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Original Article

MiR-15a/b promote adipogenesis in porcine pre-adipocyte via repressing FoxO1

Peiyue Dong, Yin Mai, Zhenyu Zhang, Lin Mi, Guofang Wu, Guiyan Chu, Gongshe Yang, and Shiduo Sun*

College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China
*Correspondence address. Tel/Fax: +86-29-8791017; E-mail: ssdsm@tom.com

Diabetes and many other metabolism syndromes are closely related to obesity. To reveal the underlying mechanism of fat deposition, an increasing number of studies are focusing on the functions of miRNAs during adipocytes development. Previous studies have proved that miR-15a/b play important roles in multiple physiological processes; however, their functions during adipogenesis remain unclear. To reveal this, we detected the expression profiles of miR-15a/b during adipogenesis in porcine pre-adipocyte, and found that their expression levels increased in the early stage of adipocyte differentiation and dropped after day 4. Moreover, over-expression of miR-15a/b in porcine pre-adipocytes promoted adipocyte differentiation and lipid accumulation. Target genes of miR-15a/b were predicted and examined, which revealed that Forkhead box protein O1 (FoxO1) is the target gene of miR-15a/b. The inhibition of FoxO1 expression level caused by miR-15a/b over-expression had a positive effect on adipogenesis. Thus, we conclude that miR-15a/b promote adipogenesis in porcine pre-adipocyte via repressing FoxO1.

Keywords miR-15a/b; porcine; pre-adipocyte; adipogenesis; FoxO1

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Introduction

Obesity has become an urgent global problem. With the abundant junk food and sedentary behaviors, the ratio of people with obesity has dramatically increased in recent years [1]. Obese people have a much higher risk of developing insulin resistance and diabetes [2]. Besides, cardiovascular disease and hepatopathy are often induced by overweight [3,4]. Obesity is caused by the excessive increase of adipocytes number or size. The mechanism of adipocytes differentiation has been demonstrated to be related to plenty of genes such as peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein beta (C/BEPβ), insulin receptor substrate (IRS) [5], etc. In recent years, researches have revealed that miRNAs also play important roles in adipogenesis.

MiRNAs are 19–22 nt non-coding RNAs which can bind to the 3′-untranslated region (UTR) of target mRNAs, leading to its digestion or impeding the transcription levels of target genes [6]. Roles of miRNAs in various physiological processes have been revealed in recent years. Previous studies showed that miRNAs had crucial roles in many aspects, like cancer [7], immunization [8], cell proliferation [9], and differentiation [10]. Nowadays, more and more studies focus on functions of miRNAs in metabolism, proliferation, and differentiation of adipocytes. It has been reported that miR-143, miR-375, and miR-103 enhanced the differentiation of adipocytes [11–13], while miR-27b, let-7, and miR-138 impaired the adipogenesis [14–16].

Previous studies have reported that the expression levels of miR-15a/b changed dramatically during adipogenesis [17,18]. MiR-15a was also reported to regulate uncoupling protein-2 (UCP-2) and promote insulin biosynthesis [19]. Another study showed that miR-15a could repress the differentiation of 3T3-L1 via Delta-like 1 homolog (Dlk1) [20]. Moreover, over-expression of miR-15b had an effect on decreasing cell proliferation and increasing intracellular triglyceride in QSG7701 cells [21]. These results hinted that miR-15a/b may play an important role in adipocyte development. However, the mechanisms of miR-15a/b during adipocytes differentiation are still unknown.

In this study, we over-expressed miR-15a/b in porcine pre-adipocytes and detected the expression of adipogenesis marker genes at the mRNA and protein levels. Results showed that the expressions of key genes and lipid accumulation increased markedly, indicating that miR-15a/b had a promoting effect on adipogenesis. Target gene analysis revealed that Forkhead O1 (FoxO1) was a potential target gene of miR-15a/b with its conserved binding site. Notably, the mRNA and protein expression levels of FoxO1 also decreased after the over-expression of miR-15a/b in porcine pre-adipocytes. In addition, the luciferase reporter assay demonstrated that FoxO1 3′UTR contained elements interacting with miR-15a/b. These results indicated that miR-15a/b

may promote adipogenesis via repressing the expression of FoxO1.

