evaluated the differentiation of pre-adipocytes. In parallel to the morphological analyses, the expression and protein distribution of SREBP-1c and PPARγ2 were determined via real-time PCR, western blotting and immune-fluorescence, respectively. These data facilitated the examination of the mechanisms of adipocyte differentiation and lipid mobilization in dairy cows.

MATERIALS AND METHODS

Materials: DMEM/F12 medium, collagenase I, bovine serum albumin, insulin, biotin and pantothenate were purchased from Gibco (Grand Island, NY, USA). The antibodies against PPARγ2 and SREBP-1c were purchased from Abcam (Cambridge, MA, USA).

Cell culture: The experimental protocol was approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China). The omental and perirenal adipose tissues were obtained via surgical excision from healthy Holstein calves. After sliced into small pieces (approximately 1 mm^3), the tissue was digested in DMEM containing 2 mg/mL type I collagenase and 2% bovine serum albumin for 90 min at 37ºC in a slightly shaking water bath. Subsequently, the cell pellet obtained from centrifuging was resuspended in basal medium (BM), which consisted of DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, seeded on a 6-well tissue culture plate ($5x10^4$ cells per well) and incubated at 37ºC in 5% CO_2. After reaching 70% confluence (approximately 5 or 6 days in culture), the cells were treated with differentiation medium (DM: BM supplemented with 10 μg/mL insulin, 33 μM biotin and 17 μM pantothenate) for 13 days.

Morphology and lipid accumulation observation and activity detection in bovine adipocytes: The morphological changes in the cells were observed using a microscope on days 1, 4, 7, 10 and 13.

An Oil Red O staining assay was used to determine the accumulation of neutral lipids in cells on days 4, 7, 10 and 13. Briefly, cells were fixed using 10% formalin for 25 min and then stained with 0.5% Oil Red O for 30 min. Then, the cells were observed and photographed using a microscope. Furthermore, the TG content of the adipocytes was detected using a tissue TG assay kit (Applygen Technologies, Inc., Beijing, China).

Adipocyte proliferation in different culture media (BM, DM or DM containing 20% FBS) were measured using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay method on days 1, 3, 5, 7, 9, 11 and 13 (Capilla *et al*., 2011). The cells were incubated in 96-well plates for 4 h at 37ºC in BM at a final concentration of 0.5 mg/mL MTT. After washing 3 times, 150 μL of dimethyl sulfoxide (DMSO) was added to each well. Then the proliferation values were obtained based on the absorbance measured at 490 nm using a spectro-photometer (ELX800, Bio-TEK, USA).

Molecular biotechnology: The mRNA expression levels were estimated via real-time PCR analysis. The following primer sequences were used for PPARγ2: forward: CGGGAAAGACGACAGACAAA; reverse: ACTGACA CCCCTGGAAGATG. The following primer sequences were used for SREBP-1c: forward: GCAGCCCATTCA TCAGCCAGACC; reverse: CGACACCACCAGCATC AACCACG.

The protein levels of PPARγ2 and SREBP-1c were measured using western blotting. Briefly, the target protein was separated by polyacrylamide gel electrophoresis and then electrotransferred onto PVDF membranes. Then immunoreactive bands were detected with enhanced chemiluminescence solution (ECL, Beyo-time Biotechnology Inc., China).

Immunofluorescence was performed to assess the nuclear localization of PPARγ2 and SREBP-1c in pre-adipocytes and mature adipocytes. Briefly, cells cultured on glass coverslips were washed with PBS and fixed using 4% paraformaldehyde for 20 min. After further washing, the cells were incubated in the primary antibody overnight at 4ºC. A Cy3-conjugated goat-anti-rabbit IgG was used as the secondary antibody. Then, the coverslips were photographed using a fluorescence microscope (Fluoview FV1200, Olympus, Japan).

RESULTS

Cell morphology characterization: The isolated cells were rounded or polygonal after washing with PBS (Fig. 1A). The originally rounded or polygonal cells became spindle-shaped 4 days after isolation (Fig. 1B). After 1 week, the cells completely converted to a spindle shape with long tenuous cytoplasmic extensions and profuse endoplasmic reticulum, which have been characterized as similar to fibroblasts in previous research (Fig. 1C). Cells

almost grew into confluence after 10 days culture (Fig. 1 D and E) and became senescence on day 15 (Fig. 1F).

