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AMFR gene silencing inhibits the differentiation of porcine preadipocytes

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ABSTRACT. Our study clarifies the role of the autocrine motility factor receptor (AMFR) gene in porcine preadipocyte differentiation. AMFR-siRNA was transfected into porcine preadipocytes and the preadipocytes were induced to differentiation. Subsequently, qRT-PCR was conducted to examine changes in mRNA expression of a series of genes in porcine preadipocytes, including AMFR, sterol-regulatory element-binding protein-1a (SREBP1a), SREBP2, insulin-induced gene 1 (Insig1), and Insig2. Expression changes in the mRNA of genes regulating adipocyte differentiation were also analyzed using qRT-PCR, including peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα), and Kruppel-like factor 2 (KLF2). Western blot analysis was conducted to examine the changes in AMFR

protein expression in porcine preadipocytes. Additionally, morphological changes in differentiated porcine preadipocytes were examined by oil red O staining, and changes in optical density (OD) values were measured using an ultraviolet spectrophotometer. At 24 h after transfection with AMFR-siRNA, AMFR mRNA expression significantly reduced ($P < 0.01$), and AMFR protein expression markedly decreased ($P < 0.05$). The mRNA expression of SREBP1a, SREBP2, Insig1, and C/EBPα was significantly reduced ($P < 0.01$), whereas the expression of KLF2 mRNA was significantly elevated ($P < 0.01$). After induction of preadipocyte differentiation, the number of lipid droplets decreased in the AMFR-silenced group, and the OD value markedly reduced ($P < 0.05$). In addition, the expression of C/EBPα mRNA significantly decreased ($P < 0.05$), whereas the expression of KLF2 mRNA considerably increased ($P < 0.05$). Taken together, silencing of the AMFR gene inhibits the differentiation of porcine preadipocytes.

Key words: Porcine preadipocytes; AMFR; Adipocyte differentiation; siRNA

INTRODUCTION

Pig is an important domesticated animal and a primary source of animal protein for human nutrition. As consumer demand for higher quality pork grows, reduction of back fat deposition in pigs has become a major goal for researchers in the field of animal genetics (Guo et al., 2012). Due to their high adipogenic capacity, and an adipogenic mode similar to that of human preadipocytes, porcine preadipocytes are recognized as a model system that is superior to rodents in the study of preadipocyte differentiation (Pang et al., 2009).

A large number of genes are involved in the process of adipocyte differentiation, such as peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα), and Kruppel-like factor 2 (KLF2) (Jiang et al., 2012). Glucocorticoids enhance the function of CCAAT/enhancer-binding protein beta (C/EBPβ) and C/EBPδ, which in turn activate PPARγ through regulation of KLF5. Once activated, PPARγ promotes the expression of C/EBPα. The above genes function jointly to induce adipocyte differentiation and fat deposition (Guo et al., 2012; Jiang et al., 2012). KLF2 is expressed at a rather high level in adipose tissue and preadipocytes. However, KLF2 expression declines rapidly in mature adipocytes. Studies have shown that KLF2 expression in preadipocytes not only inhibits the expression of PPARγ but also affects the expression of essential genes regulating adipocyte differentiation, such as C/EBPα and sterol-regulatory element-binding protein-1c (SREBP1c), thus hindering preadipocyte differentiation (Banerjee et al., 2003; Wu et al., 2005).

Autocrine motility factor receptor (AMFR, also called gp78) is an endoplasmic reticulum (ER)-anchored ubiquitin ligase (Liu et al., 2012). Since the discovery of AMFR (Silletti et al., 1991), studies have shown that AMFR is involved in a variety of important biological activities, including tumor cell migration, Huntington's disease (Romagnoli et al., 2003; Endo et al., 2006). AMFR regulates genes in the SREBP pathway and participates in fat anabolism in the liver. Using AMFR knockout mice, Liu et al. (2012) found that disruption of AMFR increases the protein expression

levels of insulin-induced gene-1 (Insig1) and Insig2. As a result, the SREBP pathway is inhibited, fat synthesis is reduced, and energy consumption is elevated. In addition, disruption of AMFR leads to resistance to diet- and age-induced obesity (Liu et al., 2012).