Materials and Methods

Construction of adenovirus vector

A 359 bp fragment of pri-miR-15a and a 442 bp fragment of pri-miR-15b encompassing the stem loop were amplified, and cloned into pAdTrack-CMV vector (Agilent, Santa Clara, USA); these vectors were named pAdTrack-CMV-15a and pAdTrack-CMV-15b. The pAdTrack-CMV-15a/b were recombined into the adenovirus vector pAd-Easy1. An adenovirus vector expressing scramble RNA was constructed as the control. The recombined plasmids were transfected into HEK293A by Roche transfection reagent (Basel, Switzerland), the recombinant adenoviruses were generated within 7–10 days, and then the adenoviruses were harvested, propagated, and concentrated. The packaged adenovirus vectors were named pAd-miR-15a and pAd-miR-15b.

Adipose tissue handing and cell culture

All animal procedures in this research were approved by Northwest A&F University Animal Care and Use Committee. Three-day-old large white piglets were sacrificed and the back fat was collected immediately. Fat tissues were cut into pieces and digested by type I collagenase (Invitrogen, Carlsbad, USA). After 1 h digestion, cells were filtered through 200 mesh sieve and then washed with phosphate-buffered saline (PBS) for three times. Cells were resuspended in DMEM/F12 medium with 10% fetal bovine serum (FBS) (Hyclone, Logan, USA), plated in 6 cm dishes at a density of 5×10^5 cells per dish, and incubated in a humidified atmosphere of 5% CO_2 at 37°C. HEK293A and HEK293T (Mucyte, Nanjing, China) cells were cultured in DMEM medium with 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C.

Induction of adipocyte differentiation

The cells were infected with adenovirus for 12 h when they grew to 80% confluency, then the medium were replaced with DMEM/F12. Twenty-four hours later, when cells grew to 100% confluency, half of them were harvested for RNA and protein extraction as the day 0 samples. The rest of them were changed to adipogenesis inducing medium I (DMEM/F12 with 10% FBS, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, 5 μg/ml insulin). After 48 h induction, the medium were changed to inducing medium II (DMEM/F12 with 10% FBS, 5 μg/ml insulin). Then, the medium II was changed every 2 days. On the sixth day, the cells were harvested for RNA and protein extraction. For miR-15a/b expression profiles analysis, cells were harvested every 2 days after induction, and the cells were cultured until the 10th day.

Oil Red O staining

The pre-adipocytes were gently washed twice with PBS and fixed with 4% paraformaldehyde for 40 min. Then, cells were washed twice with PBS and stained with 0.3% Oil Red O for 40 min. After that, cells were washed twice with water and images were captured by a microscopy imaging system (Nikon, TE2000-S, Tokyo, Japan). Isopropanol was added to dissolve the Oil Red O after the pictures were captured, and the optical density (OD) value was measured at 510 nm.

Quantitative real-time polymerase chain reaction

Total RNA was extracted by Trziol (TaKaRa, Dalian, China). To analyze miRNA expression level, total RNAs were reverse transcribed using miRNA and U6 stem-loop primer (RiboBio, Guangzhou, China) and then subject to quantitative real-time polymerase chain reaction (qRT-PCR). To measure the mRNA levels of β-actin, PPARγ, ethylene responsive element binding protein (aP2), sterol regulatory element binding protein-1c (SREBP-1C), FoxO1, IRS1, and mitogen-activated protein kinase kinase 4 (MAP2K4), total RNAs were reverse transcribed using PrimeScript™ RT reagent (TaKaRa) and then subject to qRT-PCR. U6 and β-actin were used as internal control, respectively, and the fold changes were calculated relatively to the control groups. Primer sequences are shown in **Supplementary Table S1**. The qRT-PCR was performed using One-Step SYBR PrimeScript RT-PCR Kit (TaKaRa) on Bio-iQ5 Real-Time PCR System (Bio-Rad, Hercules, USA). The procedure of qRT-PCR was: step 1, 95°C for 5 min; step 2, 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, repeat for 30 times; step 3, start at 55°C for 10 s, raise 0.5°C per cycle, repeat for 80 times.