Characterization of adipocyte differentiation: Lipid accumulation in adipocytes was determined using an Oil Red O staining assay and TG determination kits. Fig 2 shows that the lipid droplets gradually increased in the adipocytes from 4 to 15 days in culture (Fig. 2 A-E). Similarly, the TG content in the adipocytes increased from 4 to 15 days and was significantly higher at 10, 13 and 15 days than at 4 and 7 days in culture (Fig. 3) (mean±SD). These results indicate that fat deposition gradually increased as the adipocytes grew.

Cell proliferation in different culture media: An MTT assay was performed to investigate the proliferation of pre-adipocytes from dairy cows. Fig 4 shows that the proliferation of the cells cultured in BM was significantly increased on day 3 ($P<0.001$) and continued to increase until day 11. In contrast, the cells cultured in DM proliferated slowly from days 0 to 9. However, cell proliferation on day 11 was not significantly different between those cultured in BM and DM ($P=0.104$) (mean±SD). The cells cultured in DM containing 20% FBS proliferated dramatically from day 3 to 7, but their proliferation duration was short, and they matured early.

The mRNA expression of PPARγ2 and SREBP-1c in adipocytes: To investigate the mRNA expression of PPARγ2 and SREBP-1c, two important inducers of adipogenesis, the gene expression levels were assessed via real-time PCR. Fig 5 shows that PPARγ2 and SREBP-1c expression peaked on day 4. Thereafter, the expression of these two genes decreased. However, the mRNA level of SREBP-1c increased after 10 days of differentiation and was equivalent to that at day 0.

The protein levels and immunofluorescence of PPARγ2 and SREBP-1c in adipocytes: As shown in Fig 6(A), the protein levels of PPARγ2 and SREBP-1c significantly increased on day 13 compare to day 0. Immunofluorescence was performed to assess the nuclear localization of PPARγ2 and SREBP-1c. Fig 6B and C shows that the fluorescence intensity of PPARγ2 and SREBP-1c in the nucleus was significantly higher on day 13 than on day 0. PPARγ2 and SREBP-1c were primarily localized to the cytoplasm on day 0 and to the nucleus on day 13. These results indicate that PPARγ2 and SREBP-1c expression was upregulated and that these proteins translocated to the nucleus with increasing lipid deposition in adipocytes.

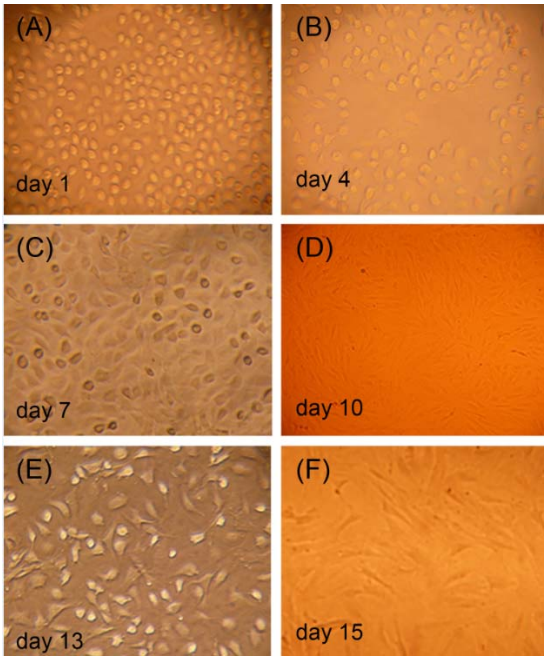

Fig 1: The morphological changes in adipocytes isolated from dairy cows. (A-E) general photomicrographs of bovine adipocytes cultured from day 1 to day 15 (×200).

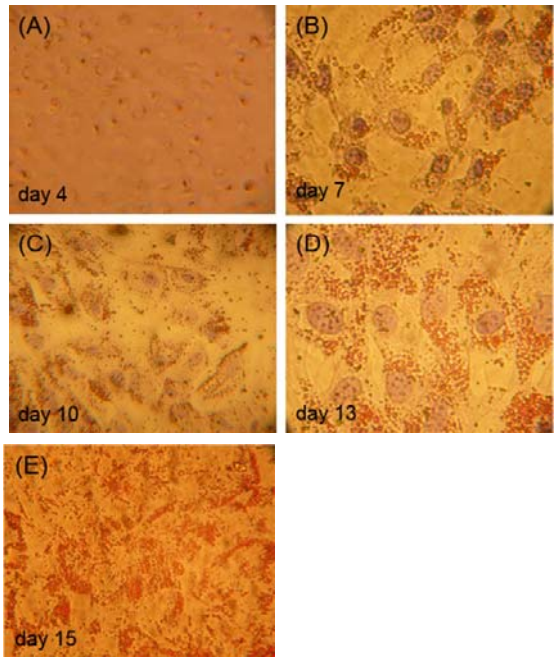

Fig 2: The process of primary pre-adipocyte culture. The cells were induced to form mature adipocytes and were stained with Oil Red O on days 4 (A), 7 (B), 10 (C), 13 (D) and 15 (E) (×200).