In the present study, RNA interference technology was employed to silence the AMFR gene in porcine preadipocytes, and changes in the expression of AMFR mRNA and protein were analyzed. In addition, changes in the mRNA expression of the genes in the SREBP pathway, and the genes regulating adipocyte differentiation, were examined. Changes in the morphological and biochemical indices of adipocytes after induction of differentiation were also analyzed. The present study aimed to reveal the role of the AMFR gene in the process of fat deposition in pigs, thus providing the basis for the use of the AMFR gene as a molecular marker of pork quality traits, and for its application in early selective breeding of pigs.

MATERIAL AND METHODS

Experimental animals

The piglets used in this study were healthy Junmu1 piglets less than 7 days old, raised in Original Breeding Pig Farm of Jilin University (Changchun, China). Experiments were performed in accordance with the guiding principles in the use of animals, adopted by the Chinese Association for Laboratory Animal Sciences. The study plan was approved by the Ethics Committee on the Use and Care of Animals, Jilin University.

Isolation and culture of porcine preadipocytes and construction of growth curves

Porcine preadipocytes were isolated and cultured, and growth curves were constructed, according to the procedures published previously by Gao et al. (2013) in our laboratory.

Transfection of porcine preadipocytes

Each 12-well plate was seeded with preadipocytes at 1 x 10^5 cells/ mL, and transfection was performed when the cells reached 70% confluence. Two different culture media were prepared by adding either 2 μL Lipofectamine™ 2000 (Invitrogen, Beijing, China) or 10.0 μL (0.008 OD/μL) AMFR-siRNA to 50 μL serum- and antibiotic-free OptiMEM medium (Gibco, Beijing, China). The two culture media were gently pipetted up and down individually for 5 min, mixed together and then incubated at room temperature for 15 min. The spent culture media on the 12-well plates was discarded, and 900 μL OptiMEM media was added to each well. The siRNA-Lipofectamine™ 2000 mixture was then dropped evenly into the complete media. After 24 h of culture, the culture media were removed, and the subsequent experiments were conducted. The siRNA sequences are shown in Table 1.

Table 1. siRNA sequence.

	Sense	Antisense
AMFR-siRNA	CGUGUGGGUUCUGGUAAAUTT	AUUUACCAGAACCCACACGTT
Negative control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Quantitative reverse transcription-PCR (qRT-PCR)

At 24 h after transfection, total RNA was extracted using the Trizol reagent (Invitrogen) according to manufacturer protocol. Subsequently, total RNA was reverse transcribed into complementary DNA (cDNA) using a reverse transcription kit (Takara, Dalian, China) in accordance with the manufacturer protocol. The qRT-PCR experiments were conducted using a fluorescence quantitative PCR kit (Takara) and the cDNAs as templates. The PCR system included 0.8 μL (10 pM) upstream primer, 0.8 μL (10 pM) downstream primer, 10 μL SYBR Premix Ex Taq II (Tli RNaseH Plus), 6.4 μL ddH_2O, and 2 μL cDNA template. The reaction conditions were as follows: 95°C, 30 s; 95°C, 5 s; and 60°C, 30 s, 40 cycles. The results were standardized to control values of *Sus scrofa* GAPDH. The experimental data were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$). Each experiment was repeated three times. The primers utilized in the present study are summarized in Table 2.

Table 2. Primers used for real-time PCR.

Gene	Forward	Reverse	Length (bp)
GAPDH	ACAGTCAAGGCGGAGAACG	GGCAGAAGGGGCAGAGAT	204
PPARγ	GTTGATTTCTCCAGCATTTCCA	GGCTCTTCGTGAGGTTTGTTG	188
C/EBPα	TGGACAAGAACAGCAACGAG	ACCTTCTGTTGAGTCTCCACG	109
SREBP1a	TCAGCGAGGCGGCTTTGGAGCAG	CATGTCTTCGATGTCGGTCAG	80
KLF2	GCACCGCCACTCACACCTG	CCGCAGCCGTCCCAGTTG	127
Insig2	CAGTGTAATGCGGTGCGTAG	CCAAGGTTGCCAAGAAAG	185
SREBP2	GCGTGCTCACTTTACCGA	GAACTCTGCTGCCCATCC	138
Insig1	CCCCGAGGAGGTTATTGC	GGTTCTCCAAGGTGGCTGT	120
AMFR	TTTGGCAACATCTGGTTATCT	AAATGGCACAGTCATCGTTA	187

The primers for GAPDH and C/EBPα refer to Guo et al., 2012. The primers for KLF2 refer to Fang and Davies, 2012.