Western blot analysis

Total proteins of porcine pre-adipocytes in different groups were extracted by using RIPA lysis buffer (Applygen, Beijing, China). Protein expression was analyzed by western blot analysis. Briefly, cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked with 5% skim milk for 2 h and then incubated with anti-PPARγ (mouse, Santa Cruz, Santa Cruz, USA), anti-Fas (rabbit, Santa Cruz), anti-aP2 (goat, Santa Cruz), anti-FoxO1 (mouse, Boster, Wuhan, China), anti-IRS1 (rabbit, Bioss, Beijing, China) antibodies, followed by the incubation with the corresponding horseradish peroxide-labeled IgG secondary antibodies (1 : 2000) for 2 h. Mouse GAPDH (Santa Cruz) was used as the internal control.

Dual-luciferase reporter assay

The partial 3′UTR of *FoxO1* (393 bp) were amplified by PCR using the primers shown in **Supplementary Table S1**, and cloned into psiCHECKTM-2 vector (Promega, Madison, USA). Three bases of seed region were mutated by overlap

PCR. HEK293 T-cells were seeded into a 24-well plate with a density of 1×10^5 cells per well. Twenty-four hours later, pAdTrack-CMV-miR-15a/b over-expression vector and psiCHECK^{TM}-2-FoxO1 vector were cotransfected into the cells by Roche transfection reagent. Cells were harvested after 48 h and the luciferase activities were measured by Dual-Luciferase Reporter Assay System (PerkinElmer, Boston, USA). The luciferase activity was normalized by the ratio of Renilla luciferase activity and Firefly luciferase activity.

Statistical analysis

Experiments were repeated at least three times. Data are expressed as mean ± standard error of the mean. Differences between groups were determined using Student's paired *t*-test by SPSS 19.0. $P < 0.05$ was considered significant difference.

Results

miR-15a/b are conserved among mammals and highly expressed during the early stage of adipogenesis

We searched the matured sequences of miR-15a/b in mammals by miRBase (Release 20). The results showed that one base deletion was found in miR-15a-5p of pig. While the seed region of miR-15a/b were 100% conserved among mammals, indicating that miR-15a/b may possess similar functions among mammals (**Fig. 1A**). To uncover the expression profiles of miR-15a/b during porcine pre-adipocyte adipogenesis, we induced the pre-adipocytes into adipocytes, harvested cells every 2 days, and detected the expression of miR-15a/b by qRT-PCR at different time points. The data showed that the expression levels of miR-15a/b reached the highest on the fourth day. After that, the expression levels of miR-15a/b decreased during 4–10 days (**Fig. 1B**). These results indicated that miR-15a/b might play a role in the early stage of adipogenesis.

miR-15a/b significantly promote the differentiation of porcine pre-adipocyte

To examine the function of miR-15a/b during adipogenesis, two over-expression adenovirus vectors were constructed (**Fig. 1C**). We used adenoviruses to transfect the cells, and the transfection efficiency in pre-adipocytes achieved almost 100% on day 0 (**Fig. 2A**). qRT-PCR showed that the expression levels of miR-15a/b after infection increased 2 and 4 folds, respectively (**Fig. 1D**). Cells were stained with Oil Red O (**Fig. 2B**) on sixth day after induction, and the results clearly illustrated that there were more lipid droplets in the miR-15a/b groups than in the GFP and control groups. The OD values at 510 nm also showed the same results (**Fig. 2C**).

Total mRNA and protein were also extracted on days 0 and 6. mRNA expression levels of PPARγ, aP2, and SREBP-1C were examined by qRT-PCR (**Fig. 3A–C**), and the protein