DISCUSSION

Most metabolic diseases in dairy cows, such as fatty liver and ketosis, occur during the peripartum period and are attributed to NEB-induced lipid metabolism dysfunction (Grummer, 1995; Katoh, 2002). Lipid mobilization is the primary mechanism by which dairy cows fulfill the energy demand during early lactation (Locher *et al*., 2011; Khan *et al*., 2013). The blood NEFA concentration subsequently increases with the mobilization of adipose tissue. Redundant NEFA cannot be oxygenized by the liver and is then esterified and stored as triacylglycerol in the liver, which leads to the development of fatty liver disease (Bobe *et al*., 2004). Ketosis, which most likely occurs secondary to fatty liver disease, is also caused by excess plasma NEFA levels (Grummer, 1993). During early lactation transition period, energy adipocytes are the principal cell for fat storage, and physiological, metabolic and endocrine adaptations occur in adipocytes to support lipid mobilization.

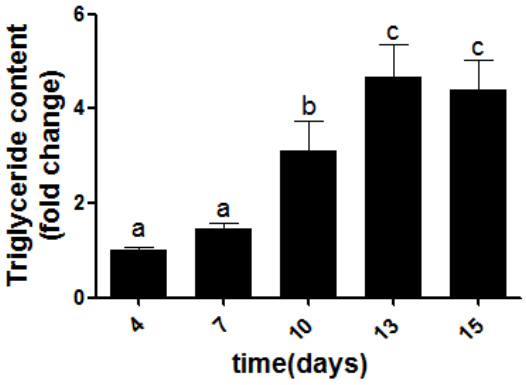

Fig 3: Lipid accumulation in bovine adipocytes during the differentiation process. The values are presented as the fold-changes relative to day 4. The different letters indicate significant differences at p<0.05 (similarly hereinafter).

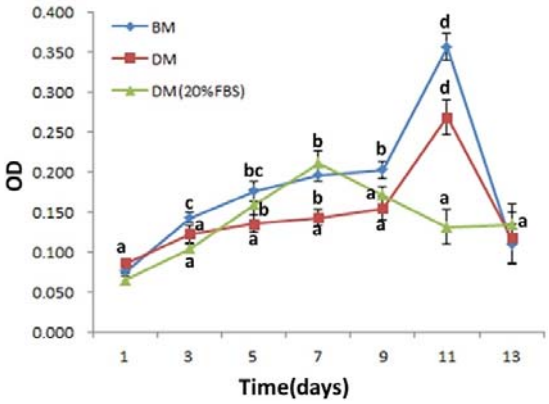

Fig 4: The profile of bovine adipocytes proliferated in different culture media was examined every two days. The proliferation of cells cultured in BM, DM or DM containing 20% FBS (DM (20% FBS)) was measured using an MTT assay.

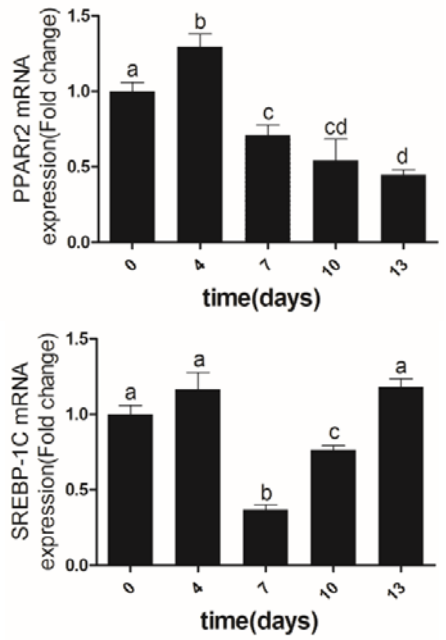

Fig 5: Adipogenesis-related gene expression during adipocyte differentiation. The expression level of each gene was normalized to the β-actin level in each sample. The values represent mean±SEM of three independent experiments, each performed in duplicate.

