MiR-9-5p promotes rabbit preadipocyte differentiation by suppressing *leptin* gene expression

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Abstract

Background: MicroRNAs are a class of small non-coding RNAs and participate in the regulation of cell differentiation programs. Previous studies have demonstrated that miR-9-5p play a key role during cancer cell research, but the mechanisms regulating of miR-9-5p in adipogenesis remain poorly understood. This study intended to investigate its significance in rabbits with high quality meat by observing the regulating effect of miR-9-5p in pre-adipocytes and finding related targets. **Methods:** In this study, a dual luciferase reporter assay was employed to validate the targeting relationship between miR-9-5p and *leptin* gene. We also utilized quantitative reverse transcription PCR (qRT-PCR), western blot, determination of triglyceride and oil red O staining assay to analyze the regulation of miR-9-5p and leptin gene during adipocyte differentiation.

Results: The analysis demonstrated that during pre-adipocyte differentiation, miR-9-5p was up-regulated and the fat formation related biomarkers fatty acid-binding protein 4(*FABP4*), CCAAT-enhancer binding protein α (*C/EBP α*), and peroxisome proliferator activated receptor (*PPAR γ*) were also up-regulated. Meanwhile, the oil red O staining assay revealed that the accumulation of lipid droplets increased. We explored the expression pattern and role of miR-9-5p in adipogenesis using white pre-adipocytes. The results showed that miR-9-5p was up-regulated during pre-adipocyte differentiation and overexpression of miR-9-5p enhanced lipid accumulation. Furthermore, we found overexpression of miR-9-5p significantly up regulated the mRNA levels of marker gene *PPAR γ* , *C/EBP α* and *FABP4*, as well as the protein levels of *PPAR γ* and content of triglyceride, the results suggested that miR-9-5p might be involved in the regulation of rabbit pre-adipocyte differentiation. We predicted *leptin* is a target of miR-9-5p by using bioinformatics tools and the conclusion was validated in a luciferase reporter assay. Finally, we verified inhibition of *leptin* by *si-leptin* promoted rabbits pre-adipocyte differentiation.

Conclusion: In a word, these results indicate that miR-9-5p influences rabbits white pre-adipocyte differentiation by targeting *leptin*.

Introduction

Adipose tissues, which may be brown, beige or white, not only maintain energy metabolism by generating heat or storing lipids but also serve as an endocrine organ. Of them, white adipose tissues are widely available and have the ability to differentiate. Adipose tissue-derived stem cells (ASCs) are initially recruited by certain stimulation, followed by being transformed into preadipocytes, and are ultimately developed into mature adipocytes(Francisco et al. 2016). Adipogenesis, a complex process in which fibroblast-like pre-adipocytes differentiate into lipid-laden and insulin-responsive mature adipocytes(Lefterova and Lazar 2009), requires sequential activation of numerous transcription factors and noncoding RNAs. During adipogenesis, several transcription factors such as peroxisome proliferator-activated receptor- γ (*PPAR γ*) and members of the CCAAT/enhancer-binding family of proteins (*C/EBP α*) participate in regulating adipogenesis(Hamam et al. 2014; Prayitno et al. 1997). Expression of *FABP4* is induced during adipocyte differentiation and is controlled by insulin and peroxisome proliferator-

activated receptor γ agonists (Hunt et al.). leptin can lead to the formation of positive feed-back between insulin and leptin, which further increases the synthesis of adipose tissue and aggravates hyperleptin (Brüning et al. 2000; Wang et al. 2001). Leptin plays a pivotal role in regulating feed intake, energy expenditure and fat deposition in mammals (Matteri 1998). Leptin can promote lipolysis and thermogenesis by influencing the sympathetic nervous system (Ekelis and N. 2004). In addition, leptin can also directly regulate metabolism of some tissues and cells (McClelland et al. 2004; Sanigorski et al. 2013). *Leptin* resulted in a dose and time-dependent increase in type1 collagen, a decrease of adiponin and *leptin* mRNA levels, and a 50% decrease in lipid droplet formation (Thierry et al. 1999). Adenovirus-induced hyperleptinemia in normal rats caused fat loss, downregulation of lipogenic enzymes, and suppression of the transcription factor peroxisome proliferator-associated receptor- γ (PPAR γ) (Zhou et al. 1999). The absence of fat in transgenic mice led to reduction in *leptin* levels and marked metabolic alterations including hyperlipidemia, insulin resistance, and diabetes (Moitra et al. 1998). Research recently showed that leptin enhanced differentiation of osteoblasts and inhibited differentiation of adipocytes in human bone marrow stromal cells (Thierry et al. 1999). Taken together, these findings showed that leptin may act locally in adipose tissue to influence the formation of mature adipocytes.