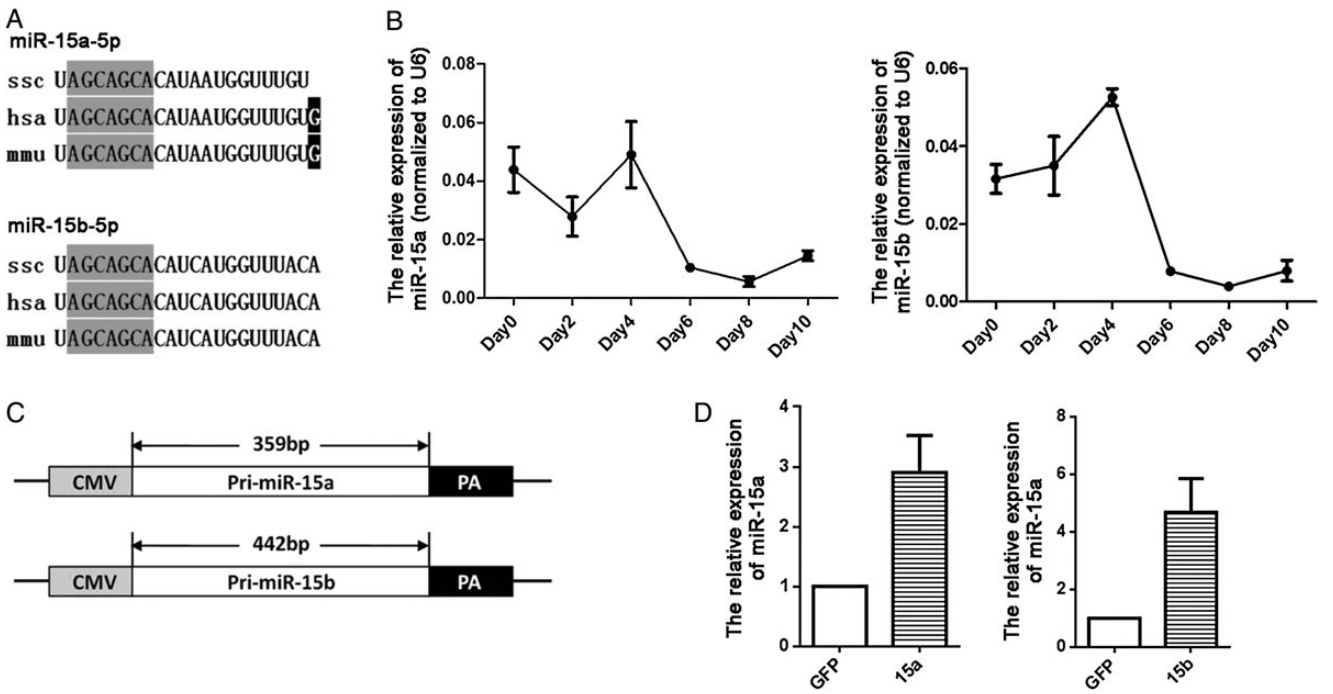


Figure 1. Expression profiles of miR-15a/b and the construction of Ad-virus (A) MiR-15a/b-5p are conserved among mammals. (B) The expression profiles of miR-15a/b during adipogenesis in porcine pre-adipocyte. (C) Structures of Pri-miR-15a/b Ad-virus, 359 and 442 bp, respectively. (D) The expression levels of miR-15a/b-5p after infected by Pri-miR-15a/b Ad-virus in porcine pre-adipocyte, the expression levels are 2.8 and 4.3 times of that of the GFP group, respectively.

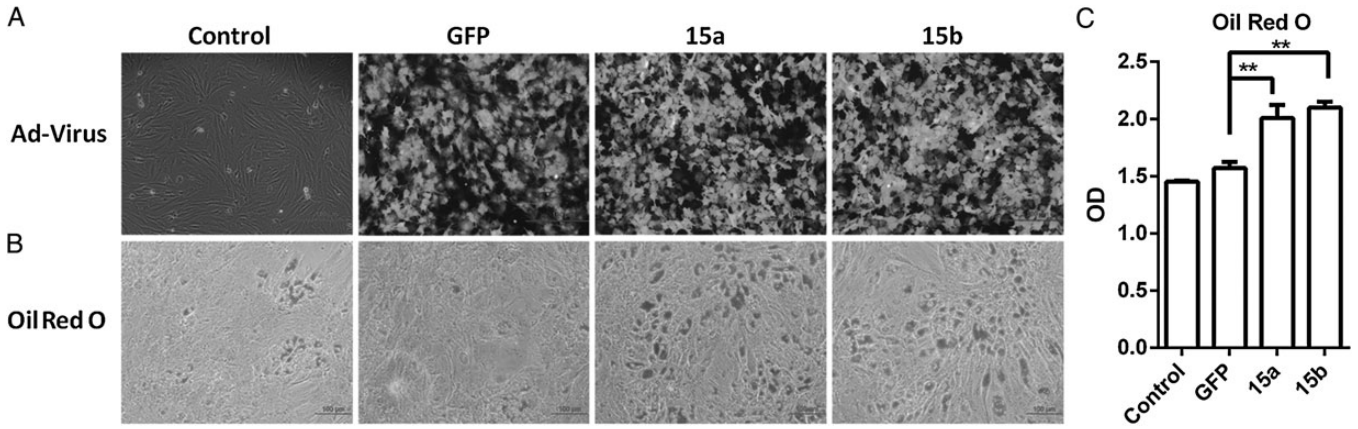


Figure 2. Porcine pre-adipocyte infected by Ad-viruses and stained by Oil Red O after induction (A) After 2 days infection with Ad-viruses, the infection efficiency were almost 100%, ×100. (B) Oil Red O staining on day 6 after induction. Apparently, the miR-15a/b-infected groups contained more lipids than the GFP and control groups, ×200; (C) The contents of lipids in different groups were expressed by $OD_{510\ nm}$. $^{**}P < 0.01$.