Western blot analysis

At 24 h after transfection, the cells were trypsinized and harvested. Total protein was extracted using the Total Protein Extraction Kit (BestBio, Shanghai, China), and the protein concentration was determined. Fifty micrograms of protein sample was mixed with 10 μL 5X sample buffer, heated at 95°C for 15 min in a metal bath, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel), and then electrically transferred to nitrocellulose membranes (pore size: 0.45 mm). The membrane was blocked with 5% non-fat milk at room temperature for 2 h, incubated with primary antibodies against AMFR (Cell Signaling Technology, USA) and β-actin at 4°C overnight on a shaker, and then incubated with either goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody at room temperature for 1 h. The target proteins were visualized and images were captured using the Tanon-5200 automated chemiluminescence image analyzer. The other antibodies were all purchased from Santa Cruz Biotechnology, Inc.

Induction of porcine preadipocyte differentiation

At 24 h after transfection, the transfection medium was removed and replaced with induction medium 1, which contained 10% fetal bovine serum, 90% Dulbecco's modified Eagle's medium (DMEM)/F12, 1.72 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 100 nM rosiglitazone and 1 μM dexamethasone. The time of addition of induction medium 1 was counted as time point 0 h. At

48 h, induction medium 2 was added, which contained 1.72 μM insulin and 1 μM dexamethasone. The media were changed every 24 h.

Oil red O staining of porcine preadipocytes

After induction of porcine preadipocyte differentiation, induction medium 2 was removed. The cells were washed three times with phosphate-buffered saline (PBS, without penicillin-streptomycin), fixed in 10% neutral buffered formalin for 30 min, and then incubated with 10% oil red O staining solution (solvent: 70% ethanol) for 15 min. After removal of the dye solution, the cells were washed three times with PBS, counterstained with hematoxylin, and again washed three times with PBS. The stained plates were observed and imaged under a microscope.

Measurement of the OD values of porcine preadipocytes

After induction of porcine preadipocyte differentiation, induction medium 2 was removed. The cells were washed three times with PBS (without penicillin-streptomycin), fixed in 10% neutral buffered formalin for 30 min, and then incubated with 10% oil red O staining solution (solvent: 70% ethanol) for 15 min. After removal of the dye solution, the cells were washed three times with PBS and then incubated with 100% isopropanol for 15 min to extract the oil red O dye from stained lipid droplets. The OD values were measured at 510 nm.

Examination of marker genes after induction of porcine preadipocyte differentiation

After induction of porcine preadipocyte differentiation, the induction medium was removed. qRT-PCR was conducted according to the procedure described above to examine the mRNA expression of the marker genes C/EBPα and KLF2 for adipocyte differentiation.

Statistical analysis

The experimental data are reported as means ± standard deviation (SD). One-way ANOVA, using SPSS18.0, was used for analysis of variance and tests of significance. The differences between the treatment groups were compared using the least significant difference test. Each experiment was repeated three times.

RESULTS

Changes in AMFR mRNA and protein expression after AMFR silencing

Fluorescence-based qRT-PCR was conducted at 24 h after transfection with AMFR-siRNA. The PCR results showed that the expression level of AMFR mRNA was significantly reduced compared with the negative control ($P < 0.01$; Figure 1). In addition, western blot analysis showed that the expression level of the AMFR protein was markedly decreased in the siRNA-transfected group compared to the negative control group ($P < 0.05$; Figure 2).

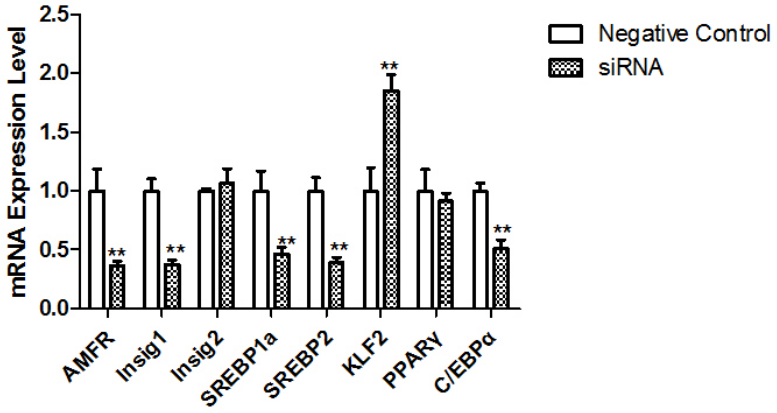

Figure 1. Changes in mRNA expression of AMFR, Insig1, Insig2, SREBP1a, SREBP2, KLF2, PPARγ, and C/EBPα at 24 h after transfection with AMFR-siRNA. mRNA expression of AMFR, SREBP1a, SREBP2, Insig1, and C/EBPα was significantly reduced ($P < 0.01$), whereas the expression of KLF2 mRNA was significantly increased ($P < 0.01$). **Indicates significant differences between the means ($P < 0.01$).