Furthermore, microRNAs, as a class of short noncoding RNA, are generally regarded to inhibit target gene expression by binding to their 3'-UTRs and have been demonstrated to regulate lipid metabolism (Bagga et al. 2005; Lagos-Quintana and M. 2001). miRNAs were also shown to be involved in cell differentiation (Zhang et al. 2017). In goats, miR-183 promoted preadipocyte differentiation by suppressing expression of Smad4 (Zhao et al. 2018) and miR-21a-5p induced pre-adipocyte differentiation by targeting Mitogen-activated protein kinase 2/ kinase 3 (MAPK2K3) through MKK3/p38/MAPK signalling pathway in mice (Xie et al. 2016). However, miR-33b downregulated the differentiation of porcine preadipocytes (Taniguchi et al. 2014). In 3T3-L1 cells, miR-26b promoted adipocyte differentiation through targeting *PTEN* (Li et al. 2017), while one study has demonstrated that miR-148a-3p impaired rabbit adipocyte differentiation by targeting *PTEN* in rabbits preadipocytes (He et al. 2018). MiR-9-5p is a well-conserved microRNA and primarily expresses in the central nervous system (Kapsimali et al. 2007; Wienholds and E.). Another study also showed that miR-9-5p can promote MSC (mesenchymal stem cells) migration by activating b-catenin signalling pathway (Li X et al. 2017). b-catenin is an integral part of Wnt signalling and play an important role in Wnt signalling. The study has found that the classical Wnt signal can inhibit fat differentiation (Ross et al. 2000). It has also been shown that *C/EBP α* or *PPAR γ* can inhibit Wnt signal, which can affect the differentiation of adipocytes by reducing the level of b-catenin (MOLDES et al. 2003). Yet the role of miR-9-5p in differentiation of adipocytes remains unknown.

In this study, we isolated and cultured rabbit perirenal adipose cells. Then, we studied the effect of miR-9-5p on adipocyte differentiation by overexpression and inhibition of miR-9-5p expression. In addition, we also predicted and verified that miR-9-5p is a target gene of *leptin*. Finally, we found miR-9-5p can influence differentiation of adipocyte by downregulating the mRNA expression levels of *leptin*.

Materials And Methods

2.1. Animals and tissues collection

All the experimental procedures using rabbits in this study were conducted under a protocol approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Sichuan Agricultural University, China. Perirenal adipose tissues were collected from three newborn Zealand rabbits, which were raised under standard condition at the Sichuan Agricultural University farm (Yaan, Sichuan, China).

2.2. Cell isolation, culture and induction of adipogenesis

Adipose tissue was rinsed three times in PBS then minced and digested with 0.25% collagenase type I (Gibco, Carlsbad, CA, USA) at 37°C for 1 h. The mixture was mixed with complete medium (CM; DM/F12, 10% fetal bovine serum, 2% penicillin-streptomycin) (Gibco) followed by filtration through 70- and 40-nm cell sieve, respectively. Finally, the mixture solution was centrifuged at 1200 ×g for 5min to collect the preadipocytes. The preadipocytes were seeded in culture flask with complete medium and the flask was placed at 37°C in a humidified incubator with 5% CO₂. Culture medium was changed every 2 days and the cell was frozen for future studies when they were passed to the third generation. To induce differentiation, an adipogenic inducer cocktail (0.5 mM 3-isobutyl-1-methylxanthine, 10% FBS, 1 μM dexamethasone, and 1.7 μM insulin) was added into the growth medium when cell density reached about 70% confluence and the cells were subsequently cultured for a further 72 h, after which the medium was replaced with maintenance medium (growth medium supplemented with 1.7 mM insulin per 50 mL) for an additional 72 h. Thereafter, the cells were cultured in growth medium until maturation at ten days.

