

Effects of PSMA1 on the differentiation and lipid deposition of bovine preadipocytes

Sheng Li¹ , Chengzhen Chen^{1*} , Hao Jiang^{1,2} , Jiabao Zhang¹ , Qian Zhou¹ , Yan Gao¹ , Bao Yuan¹ , Mingjun Zhang¹ 

¹ Jilin University, College of Animal Sciences, Department of Laboratory Animals, Changchun, Jilin, China.

² Chungbuk National University, Department of Animal Science, Cheongju, Chungbuk, Korea.

*Corresponding author:
1651001215@qq.com

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ABSTRACT - In this study, our goal was to clarify the role of proteasomal subunit α -1 (PSMA1) in both the differentiation of preadipocytes and the accumulation of lipids in adipocytes. Preadipocytes from healthy one-day-old calves were collected, isolated, and cultured *in vitro*. The expression pattern of the PSMA1 gene was explored during the differentiation of bovine preadipocytes firstly. Then, the expression of the PSMA1 gene was inhibited by transfection of a chemically synthesized small interfering RNA (siRNA) before differentiation. After induction of differentiation, the mRNA levels of key regulating genes involved in preadipocyte differentiation and the lipid content of mature adipocytes with and without inhibition of PSMA1 were detected by qRT-PCR and oil red O staining, respectively. The data showed that PSMA1 mRNA was differentially expressed during the differentiation of bovine preadipocytes under normal culture conditions *in vitro*. The expression level of peroxisome proliferator-activated receptor gamma (PPAR γ), CCAAT enhancer-binding protein alpha (C/EBP α), and lipoprotein lipase (LPL) were significantly decreased in the transfected PSMA1-siRNA group compared with those in the control group, and the mRNA levels of the preadipocyte factor-1 (Pref-1) were significantly upregulated in the transfected PSMA1-siRNA group compared with those in the control group. In addition, significantly fewer lipid droplets were formed by adipocytes transfected with PSMA1-siRNA than by the negative control group (adipocytes transfected with NC-siRNA). Therefore, PSMA1 plays an important role in differentiation and lipid deposition.

Keywords: gene expression, siRNA, transfection, triglyceride

Introduction

As the main organ of energy storage in animal bodies, adipose tissue plays an important role in regulating metabolism. Moreover, fat (or fatty acid) content has an important influence on meat quality and flavor (Lee et al., 2010; Corino et al., 2008). In recent years, the effects of molecular genetics, management, nutrition, and feeding methods on fat content have been studied (Ceciliani et al., 2015; Koltjes et al., 2017), and studying the biological process of bovine fat formation from the perspective of molecular genetics can save costs and be more effective (Mehta et al., 2019).

Proteasome subunit alpha type 1 (PSMA1) is a member of the 20S proteasome α subunit. The 20S proteasome core is responsible for dissociating the protein substrate. PSMA1 is an important component of the proteasome and plays an important role in regulating the physiological state of the body and the occurrence of cancer (Lee and Ryu, 2017; Wang et al., 2018). Recent studies have found that PSMA1 is differentially expressed during fatty acid metabolism and lipid production (Seong et al.,

2016) and in the non-alcoholic fatty liver (Wang et al., 2016) and an obese rat model (Sakamuri et al., 2016). This pattern suggests that PSMA1 may play an important role in regulating fat formation and metabolism. However, the specific physiological role of PSMA1 in preadipocytes and whether it can affect the differentiation, lipid generation, and lipid deposition of preadipocytes are rarely studied.

In adipose tissue, many cytokines, including preadipocyte factor-1 (Pref-1), peroxisome proliferator-activated receptor gamma (PPAR γ), lipoprotein lipase (LPL), and CCAAT enhancer-binding protein alpha (C/EBP α), can regulate preadipocyte differentiation, lipid accumulation, and adipogenesis (Issemann and Green, 1990; Lim et al., 2015). Preadipocyte differentiation requires exogenous adipogenic stimulators such as glucocorticoid and insulin (Zebisch et al., 2012). Once preadipocytes begin to transform into adipocytes, some transcription factors, such as C/EBP α , are activated to induce the expression of metabolic genes and adipokines (Lefterova and Lazar, 2009). Pref-1, PPAR γ , LPL, and C/EBP α all play a key role with different regulatory effects (Guo and Liao, 2000). Pref-1 is a member of the EGF-like gene family homeobox that inhibits the differentiation of preadipocytes (Smas et al., 1994; Smas et al., 1997). PPAR γ mainly plays a role in regulating adipocyte differentiation, lipid metabolism, glucose homeostasis, and insulin sensitivity (Chawla et al., 1994; Tontonoz et al., 1998); it is known as an intrinsic determinant of adipocyte differentiation and can initiate the expression of adipose tissue-specific genes (Gregoire et al., 1998).