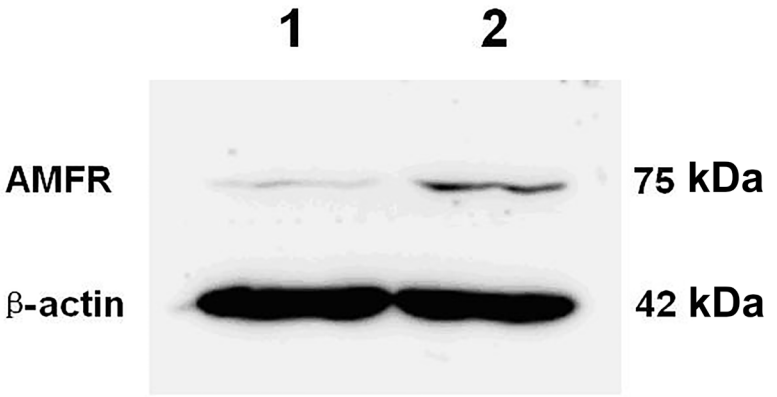

Figure 2. Changes in AMFR protein expression in porcine preadipocytes at 24 h after transfection with AMFR-siRNA. *Lane 1* represents the siRNA-transfected group; *lane 2* represents the negative control group. AMFR protein expression was markedly reduced in the siRNA-transfected group ($P < 0.05$).

Changes in mRNA expression of genes in the SREBP pathway, PPARγ gene, C/EBPα gene, and KLF2 gene after silencing of AMFR

At 24 h after silencing of AMFR, the expression level of KLF2 mRNA was significantly increased compared with the negative control group ($P < 0.01$; Figure 1), whereas the mRNA expression level of C/EBPα, Insig1, SREBP1a, and SREBP2 was significantly reduced ($P < 0.01$; Figure 1). The mRNA expression level of PPARγ and Insig2 showed no significant changes (Figure 1).

Morphological changes of porcine preadipocytes after silencing of AMFR

The results of the morphological examination showed that the size of the lipid droplets decreased, and the number of lipid droplets was reduced in the AMFR-silenced group (Figure 3B). In contrast, larger and an increased number of lipid droplets were observed in the negative control group (Figure 3A). The results showed that morphologically, silencing of AMFR reduced lipid droplet biogenesis in porcine preadipocytes.

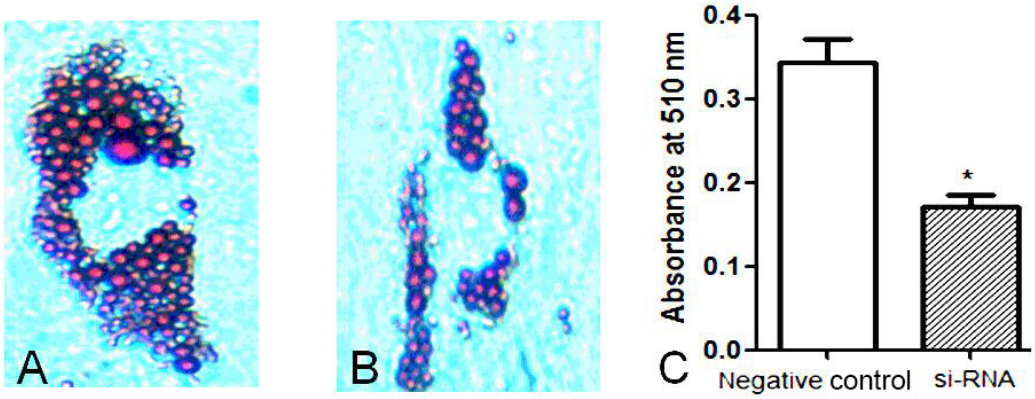

Figure 3. Changes in the cell morphology and OD value following induction of porcine preadipocyte differentiation at 24 h after silencing of AMFR. **A.** Oil red O staining of porcine preadipocytes in the negative control group (400X). Larger and higher numbers of lipid droplets were observed. **B.** Oil red O staining of porcine preadipocytes in the siRNA-transfected group (400X). The size of the lipid droplets decreased, and the number of lipid droplets was reduced. **C.** OD values measured at the 510 nm wavelength. The OD value of the negative control group was significantly higher than that of the siRNA-transfected group. *Indicates a statistically significant difference between the means ($P < 0.05$).