2.3. Cell transfection

For transfection, preadipocyte cells were seeded into 24-well or 6-well plates and transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions after the cells had reached 70% confluency. The final concentrations of the miRNA mi-mics [mimics NC(negative control)], inhibitor (inhibitor NC), and leptin siRNA (siRNA NC) were 50 nM, 100nM, and 100 nM, respectively. Three independent repetitions were performed for each treatment group. The cells were harvested at different time after transfection and used to study adipogenic differentiation.

2.4. RNA isolation and qPCR analysis

Total cellular RNA was isolated with RNAiso Plus reagent (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. RNA purity was determined by OD 260nm /OD 280nm and OD 260nm /OD 230nm ratios with the Nano Drop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand cDNA of total RNA and small RNA was synthesized using the Prime Script RT reagent Kit (Takara, Japan) and the SYBR® Prime Script™ miRNA reverse transcription Kit (Takara), respectively, according to the manufacturer's protocol. The corresponding cDNA was stored at -20 °C. qPCR used the

SYBR premix Ex Taq II (Tli RNase H Plus) (Catalog No. RR820A; Takara) on a CFX96 system (Bio-Rad, Hercules, CA, USA). U6 was selected to normalize the expression of miRNA, while the internal reference used for mRNA expression was actin. The RT-qPCR procedure was as follows: 3 min at 95 °C, 39 cycles of 95 °C for 30 s, 30 s at the optimum annealing temperature, and a final extension for 66 s with temperature in increments of 0.5°C/s from 65°C to 95°C. The $2^{-\Delta\Delta C_t}$ method was used to analyze the relative expression of each gene. The sequences of the primers used for qPCR are shown in Table 1.

2.5. Western blotting

Total protein of cell samples was extracted with the Total Protein Extraction Kit (Sangon, Shanghai, China) according to the manufacturer's protocol and protein content was quantified with the BCA Protein Assay Kit. Forty micrograms of proteins were resolved by 8%-12% SDS-polyacrylamide gels and transferred to a PVDF membrane (Bio-Rad). The PVDF membrane was rinsed with TBS-Tween20 (TBST) and blocked for 2 h in skimmed milk. Then, the membranes were incubated with anti-PPAR γ (Santa Cruz Biotechnology,) and anti- β -actin (Abs, Beijing, China) according to the respective instructions overnight. the PVDF membranes were incubated with the secondary goat anti-mouse IgG (H + L) (Transgen, IllkirchGraffenstaden France) for 2 h after being washed three times. The protein bands were incubated with chemiluminescence reagents (Beyotime) after being washed four times. The images were obtained with a Bio-Rad GelDoc system equipped with a Universal Hood III (Bio-Rad), and the integrated optical density (IOD) was calculated using Gel-Pro Analyzer 4.0.0.4. Actin was used as an internal control.

2.6. Oil Red O staining and determination of triglyceride content

Adipocytes were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. After being washed three times with PBS, the cells were stained with 1% filtered oil red-O solution for about 30 min in the dark. Then, adipocytes were washed and observed using a phase contrast microscope. Finally, oil Red O was eluted from the stained cells with isopropanol and quantified by measuring the optical density values at 510nm wavelength. Intracellular triglyceride (TG) content was quantified with the TG Assay Kit (Applygen, Beijing, China) according to the manufacturer's protocol. The TG content (nmol/mg protein) was normalized to the cellular protein concentration, and the protein concentrations were measured with the BCA Protein Assay Kit (Beyotime, Shanghai, China).

2.7. Prediction and verification of the target gene of miR-9-5p

MiRwalk (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) was employed to predict the target genes of miR-9-5p and *leptin* was the target gene of miR-9-5p. The 3'UTR of *leptin* containing the miR-9-5p target site was cloned into the Sac I-Xba I site of the pmirGLO Vector (Promega, Madison, WI) to construct luciferase reporter plasmids. The primers used for plasmid construction are listed in Table 1, and multiple cloning sites were located downstream of firefly luciferase gene. Hela cells were seeded into 24-well plates in triplicate. Then, the pmirGLO-leptin-3' UTR of wild or mutant type was co-transfected with the synthetic miR-9-5p mimic into Hela cells after the cell density reached 70–80%. Firefly luciferase (luc2) activity was measured 48 h after transfection and normalized to Renilla luciferase activity

according to the TransDetect® Double Luciferase Reporter Assay Kit instructions (Transgen, Beijing, China).