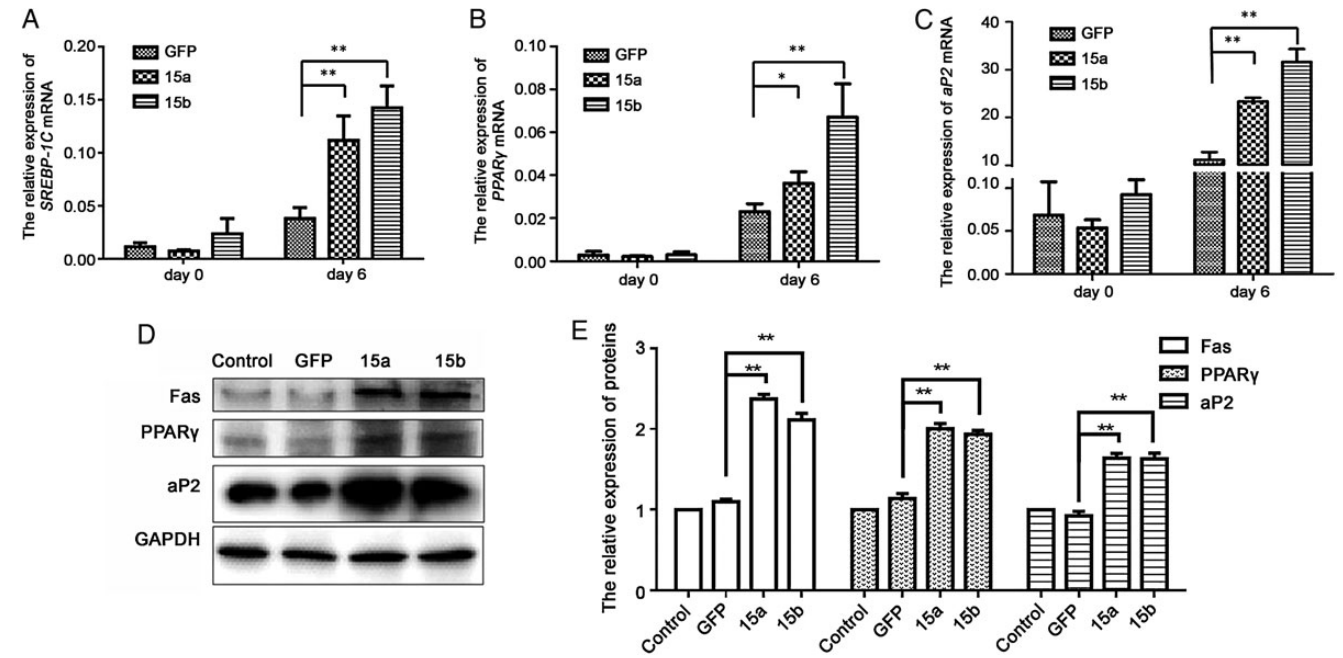


Figure 3. The expression levels of key genes in different groups (A–C) The expression levels of key genes *SREBP-1C*, *PPARγ*, and *aP2* during adipogenesis in different groups. (D) On the sixth day after induction, the expression of Fas, PPARγ, and aP2 were examined by western blot. (E) Quantification of Fas, PPARγ, and aP2 protein expression levels. $^{*}P < 0.05$; $^{**}P < 0.01$.

expression levels of PPARγ, aP2, and fatty acid synthase (Fas) on day 6 were detected by western blot (**Fig. 3D,E**). The results showed that the over-expression of miR-15a/b could dramatically promote the mRNA and protein levels of key adipogenesis genes.