There is a correlation between the expression of LPL and the intermuscular fat content in the *longissimus* muscle of beef cattle (Li et al., 2017a), and its activity also affects the intermuscular fat content and meat quality (Soret et al., 2016). C/EBP α regulates adipogenesis by assisting lipogenesis gene expression and influencing glucose uptake in adipocytes, and it plays an important role in different stages of preadipocyte differentiation (Madsen et al., 2014; Zaini et al., 2018). In addition, other studies have shown that many cytokines or endogenous chemicals can affect the differentiation and lipid accumulation of preadipocytes (Chen et al., 2016; Ruiz-Ojeda et al., 2016). However, whether these key cytokines and genes are affected by PSMA1 is still unclear.

Therefore, this study aims to explore the effect of PSMA1 on the differentiation and lipid deposition of bovine preadipocytes. We reveal the effect of PSMA1 on fat formation at the gene level and provide a theoretical basis for the improvement of beef meat quality.

Material and Methods

In this study, three healthy one-day-old calves that did not eat colostrum were selected. All experiments were conducted in strict accordance with the recommendations of the local Guidelines for the Care and Use of Laboratory Animals (IACUC-20170355) in Changchun, Jilin Province of China (43.919825° N, 125.273824° E).

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless expressly stated otherwise.

Preadipocytes were obtained as previously reported (Hong et al., 2006; Karagiannides et al., 2006; Gupta et al., 2008; Nomura et al., 2011). Briefly, the bovine mesentery was removed in a sterile environment, placed in phosphate buffer saline (PBS) containing a 5% penicillin/streptomycin mixture (Hyclone Laboratories Inc, Utah, USA), and flushed gently with a pipette to remove the attached blood. Two layers of the mesentery were separated by using ophthalmic forceps, and the adipose tissue attached to the mesentery was separated and collected. After being washed several times with a pipette in PBS, the adipose tissue was placed in a 10-mL centrifuge tube and cut into 1-mm³ pieces with ophthalmic scissors. Collagenase (GIBCO, NY, USA) was added, and the adipose tissue was digested at 37 °C in a water bath for 1.5 h, filtered through 80 mesh and 200 mesh cell strains, and centrifuged at 1500 × *g* for 15 min. The cell pellet was mixed with complete medium (DMEM/F12 medium containing 10% FBS and 1% penicillin/streptomycin) and placed in a cell culture flask. The complete medium was changed every two days after the cells became adherent.

Images of cells were obtained using a stereoscopic microscope (Olympus, Tokyo, Japan) (Gálvez-Prieto et al., 2008; Chen et al., 2013).

The bovine preadipocytes were seeded into six-well plates. When the cells grew to 0.6 confluence, the complete medium was changed to basic DMEM/F12 medium without serum and double antibodies. After 6 h of incubation in DMEM/F12 medium, the cells were prepared for transfection. For transfection of cells, the original culture medium was first removed from the cell culture well, and 5 μ L of the transfected culture medium, Opti-MEM medium (Life Technologies, Inc., MD, USA) with 5 μ L of Lipofectamine™ 2000 (Life Technologies), and 100 pmol siRNA (GenePharma, Shanghai, China) was prepared. Next, 1.9 mL of complete medium was added to the culture wells, and the components were gently mixed. After 48 h of cultivation, the culture medium was replaced.

The siRNA sequences used were as follows: siRNA696 sense strand - 5'-GGUAAAGACUUGGAGUUUATT-3'; antisense strand - 5'-UAAACUCCAAGUCUUUACCTT-3' for transfection group; negative control (NC) sense strand - 5'-UUCUCCGAACGUGUCACGUTT-3'; and NC antisense strand - 5'-ACGUGACACGUUCGGAGAATT-3' for negative control group (no gene expression were changed with NC-siRNA transfection).

Briefly, the isolated primary preadipocytes were inoculated into culture medium in 25 cm² cell culture flasks. After the cells were grown to a confluence of 60% or 48 h after transfection (due to requirements of the experiment), the original medium was removed, and the induction medium (10 μ g/mL insulin, 33 μ mol/L calcium pantothenate, and 17 μ mol/L transferrin in 50 mL complete medium) was added to induce the bovine preadipocytes. This medium was changed every two days. The time was recorded as induction day 0. Images were obtained using a stereoscopic microscope (Olympus).