2.8. Statistical analysis

The RT-qPCR results of mRNA expression were normalized to the geometric average of *Actin* identified the stability, and the expression level of miRNA was normalized to the expression of U6 snRNA, by an optimized comparative Ct ($2^{-\Delta\Delta Ct}$) value method. All data are presented as mean $\pm$ standard error (SEM). The student's t-test in GraphPad Prism7 (GraphPad Software, La Jolla, CA, USA) was used to assess the difference between groups. $P < 0.05$ and $P < 0.01$ were deemed to be significant and highly significant, respectively.

Results

3.1 Expression profiling of miR-9-5p during rabbit preadipocyte differentiation

In the process of inducing differentiation, the adipocytes were fully differentiated, depositing large lipid droplets (Fig. 1A). Results of oil red-O staining showed that lipid droplets rapidly increased during preadipocyte cell differentiation (Fig. 1B). Simultaneously, the expression of miR-9-5p grew rapidly after three days of induction and the expression on the ninth day was significantly higher than that on the other days (Fig. 1C). Correspondingly, Adipogenic marker gene *FABP4* had the highest mRNA expression on the sixth day whereas *PPAR γ* and *CEBPa* had the highest expression on the third day after induction (Fig. 1D, E, F).

3.2. Upregulation of miR-9-5p promotes rabbit preadipocyte differentiation

To investigate the function of miR-9-5p in rabbit preadipocyte cell differentiation, we first detected the expression level of miR-9-5p in the cells transfected with miR-9-5p mimics and NC during differentiation and the miR-9-5p showed a higher expression level in the mimic group than that of the control after induction ($P < 0.01$) (Fig. 2A). We found lipid accumulation was more in mimic group than the control by oil red-O staining and quantitative detection of oil red O staining ($P < 0.01$) (Fig. 4B, C). In addition, A significant increase in triglyceride after overexpression of miR-9-5p (Fig. 2D). The mRNA levels of adipogenic markers including *PPAR γ* and *C/EBPa* were higher significantly in the mimic group than NC group on the first and third days ($P < 0.01$) (Fig. 2E, F, G). Western blot analysis further indicated that *PPAR γ* protein levels were higher significantly in the mimic group than NC group after two days of transfection ($P < 0.05$) (Fig. 2H, I).

3.3. Downregulation of miR-9-5p inhibited rabbit preadipocyte differentiation

We next examined differentiation of preadipocytes when endogenous miR-9-5p was effectively inhibited with the miR-9-5p inhibitor. As shown in Figure 3A, the expression levels in the inhibitor groups were lower significantly than that of the control on the 3rd, 5th, 7th and 9th day after transfection ($P < 0.01$). We found that down-regulation of miR-9-5p decrease lipid accumulation in the preadipocytes cells as

detected by Oil Red O staining (Fig. 3B, C) and TG content (Fig. 3D). The mRNA expression levels of the common differentiation-related genes (*PPAR γ* and *C/EBP α*) on the 1st and 3rd day after transfection had lower levels of expression in the inhibition groups (Fig. 3E, F), while the common differentiation-related genes *FABP4* on the 5th and 7th day after transfection had lower levels of expression in the inhibition groups ($P < 0.01$) (Fig. 3G). In addition, *PPAR γ* protein expression level decreased after miR-9-5p inhibition (Fig. 3H, I).

3.4. *Leptin* is a target gene of miR-9-5p

To further determine the function of miR-9-5p, bioinformatics analysis was carried out to predict direct targets of miR-9-5p. MiRWalk2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) software revealed that *leptin* contains the target sites for miR-9-5P (Fig. 4A). To verify existence of the direct binding between miR-9-5p and *leptin*, the luciferase reporter assay showed the firefly luciferase activity significantly reduced during cotransfection of pmirGLO-LEPTIN-3' UTR and miR-9-5p agomirs (Fig. 4B).

3.5. MiR-9-5p downregulated *leptin* mRNA expression

To better understand the mechanism of miR-9-5p during preadipocyte differentiation, we further validated the effect of miR-9-5p on the expression of *leptin*. As shown in Figure 5A, overexpression of miR-9-5p led to an obvious decrease in the mRNA levels of *leptin*. In contrast, when the endogenous miR-9-5p was inhibited with the miR-9-5p inhibitor, the expression level of *leptin* was increased, compared with the INC group (Fig. 5B).