Adipocyte differentiation is accompanied by fat deposition. Adipocytes store lipids as TG and mobilize TG to balance the energy supply. Therefore, it is necessary to investigate the differentiation of pre-adipocytes isolated and cultured from the adipose tissue of dairy cows. However, currently, there is no data available on adipocyte differentiation in dairy cows.

There are two primary methods to study adipose metabolism *in vitro*. One method is tissue piece incubation. This method has been used to examine the effects of growth hormone (GH) and prolactin (PRL) on human adipose tissue (Ottosson *et al.*, 1995; Ling *et al.*, 2003). This method does not require cell isolation, is easy to perform, and significantly decreases the length of the experiment. Despite these advantages, this method has some drawbacks, such as low adipocyte purity. Hence, the results of experiments employing tissue piece incubations are limited. For the second method, primary cell isolation and culture is necessary. Primary cells can be obtained at a certain purity and can be separated from the animal directly without significant biological changes, reflecting the state of the animal. In this study, we have established a method to isolate pre-adipocytes from adipose tissue, profiting the basis for further *in vitro* investigation of adipose metabolism in dairy cows. These pre-adipocytes were rounded or polygonal upon attachment to the wall of the dish. Then, these cells displayed a spindle fibroblast-like morphology when completely spread, which conformed to the previously described characteristics of these cells (Napolitano, 1963).

Because of the species-specificity of and variation in adipocyte function, the induction protocol of adipocyte differentiation might differ. Currently, the most popular induction method is Cocktail differentiation (1-methyl-3-iso-butyl-xanthine (IBMX)) , dexamethasone (DEX), insulin), which is widely used to induce adipose cell lines (3T3-L1, 3T3-F442 and ob17) and primary culture adipocytes (human, large yellow croaker, sea bream) (Yoshimura *et al*., 2006; Capilla *et al*., 2011; Wang *et al*., 2012). In ruminants, bovine metabolism displays unique characteristics compared to monogastric animals. Therefore, much attempt has been made to find an optimum way for differentiation induction of bovine adipocyte. Wan *et al*. (2009) found that propionate affected the adipogenesis of adipocytes in intramuscular adipose tissue obtained from Luxi adult yellow steers (Wan *et al*., 2009). Another study by Grant *et al*. (2008) demonstrated that the combination of 280 *nM* insulin, 20 µL/mL serum lipid supplement, 40 *µM* troglitazone (TRO) and 0.25 *µM* DEX significantly increased glycerol-3-phosphate dehydrogenase (GPDH) activity in subcutaneous (s.c.) bovine pre-adipocytes compared to other treatments and that the omission of TRO or insulin from this medium reduced GPDH activity by 68% (Grant *et al*., 2008). In this study, we found that the Cocktail differentiation method did not effectively induce bovine adipocyte differentiation. Additionally, considering that DEX is a hormone that regulates glycolipid metabolism and may affected the results, we used insulin as the only factor to maintain pre-adipocyte differentiation, along with supplemental nutrition using FBS, biotin and pantothenate. We found that lipid droplets clearly appeared and gradually increased in the DM after 4 days