To clarify the expression pattern of PSMA1 during the differentiation of preadipocytes, we collected cells at 0, 4, 8, and 12 days after the induction of their differentiation into bovine preadipocytes to detect the mRNA expression levels of PSMA1 at different time points. Then, we collected bovine cells on day 4 after the transfection of PSMA1-siRNA and NC-siRNA to detect the mRNA expression levels of Pref-1, PPAR γ , LPL, and C/EBP α . Cells were digested with 0.0025 trypsin, and total RNA was extracted using Tripure Isolation Reagent (Roche, Mannheim, Germany). Total RNA was reverse transcribed into cDNA using a reverse transcription kit, and the resultant cDNA was used as a template for qRT-PCR. The qRT-PCR reaction system consisted of 10 μ L of RealMasterMix (Tiangen, Beijing, China), 0.5 μ L each of the upstream and downstream primers (10 pmol/ μ L, all sequences shown in Table 1), 2 μ L of the c-DNA template, and 7 μ L of ddH₂O. The qRT-PCR conditions were as follows: predegeneration at 95 °C for 5 min and 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. The mRNA expression level was analyzed by an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with relative quantitation ($2^{-\Delta\Delta C_t}$ method) (Pfaffl et al., 2002) with GAPDH as the standard. Three biological duplications were performed following the same procedure with three calves, and three technical repetitions were performed for each biological repetition. The mRNA expression level

Table 1 - Primers used for real-time PCR

Gene	Sequence (5' to 3')
PSMA1	Forward: GAAGCAAAACCCAGATACCAACA
	Reverse: TCCAAGTAAGTACGAGCTGATTGA
LPL	Forward: GTACAAGAGGGAACCGGACTC
	Reverse: AGGAGAAAGCGACTTGG
Pref-1	Forward: AAGCACCGGCAGACAAGA
	Reverse: CAGAGGAGGGAGTCGTCAGTA
PPAR γ	Forward: GCGAGGAGGACGAAGCGAAG
	Reverse: GCGCAGGCGGTCATTGTC
β -actin	Forward: AGGTCATCACCATCGGCAATG
	Reverse: ACAGCACCGTGTGGCGTAGAG

of the three repeated experiments was first calculated using the $2^{-\Delta\Delta Ct}$ method, and then the value of the relative expression level in the control group (the transfected PSMA1-siRNA negative control group at day 0) was set to 1 according to the mean value and standard deviation. Finally, the relative mRNA expression levels of the other groups were calculated.

Oil red O staining was performed on day 4 after the transfection of bovine preadipocytes. In cell culture plates, the negative control cells and the transfected cells were fixed with 10% neutral formalin for 30 min, washed with PBS, and stained with an Oil Red Staining Kit (Solarbio, Beijing, China). The oil red O staining solution was removed, and then the cells were washed three times with PBS. After the stained cells were fixed in isopropanol for 15 min, images were obtained using a stereoscopic microscope (Olympus) (Puri et al., 2012; Kim et al., 2013), and absorbance values were recorded using a visible spectrophotometer at a wavelength of 490 nm. The triglyceride content was determined according to instructions of the Triglyceride Detection Kit (E1012, Applygen, Beijing, China). Triglyceride detection was performed on the fourth day after PSMA-1 siRNA and NC-siRNA transfection.

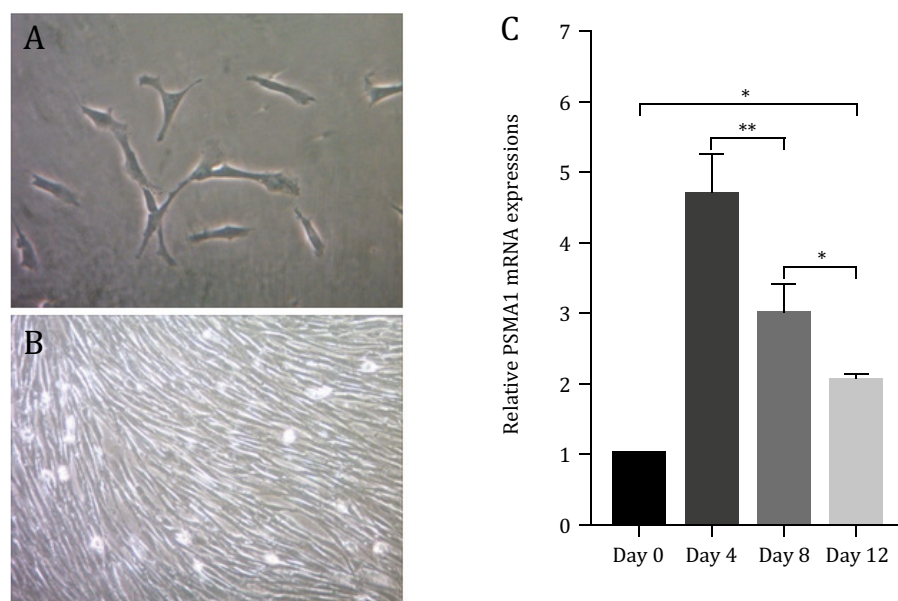
All statistical analyses were performed using SPSS version 22.0 (IBM, IL, USA) software. Data obtained from two groups were compared using the t test. Differential PSMA1 mRNA expression levels on different days were analyzed using a one-way ANOVA. The following statistical model used was as follows:

$$Y_{ij} = \mu + a_i + e_{ij}$$

in which Y_{ij} = observed variable, μ = overall mean, a_i = the sample processing effect of the cell culture time i (1, 2, 3, and 4 represent days 0, 4, 8, and 12, respectively), and e_{ij} = random error. Significant differences were determined using a protected Tukey's test after one-way ANOVA. Significant differences are represented with * ($P < 0.05$) and ** ($P < 0.01$).

Results

The isolated bovine preadipocytes were cultured for 24 h and began to adhere. After 48 h, they began to divide and grow in large numbers and gradually developed a stable, long, spindle-shaped cell morphology (Figure 1A, B). However, the expression levels of PSMA1 in bovine preadipocyte



A and B: fusiform preadipocytes cultured for 24 and 192 h, respectively (200 X). C: changes in PSMA1 mRNA expression of mature adipocytes after induction for differentiation.

Figure 1 - Bovine preadipocytes and relative mRNA expression of PSMA1 in preadipocytes at 0, 4, 8 and 12 days of differentiation.

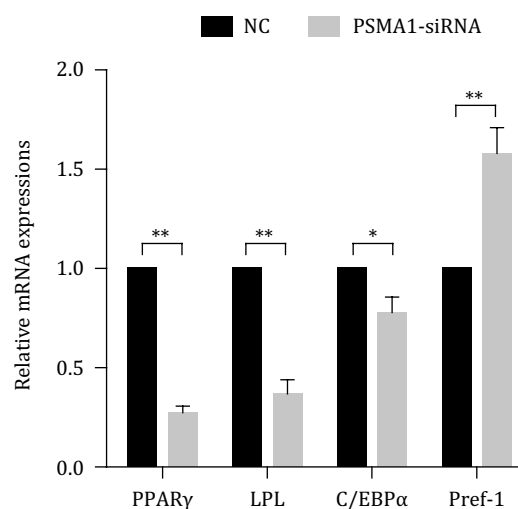
differentiation at days 0, 4, 8, and 12 first increased and then decreased (the relative expression of PSMA1 was expressed as mean \pm SD; the expression levels were 1.000 \pm 0.000, 4.698 \pm 0.563, 3.012 \pm 0.389, and 2.059 \pm 0.057, respectively), and the expression was the highest on the fourth day following the induction of differentiation (Figure 1C). The expression on the 12th day following induction was significantly decreased compared with that on the fourth day but was still significantly higher than that on day 0.

Since the expression level of PSMA1 was the highest at four days after induction under normal culture conditions, the key regulatory factors (Pref-1, PPAR γ , C/EBP α , and LPL) of preadipocyte differentiation and lipid deposition in preadipocyte that was transfected with PSMA1-siRNA changed. Their mRNA levels were tested on the fourth day following the induction of differentiation (Figure 2). Compared with those in the negative control group, the expression levels of PPAR γ , C/EBP α , and LPL, which promote the differentiation of adipocytes and lipid deposition, were significantly decreased in the transfected PSMA1-siRNA group, and the mRNA levels of the differentiation factor Pref-1 were significantly upregulated (the relative expression levels of PPAR γ , LPL, C/EBP α , and Pref-1 in the transfected PSMA1-siRNA group were 0.258 \pm 0.041, 0.353 \pm 0.076, 0.765 \pm 0.080, and 1.572 \pm 0.132, respectively; the expression levels of these mRNA in the negative control group were all set to 1.000 \pm 0.000). Thus, transfection with PSMA1-siRNA may inhibit preadipocyte differentiation and lipid deposition.