3.6. Downregulation of *leptin* expression promoted rabbit preadipocyte differentiation

To further confirm whether *leptin* is involved in the differentiation of rabbit preadipocytes, si-*leptin* was utilized to knockdown endogenous *leptin*. We found lipid accumulation and accumulation of triacylglycerol accelerated in the preadipocytes cells (Fig. 6A, B, C). When *leptin* was knocked down, the mRNA levels of *PPAR γ* and *C/EBP α* rapidly increased on the 1st and 3rd day after transfection, whereas *FABP4* rapidly increased on the 5th and 7th (Fig. 6D, E, F). Furthermore, *PPAR γ* protein expression level was higher significantly in si-*leptin* group than NC group after transfection 3 days ($P < 0.05$) (Fig. 6G, H). Additionally, we found the expression level rapidly decreased after knocking down endogenous *leptin* (Fig. 6I).

Discussion

In recent years, miRNAs were reported to be expressed in mammalian adipocytes and played an important role in the regulation of adipogenesis (Xu P et al. 2003). Previous studies have discovered a functional interplay among glucose-dependent insulin secretion, mir-9 levels and *sirt1* protein in pancreatic islets β -cells (Ramachandran et al. 2011). It has also been found that miR-9 and *sirt1* are involved in glucose dependent insulin secretion of islet β cells, and it has been proved that *sirt1* is a target gene of miR-9 (Plaisance et al. 2006b). In addition, miR-9-5p could negatively regulate *sirt1* expression

and played an important role in the regulation of cells proliferation and migration(Qi et al. 2015). *Sirt1* can bind to two coenzyme factors of *PPAR γ* , which inhibits the binding of *PPAR γ* to the target gene and the differentiation of adipocytes(Picard et al. 2004). It has also been shown that miR-9-5p promotes the secretion of Granuphilin-a and reduces the secretion and synthesis of insulin by targeting *Onecut2*(Plaisance et al. 2006a). *PPAR γ* can increase insulin sensitivity of adipose tissue through adipose tissue specificity, since ectopic production of Wnt-1 in preadipocytes blocked the induction of *PPAR γ* gene expression(MOLDES et al. 2003), and *PPAR γ* is a marker gene of preadipocyte differentiation. So, miR-9-5p may regulate the differentiation of adipocytes.

We found triglyceride content was more and lipid droplets was larger after transfection of miR-9-5p. The expression levels of adipogenic marker gene *FABP4*, *PPAR γ* and *CEBPa* increased after over-expression miR-9-5p, while downregulation of miR-9-5p using the synthetic inhibitor markedly reduced the formation of neutral lipid droplets and suppressed the expression of marker genes at both the *PPAR γ* mRNA and protein levels. These results suggested that miR-9-5p can promote the differentiation of rabbit preadipocytes. Previous studies demonstrated that NADPH oxidase 4 (*NOX4*) is a target gene of miR-9-5p(Fierro-Fernandez et al. 2015) and *NOX4* is the key enzyme of reactive oxygen species (ROS). It was reported that insulin release is inhibited by increasing ROS of islet B cells(Anvari et al. 2015; Den Hartigh et al. 2017) and the content of leptin is positive correlated with insulin (Anderwald et al. 2002; Papaspyrou-Rao et al. 1997). Then, a dual luciferase reporter assay was employed to validate that *leptin* is a target gene of miR-9-5p. The result suggested miR-9-5p can negatively regulate *leptin* gene expression. Previous studies have shown miR-9-5p can regulate secretion and synthesis of insulin. Insulin can stimulate secretion of leptin and increase the mRNA expression of *leptin*, meanwhile, leptin can directly inhibit the secretion of insulin(Emilsson et al. 1997). Studies have shown that insulin can improve the differentiation rate of preadipocytes and promote the accumulation of fat(Sato et al. 1996). It has also been found insulin may affect the expression of *PPAR γ* by affecting Sterol regulatory element-binding protein-1c (*SREBP-1c*)(Garcia-Escobar et al. 2011). Leptin can also regulate differentiation of adipocytes and previous studies have demonstrated leptin can inhibit the synthesis of lipids and promote the decomposition of triglycerides in adipocytes(Flier and Spiegelman 2001). So, miR-9-5p may target *leptin* to promote the differentiation of rabbit preadipocytes.