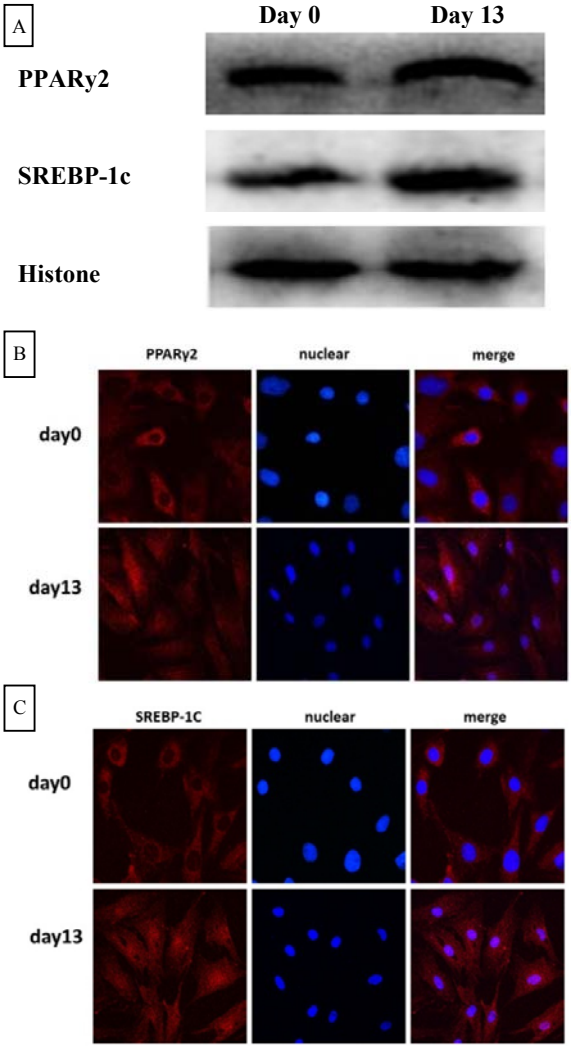

Fig 6: The protein levels and nuclear localization of PPARγ2 and SREBP-1c in undifferentiated and mature bovine adipocytes. (A) The protein levels of PPARγ2 and SREBP-1c in bovine adipocytes on day 0 and day 13 were detected by Western Blotting experiments. The level of protein Histone was used as a contrast. (B) Immunofluorescence staining for PPARγ2 in bovine adipocytes on days 0 and 13. (C) Immunofluorescence staining for SREBP-1c in bovine adipocytes on days 0 and 13. The nucleus were stained with Hoechst33258 (blue).

of culture. Similarly, the TG content also gradually increased over time. These results indicated that lipid deposition was significantly increasing during the adipocyte differentiation process and that insulin alone maintains pre-adipocyte differentiation in dairy cows.

Based on the results of the MTT assay, we showed that bovine adipocytes reached maximal proliferation after 11 days in culture. We also noted that a 10% concentration of FBS is most suitable for adipocyte proliferation. Based on the comparison of the proliferation of cells cultured in BM and DM, we found that the cell proliferation rate was significantly lower in DM than in BM. This result indicates that insulin may inhibit the proliferation rate of bovine pre-adipocytes, which was not consistent with previous research regarding the effect of insulin on other cells (Yang *et al*., 2010; Capilla *et al*., 2011; Wang *et al*., 2012). A possible explanation for this result is that insulin stimulated pre-adipocyte differentiation, potentially decelerating cell proliferation.

PPARγ and SREBP-1c have been identified as adipogenic regulatory factors involved in adipocyte differentiation and lipid metabolism. PPARγ2 is the adipocyte-specific and functional isoform that regulates adipocyte differentiation (Gurnell, 2005; Lehrke and Lazar, 2005). Ren *et al*. (2002) showed that the type-2 isoform of PPARγ is necessary for adipogenesis using a PPARγ2-deficient 3T3-L1 adipose cell line. Conversely, the exogenous delivery of PPARγ2 to PPARγ-deficient cells completely restores adipogenesis (Ren *et al*., 2002). SREBP-1c has been shown to regulate adipogenesis by increasing PPARγ ligand production (Fève, 2005; Rosen and MacDougald, 2006). Overexpressed wild-type and dominant negative forms of SREBP-1c in the 3T3-L1 cell line using retroviral vectors revealed that the simultaneous expression of SREBP-1c and PPARγ increases the transcriptional activity of this adipogenic nuclear hormone receptor, indicating that SREBP-1c plays an important role in adipocyte gene expression and differentiation (Kim and Spiegelman, 1996). In this study, we found that the mRNA expression level of PPARγ2 and SREBP-1c increased during the initial 4 days of differentiation induction and decreased thereafter. In contrast to PPARγ2, the expression of SREBP-1c increased after 10 days of differentiation and was equivalent to that at day 0. The PPARγ2 and SREBP-1c proteins were primarily localized to the cytoplasm of pre-adipocytes and were translocated into the nuclei of mature adipocytes and their protein levels were significantly increased. These results indicate that PPARγ2 and SREBP-1c affect adipocyte differentiation.

Conclusion: The present study has established and characterized a pre-adipocyte culture from dairy cows and has demonstrated that insulin alone induces and maintains dairy cow pre-adipocyte differentiation. Furthermore, SREBP-1c and PPARγ2 were increased after adipocyte differentiation, which supported the previous theory that SREBP-1c and PPARγ were involved in pre-adipocyte differentiation. The isolated and induced adipocytes can be used as a cellular model for the investigation of metabolic diseases in dairy cows.

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