After the siRNA-induced interference was confirmed to be effective, we transfected siRNA into preadipocytes by LipofectamineTM 2000 and then cultured the cells in induction medium. When the induction medium was changed, it was recorded as day 0, and oil red O staining was performed on the 12th day. There were significantly fewer lipid droplets formed by adipocytes transfected with PSMA1-siRNA than those in the NC group (Figure 3), which indicates that PSMA1 inhibits lipid droplet formation in adipocytes (Figure 3A-D). At the same time, the triglyceride content in the PSMA1-siRNA transfection group was significantly lower than that in the NC group (the relative triglyceride contents of the NC group and the transfected PSMA1 group were expressed as the mean \pm SD; these values were 1.000 \pm 0.000 and 0.706 \pm 0.165, respectively) (Figure 3E). These results show that transfection of PSMA1 with siRNA inhibits the production and accumulation of triglycerides in mature adipocytes.

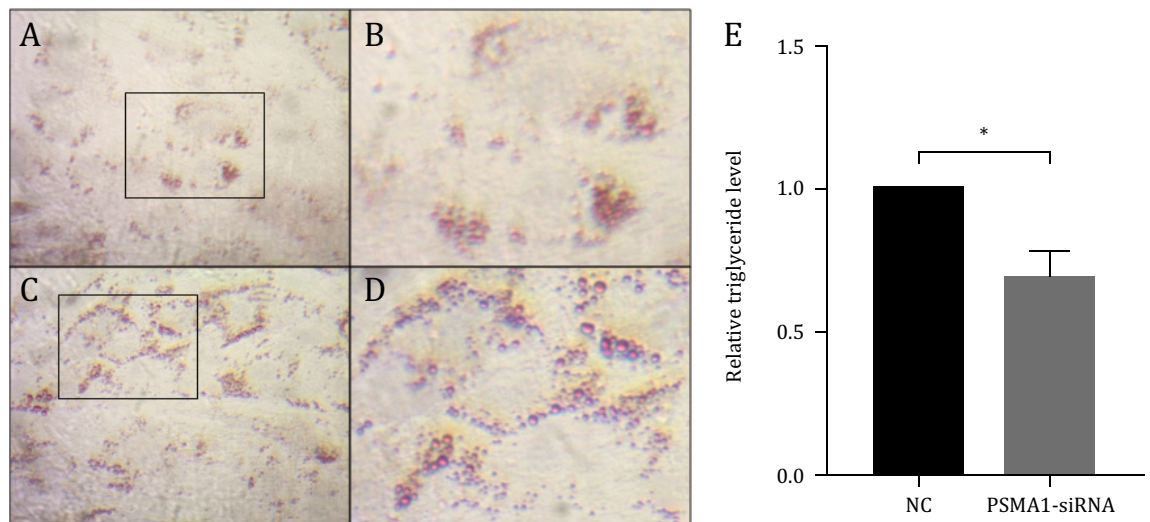
Discussion

Unlike porcine and mouse cells, the growth and differentiation of bovine preadipocytes was relatively slow. Porcine and mouse preadipocytes differentiated into more mature adipocytes after two days of



Differential gene mRNA expression levels obtained from negative control (NC) and PSMA1-siRNA groups were compared by the t test.

Figure 2 - Changes in Pref-1, PPAR γ , C/EBP α , and LPL mRNA expression after inhibition of PSMA1.



A and C show the lipid deposition in the adipocytes of negative control (NC) group and PSMA1-siRNA group under the microscope (200 X), respectively; B and D are the enlarged image of selected area from A and C, respectively; E is the relative triglyceride content level in the NC and PSMA1-siRNA groups.

Differential relative triglyceride content obtained from NC and PSMA1-siRNA groups were compared by the t test.

Figure 3 - Effects of PSMA1 inhibition on lipid deposition of bovine adipocytes.

incubation (Wang et al., 2010; Shi et al., 2014). However, only small numbers of bovine preadipocytes were observed on the second day in this study. The cells began to gradually differentiate and adopted a long, spindle-like shape after seven days of incubation. This might be one of the reasons why bovine intermuscular fat exhibits slower lipid deposition than the intermuscular fat of mice and pigs (Kratchmarova et al., 2002; Luo et al., 2008).