Leptin is secreted by adipocytes and the mRNA level of *leptin* decreased at the later stage of adipocyte differentiation(Ailhaud et al. 1992). Previous studies have been shown that leptin involved in a local ultrashort negative feedback loop and the loop regulated the differentiation of preadipocytes(Thierry et al. 1999). A study has also been shown that leptin suppresses specific biochemical processes, which contribute to lipid accumulation and adipocyte differentiation(Shimabukuro et al. 1997; Zhang and S. 1996). Furthermore, leptin can accelerate the proliferation of preadipocytes, inhibit the synthesis of lipids and promote the decomposition of triglycerides(Flier and Spiegelman 2001). We found the suppressed expression of *leptin* dramatically promoted the differentiation of preadipocytes. This finding is similar to previous study that leptin inhibits differentiation of preadipocytes. Therefore, we speculated that miR-9-5p positively regulates rabbit preadipocyte differentiation by inhibiting *leptin*. However, the biological basis and mechanism of this phenomenon need further research to get rabbits of high intramuscular fat

content. In recent years, many studies have been done to get animals with high intramuscular fat content in cattle and pigs, but it is little progress. Because we need to reduce body fat and increase muscle fat in the same animal at the same time. However, rabbits are an animal with particularly low-fat content. So, we can increase the body fat and intramuscular fat at the same time, which brings hope to cultivate a new kind of rabbit with high intramuscular fat content.

Conclusions

In summary, our data demonstrated that miR-9-5p can promote the differentiation of rabbit preadipocytes and leptin acts as one of the downstream targets of miR-9-5p. In addition, knockdown of *leptin* promoted adipogenic differentiation. Taken together, we concluded that miR-9-5p influences rabbit preadipocyte differentiation by suppressing expression of *leptin* mRNA.

Abbreviations

FABP4: fatty acid-binding protein 4; C/EBP α : CCAAT-enhancer binding protein α ; PPAR γ : peroxisome proliferator activated receptor; ASCs: Adipose tissue-derived stem cells; MAPK2K3: Mitogen-activated protein kinase 2/ kinase 3; 3T3-L1: Mouse Embryonic mouse fibroblast; PBS: phosphate-buffered saline; NOX4: NADPH oxidase 4; ROS: reactive oxygen species; SREBP-1c: Sterol regulatory element-binding protein-1c.

Declarations

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Authors' contributions

GL, JW, SJL designed and directed the study. GL, TFL, LW performed the experiments, data analysis and drafted the manuscript. GL contributed to the analysis and writing of the manuscript. QSH critically reviewed drafts of the manuscript and made comments to improve clarity. All authors approved the final version of this article.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Animal ethical approval

All experimental operations are carried out in strict accordance with the Experimental Animal Operation Regulations (No.18 [2014] of Sichuan Agricultural University), which formulated by the Sichuan Agricultural University experimental animal operation regulations and welfare management committee. Surgical procedures involving rabbits were performed according to the approved protocols of the Biological Studies Animal Care and Use Committee, Sichuan Province, China. Rabbits had free access to food and water under normal conditions and were humanely sacrificed as necessary to ameliorate suffering.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1 Primers used in this study

Gene name	Primer sequence (5'-3')	Tm/°C	Product size/bp
<i>leptin</i>	CTGTGCCCATGCGGAAAGTC AGTCCAAACCGACGACCCTC	61.4	104
β -actin	GGAGATCGTGCGGGACAT GTTGAAGGTGGTCTCGTGGAT	61.4	318
<i>PPARγ</i>	GAGGACATCCAGGACAACC GTCCGTCTCCGTCTTCTTT	61	168
<i>FABP4</i>	GGCCAGGAATTTGATGAAGTC AGTTTATCGCCCTCCCGTT	61.4	140
<i>C/EBPα</i>	GCGGGAACGAACAACAT GGCGGTCATTGTCACTGGTC	64	172
miR-9-5p mimic	UCUUUGGUUAUCUAGCUGUAUGA AUACAGCUAGAUAAACCAAAGA		
Negative Control (NC)	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT		
inhibitor-9-5p	UCAUACAGCUAGAUAAACCAAAGA		
inhibitor NC	CAGUACUUUUGUGUAGUACAA		
si- <i>leptin</i> -1	CCACAAUGGACCAGACGUUTT AACGUCUGGUCCAUUGUGGTT		
si- <i>leptin</i> -2	CCAUUGUCACCAGGAUCAGTT CUGAUCCUGGUGACAAUGGTT		
si- <i>leptin</i> -3	CCGAAAUGUGAUCCAAAUATT UAUUUGGAUCACAUUUCGGTT		
Primers for gene 3'UTR cloning <i>leptin</i>	ATGGGTAAGTACATCAAGAG GAGGTCCGAAGACTCATTT		