Changes in the OD value of porcine preadipocytes after silencing of AMFR

The OD values were measured, and the results showed that the OD value of the negative control group was significantly higher than that of the AMFR-silenced group ($P < 0.05$; Figure 3C), indicating that the triglyceride content was higher in the negative control group compared with the AMFR-silenced group. The results showed that silencing of AMFR reduced lipid droplet synthesis in porcine preadipocytes based on biochemical indices.

Changes in marker gene expression after silencing of AMFR and induction of porcine preadipocyte differentiation

qRT-PCR was conducted to examine the expression of marker genes for adipocyte differentiation after induction of porcine preadipocyte differentiation. The results showed that the mRNA expression of KLF2, a gene that negatively regulates adipocyte differentiation, was significantly increased in the AMFR-silenced group ($P < 0.05$), whereas the mRNA expression of C/EBPα, an adipocyte differentiation-promoting gene, was markedly reduced ($P < 0.05$; Figure 4). The results showed that silencing of AMFR inhibited the differentiation of porcine preadipocytes at the mRNA level.

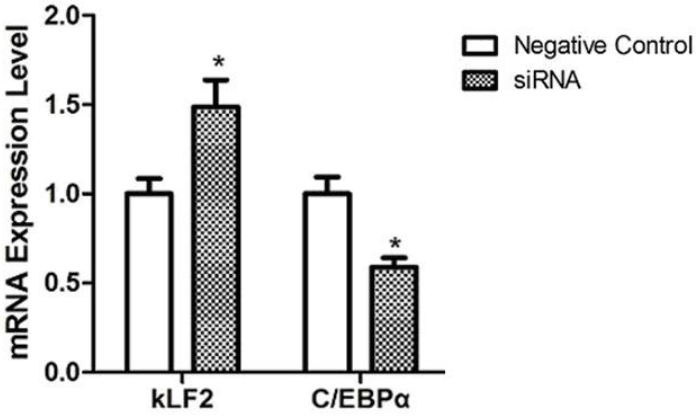

Figure 4. Changes in mRNA expression of KLF2 and C/EBPα after induction of porcine preadipocyte differentiation. KLF2 expression was significantly elevated in the siRNA-transfected group ($P < 0.05$), whereas C/EBPα expression was markedly reduced ($P < 0.05$). *indicates significant differences between the means ($P < 0.05$).

DISCUSSION

SREBPs consist of three isoforms: SREBP1a, SREBP1c, and SREBP2. SREBP1a and SREBP2 are the main forms of SREBP in cultured cell lines, whereas SREBP1c and SREBP2 are the predominant forms of SREBP in the liver and other intact tissues (Shimomura et al., 1997). SREBP2 is mainly involved in cholesterol metabolism, and SREBP1a is mainly involved in fatty acid and cholesterol metabolism, while SREBP1c is mainly involved in fatty acid metabolism and adipocyte differentiation (Horton et al., 2002). A study of SREBP1 knockout mice showed that the loss of SREBP1 can be partially compensated by SREBP2. In contrast, deletion of SREBP2 leads to early embryonic death in mice; SREBP1 fails to compensate for the loss of SREBP2 (Horton et al., 2002). In the present study, AMFR mRNA expression was drastically reduced after AMFR silencing ($P < 0.01$; Figure 1), which was accompanied by a significant decrease in the mRNA expression of SREBP1a and SREBP2 ($P < 0.01$; Figure 1). The results are consistent with those observed in the liver cells of AMFR knockout mice (Liu et al., 2012). However, the study conducted by Fisher et al. (2011) suggests that silencing of AMFR has no significant effect on SREBP1a expression in the HepG2 cell line. Fisher's result and our result were obtained from studies using the HepG2 cell line (human liver cell line) and porcine preadipocytes, respectively, which may have resulted in the different conclusions.