Similar to the results of other studies, bovine preadipocytes began to differentiate into mature adipocytes, accumulate large amounts of triglycerides, and form lipid droplets two to four days after induction (Hirai et al., 2007; Yang et al., 2017). Our study showed that the expression of PSMA1 after the differentiation of bovine preadipocytes first increased and then decreased, and the expression of PSMA1 was the lowest on the initial day of the experiment. Over time, the expression of PSMA1 reached its highest value at day 4 after induction and then gradually decreased. During this process, the proportion of undifferentiated preadipocytes gradually decreased, while the proportion of differentiated mature adipocytes and the intracellular triglyceride content gradually increased, suggesting that PSMA1 may be involved in the differentiation of bovine preadipocytes.

To clarify the specific role of PSMA1, we first examined the role of PSMA1 in regulating the key factors (PPAR γ , LPL, C/EBP α , and Pref-1) involved in the differentiation of preadipocytes and lipid deposition in mature adipocytes. Studies have shown that Pref-1 and PPAR γ play important roles in the differentiation of preadipocytes (Yeh et al., 1995; El-Jack et al., 1999; Moon et al., 2002; Kim et al., 2007a; Wang and Sul, 2009). Pref-1 is highly expressed in preadipocytes (Moon et al., 2002). As the cells differentiate, the expression level of Pref-1 gradually declines. Finally, Pref-1 is poorly expressed in mature adipocytes (Wang et al., 2006). PPAR γ is the major regulator of adipocyte differentiation and gene expression and plays an important role in adipocyte differentiation (Chawla et al., 1994) and can initiate the expression of adipose tissue-specific genes (Gregoire et al., 1998). In this study, the mRNA level of Pref-1 in the PSMA1 inhibition group was significantly higher than that of the control group four days after the differentiation of preadipocytes. This indicates that the inhibition of PSMA1 affects Pref-1 expression. At the same time, the inhibition of PSMA1 also significantly reduced the expression of PPAR γ . Combined with the biological effects of Pref-1 and PPAR γ , this is consistent with some studies that show that PSMA1 potentially regulates the c-Myc signaling pathway (Kulichkova et al., 2010; Li et al., 2017b), Notch3 (Ogura et al., 2003; Zhang et al., 2007), and Pde3b (Hopitzan et al.,

2000; Ogura et al., 2003) to promote the differentiation of bovine preadipocytes by downregulating the expression of Pref-1 and upregulating the expression of PPAR γ .

LPL and C/EBP α have important roles in preadipocyte differentiation and cellular lipid accumulation. The expression of these two genes begins with lipogenesis and induces lipoprotein storage and lipid metabolism (Bauer et al., 2015; Rozovski et al., 2016; Glatz and Luiken, 2017; Hu et al., 2018). Consistent with this, the expression of PSMA1 is potentially associated with LXR α , which is the upstream regulator of LPL and C/EBP α (Zhang et al., 2001; Steffensen et al., 2002; Kim et al., 2007b; Han et al., 2009). In this study, the expression levels of LPL and C/EBP α were significantly reduced after PSMA1 was inhibited. In the inhibited group, both the degree of preadipocyte differentiation and the ability of mature adipocytes to accumulate triglycerides were significantly reduced. These results indicated that PSMA1 has a regulatory effect on LPL and C/EBP α and that PSMA1 not only affects adipocyte differentiation but also has a potential effect on lipid production and metabolism in mature adipocytes.

Conclusions

Inhibition of PSMA1 can decrease the mRNA expression of C/EBP α , PPAR γ , and LPL and enhance the mRNA expression of Pref-1 in preadipocytes, which in turn inhibits the differentiation of preadipocytes into mature adipocytes. Moreover, PSMA1 can also influence the production of triglycerides in mature adipocytes, thereby affecting the ability of adipocytes to deposit lipids.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: S. Li, C. Chen, H. Jiang and Q. Zhou. Data curation: S. Li, C. Chen and Q. Zhou. Formal analysis: S. Li, C. Chen and Q. Zhou. Funding acquisition: S. Li, C. Chen, H. Jiang and J. Zhang. Investigation: S. Li and C. Chen. Methodology: S. Li, C. Chen, H. Jiang, J. Zhang, Y. Gao and M. Zhang. Project administration: S. Li and C. Chen. Resources: S. Li and J. Zhang. Software: S. Li, H. Jiang, Y. Gao and B. Yuan. Supervision: S. Li, H. Jiang, J. Zhang and B. Yuan. Validation: S. Li and J. Zhang. Visualization: S. Li and Q. Zhou. Writing-original draft: S. Li. Writing-review & editing: S. Li, C. Chen, H. Jiang and M. Zhang.

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