Figures

A

B

C

D

E

F

Figure 1

Rabbit preadipocyte differentiation model (A) Oil Red-O staining of lipid droplets ; (B) quantitative detection of oil red O staining; (C) MiR-9-5p expression levels during preadipocyte differentiation under normal culture conditions; (D) FABP4 expression levels during preadipocyte differentiation under normal culture conditions; (E) CEBPa mRNA expression levels during preadipocyte differentiation under normal culture conditions; (F) PPAR γ expression levels during preadipocyte differentiation under normal culture conditions ("*", $P \leq 0.05$; "**", $P \leq 0.01$)

Figure 2

Overexpression of miR-9-5p promoted rabbit preadipocyte differentiation (A) MiR-9-5p expression levels during preadipocyte differentiation after transfecting with miR-9-5p mimics and NC, (B) Oil Red-O staining of lipid droplets on day 9; (C) Quantitative detection of oil red O staining on day 9; (D) Accumulation of triacylglycerol on day 9; (E) PPARγ expression levels during preadipocyte differentiation after transfecting with miR-9-5p mimics and NC; (F) CEBPα expression levels during preadipocyte differentiation after transfecting with miR-9-5p mimics and NC; (G) FABP4 expression levels during preadipocyte differentiation after transfecting with miR-9-5p mimics and NC (H, I) PPARγ protein levels during preadipocyte differentiation after transfecting with miR-9-5p mimics and NC (“*”, $P \leq 0.05$; “**”, $P \leq 0.01$)

Figure 3

Inhibition of miR-9-5p inhibited rabbit preadipocyte differentiation (A) MiR-9-5p expression levels during preadipocyte differentiation after transfecting with the miR-9-5p inhibitor and INC, (B) Oil Red-O staining of lipid droplets on day 9; (C) Quantitative detection of oil red O staining on day 9; (D) Accumulation of triacylglycerol on day 9; (E) PPAR γ expression levels during preadipocyte differentiation after transfecting with the miR-9-5p inhibitor and INC; (F) CEBP α expression levels during preadipocyte differentiation after transfecting with the miR-9-5p inhibitor and INC; (G) FABP4 expression levels during preadipocyte differentiation after transfecting with the miR-9-5p inhibitor and INC (H, I) PPAR γ protein levels during preadipocyte differentiation after transfecting with the miR-9-5p inhibitor and INC ("*", $P \leq 0.05$; "**", $P \leq 0.01$)

A

Conserved

		Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	PT
Position 181-188 of LEP 3' UTR	5'	...CUCCUUGUCUGAA-AACAAAGA...						
hsa-miR-9-5p	3'	AGAAUGGCAAGCAUAGGUGUCU	8mer	-0.44	99	-0.44	3.750	0.75

Figure 4

Leptin is a target miR-9-5p gene. (A) prediction of target gene (B) PmirGLO and pmirGLO-leptin 3' UTR were cotransfected with the mimic into Hela cells, and normalized firefly luciferase activity was determined (n=3). (** P<0.01).

Figure 5

MiR-9-5p negatively regulated leptin expression: (A) Leptin expression based on quantitative real-time polymerase chain reaction (QRT-PCR) during adipogenesis with miR-9-5p overexpression. (B) Leptin expression based on QRT-PCR during adipogenesis with miR-9-5p knockdown. ("**", $P \leq 0.01$)

Figure 6

Inhibition of leptin gene promoted rabbit preadipocyte differentiation (A) Oil Red-O staining of lipid droplets on day 9; (B) Quantitative detection of oil red O staining on day 9; (C) Accumulation of triacylglycerol on day 9; (D) PPAR γ expression levels during preadipocyte differentiation after transfecting with si-leptin and NC; (E) CEBP α expression levels during preadipocyte differentiation after transfecting with si-leptin and NC; (F) FABP4 expression levels during preadipocyte differentiation after transfecting with si-leptin and NC; (G, H) PPAR γ protein levels during preadipocyte differentiation after transfecting with si-leptin and NC. (I) Leptin expression levels during preadipocyte differentiation after transfecting with si-leptin and NC (“*”, $P \leq 0.05$; “**”, $P \leq 0.01$)