Down-regulation of FoxO1 through over-expression of miR-15a/b

To identify the target gene of miR-15a/b, we performed a bioinformatics analysis using TargetScan, RNA22, PITA, and miRanda. Over thousands of potential target genes were predicted. The common target genes associated with adipogenesis predicted by at least three programs were FoxO1 (predicted by TargetScan, RNA22, and miRanda) and IRS1 (predicted by TargetScan, PITA, and miRanda). The latter one was demonstrated to be a pro-adipogenesis gene in previous studies [22], which was contrary to our results if it is the target gene of miR-15a/b. Furthermore, the mRNA and protein expression levels of IRS1 were similar in different groups (**Supplementary Fig. S1A–C**), and the luciferase reporter assays also confirmed that IRS1 was a pseudotarget (**Supplementary Fig. S1D**). The 3′UTR of FoxO1 was predicted to complement the seeds of miR-15a/b perfectly by M fold [23] (**Supplementary Fig. S1E,F**) and

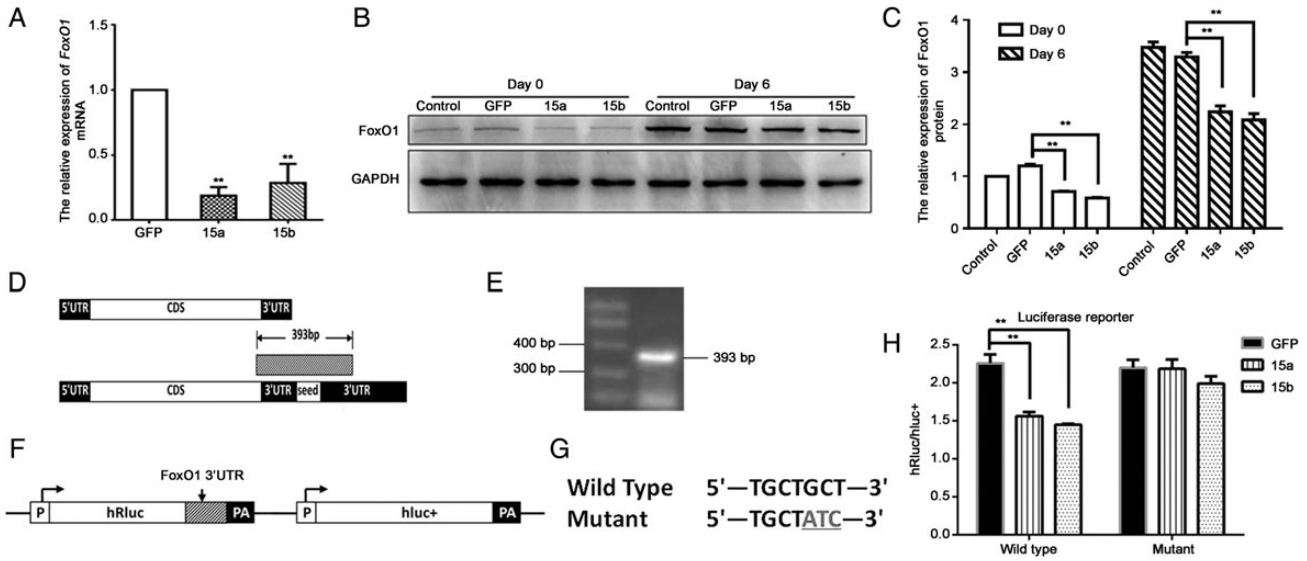


Figure 4. *FoxO1* is the target gene of miR-15a/b (A) qRT-PCR of FoxO1 on day 0. (B) The expression levels of FoxO1 during adipogenesis were detected by western blot. (C) Quantification of FoxO1 protein expression levels in different groups. (D) Amplification region of *FoxO1* 3′UTR. (E) Result of target fragment by PCR. (F) Structures of FoxO1 luciferase reporter plasmid. (G) Difference between wild-type and mutant reporter in seed region. (H) Results of *FoxO1* 3′UTR luciferase reporter assay. $^{**}P < 0.01$.

the down-regulation of FoxO1 had a promoting effect on adipogenesis. Collectively, these results suggested that FoxO1 might be the target of miR-15a/b.

To verify this hypothesis, the mRNA expression level of FoxO1 was detected on day 0, when the over-expression levels of miR-15a/b vectors were the highest. The results showed that the transcriptional level of FoxO1 was dramatically decreased (**Fig. 4A**). Similar results were also obtained at the protein level by western blot (**Fig. 4B,C**). Therefore, our results indicated that the over-expression of miR-15a/b could suppress the expression of FoxO1.