A study on fasted mice showed that the Insig1 expression level is increased and that Insig2 expression is suppressed when the insulin concentration is high, while the Insig2 expression level is elevated and the Insig1 expression level is reduced when the insulin concentration is relatively low (Yabe et al., 2003). Li et al. (2003) conducted a study using the cell line 3T3-L1 and demonstrated that adipocyte differentiation is accompanied by elevated expression levels of Insig1 and Insig2. A previous study also showed that overexpression of Insig1 and Insig2 inhibits adipocyte differentiation (Takaishi et al., 2004). Studies indicated that Insig1 and Insig2 negatively regulate SREBP (Yabe et al., 2002; Yang et al., 2002). In addition, Insig1 is regulated by SREBP. A high level of SREBP expression inhibits the expression of Insig1, whereas a low level of SREBP expression promotes the expression of Insig1. In contrast, Insig2 is not regulated by SREBP

(Goldstein et al., 2006). After silencing of AMFR, the expression of AMFR mRNA was markedly reduced, and accompanied by a significant decrease in Insig1 mRNA expression. No significant change was observed in the expression of Insig2 mRNA (Figure 1). The results are consistent with the findings reported by Liu et al. (2012).

Studies show that KLF2 expression is reduced in the process of adipocyte differentiation, indicating that the KLF2 gene is a negative regulatory gene in adipocyte differentiation (Banerjee et al., 2003; Wu et al., 2005). At 24 h after silencing of AMFR, a significant increase in KLF2 expression was observed ($P < 0.01$; Figure 1). Studies demonstrate that KLF2 is regulated by the ubiquitin system involving a WW domain containing E3 ubiquitin protein ligase 1 [WWP1, which is homologous to the E6AP carboxyl terminus (HECT) domain E3 ligase] or p62 ubiquitin-binding protein (Zhang et al., 2004; Guo et al., 2013). AMFR is an important ubiquitin ligase (RING-finger E3 ubiquitin ligase), which is capable of regulating 3 hydroxy 3 methylglutaryl CoA reductase (HMGCR) and cytochrome P450 3A4 (CYP3A4) through ubiquitination (Pabarcus et al., 2009; Liu et al., 2012). E3 ubiquitin ligases regulate KLF2 synergistically (Zhang et al., 2004). After interference, a negative correlation was detected between the AMFR and KLF2 expression levels, which may result from AMFR-mediated ubiquitination and regulation of KLF2. This is another way in which KLF2 also may be regulated by AMFR at the transcriptional level.

In the present study, no significant change was observed in PPARγ expression at 24 h after silencing of AMFR (Figure 1), which is consistent with the results of Fisher et al. (2011). A study conducted by Guo et al. (2013) showed that KLF2 is degraded through the activity of the ubiquitin system, which promotes the expression of PPARγ and C/EBPα. In the present study, a significant change was detected in the expression of C/EBPα at 24 h after silencing of AMFR ($P < 0.01$; Figure 1). It is likely that AMFR regulates KLF2 through the ubiquitin system, thereby inducing changes in C/EBPα expression.

Lipid droplet formation is a complex process. First, neutral lipids are synthesized by many enzymes localizing to the ER, and when neutral lipids are at low concentrations, they will accumulate to form a lipid lens within the bilayer of the ER. As additional neutral lipids gather to the bilayer, they form a sizeable lipid lens and cause the bilayer to deform. This results in lipid droplets being released into the cytoplasm (Wilfling et al., 2014). With the storage of lipids, the adipocyte matures (Rayalam et al., 2008). Fat and lipid droplets absorb the oil red O dye and appear red under a microscope. Therefore, porcine preadipocyte differentiation can be determined by the morphological appearance of the cells. After AMFR silencing, the size of the lipid droplets decreased, and the number of lipid droplets was reduced (Figure 3B). In addition, the OD value significantly decreased ($P < 0.05$; Figure 3C). These results indicate that the content of biosynthesized triglycerides was markedly reduced after AMFR silencing. The qRT-PCR results show that the expression of KLF2, a gene negatively regulating adipocyte differentiation, was significantly elevated ($P < 0.05$; Figure 4). In contrast, the expression of the adipocyte differentiation-promoting gene, C/EBPα, was noticeably reduced ($P < 0.05$; Figure 4). The results indicate that silencing of AMFR inhibits the differentiation of porcine preadipocytes.

In summary, silencing of AMFR inhibits the mRNA expression of Insig1, SREBP1a, SREBP2, and C/EBPα, and promotes the expression of KLF2 mRNA, thus affecting porcine preadipocyte differentiation and reducing triglyceride synthesis.

Conflicts of interest

The authors declare no conflict of interest.

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