FoxO1 is a direct target gene of miR-15a/b

To confirm that *FoxO1* was the target gene of miR-15/b, we tried to construct a luciferase reporter vector first, but the full length of porcine *FoxO1* mRNA was unavailable in NCBI, only coding sequence (CDS) region and ~140 bp length 3′UTR were available. The prediction programs predicted that the target site was ~220 bp far from CDS region in human *FoxO1* mRNA. So we compared the genome sequence of pig with human's and found that they shared the same target site in similar region. Then, we designed the primers to amplify the target site. The forward primer was designed at 32 bp upstream of the end of CDS, and the reverse primer was designed at 342 bp downstream of the CDS (**Fig. 4D**), which contained the seed region not found in NCBI database. The PCR and agarose gel electrophoresis results showed that the target region existed in the porcine *FoxO1* mRNA (**Fig. 4E**). The amplified region was inserted into Check-2 vector at the 3′UTR of Renilla luciferase (**Fig. 4F**), and a mutant vector was also constructed (**Fig. 4G**). The results of dual-luciferase reporter assay demonstrated that the over-expression of miR-15a/b interacted with the target region of FoxO1 markedly and the expression of Renilla luciferase was decreased dramatically (**Fig. 4H**), which indicated that *FoxO1* is a direct target gene of miR-15a/b.

Discussion

In this study, we demonstrated that miR-15a/b are positive regulators during the differentiation of porcine pre-adipocytes. The expression levels of miR-15a/b were higher in the early stage of adipogenesis and declined 4 days later, which was similar to the result of a previous study in 3T3-L1 cells [20]. These results suggested that miR-15a/b might play an important role in the early phase of adipogenesis. Over-expression of miR-15a/b in the porcine pre-adipocytes led to the up-regulation of key adipogenesis genes at both transcriptional and translational levels. The Oil Red O staining and OD detection also proved that increased miR-15a/b levels could enhance the accumulation of triglyceride, which was in accordance with the results from hepatic QSG7701 cells [21]. Collectively, these results indicated that miR-15a/b play a positive role in adipogenesis. To explore the underlying mechanism in this process, two possible target genes, *FoxO1* and *IRS1*, were selected. Both of them are important genes during the early stage of adipogenesis. FoxO1 inhibits the differentiation of adipocytes while IRS1 possesses the opposite activity. IRS1 is the up-regulator of phosphatidylinositol 3 kinase-protein kinase B (PI3K-AKT) signal pathway. The inhibition of IRS1 was reported to

repress adipogenesis [22], which is contrary to our results. Consistently, the expression levels of IRS1 mRNA and protein were stable and dual-luciferase reporter analysis also confirmed that IRS1 is not the target gene of miR-15a/b. We further demonstrated that the mRNA levels of *FoxO1* in the miR-15a/b over-expression groups were dramatically decreased on day 0 and the protein levels of FoxO1 were also decreased on days 0 and 6. Furthermore, the dual-luciferase reporter analysis revealed that *FoxO1* 3′UTR possesses the binding site of miR-15a/b, which meant that FoxO1 is the target gene of miR-15a/b. Another potential target gene of miR-15a/b was *MAP2K4*. A previous study revealed that MAP2K4 could regulate the expression of FoxO1 via Jun N-terminal kinase (JNK) signaling pathway [24]. But an even earlier article also declared that miR-15b and other miRNAs jointly, not individually, suppress the translation of MAP2K4 [25]. We detected the *MAP2K4* mRNA expression levels of different groups, but no significant difference was found (**Supplementary Fig. S1G**). So we consider that MAP2K4 may not be a key target gene of miR-15a/b.

FOXO1 transcription factor has been reported to be relevant to cell metabolism, proliferation, differentiation, and apoptosis [26]. Previous studies have revealed that FoxO1 represses the expression of PPARγ by targeting its promoter [27]. PPARγ is a crucial gene during adipogenesis, and its repression might lead to a negative effect on the differentiation of pre-adipocyte. In the presence of insulin, FoxO1 is transferred to cytoplasm from nucleus, which rescues the expression of PPARγ [28,29]. Another study also reported that constitutively active FoxO1 prevented the differentiation of pre-adipocytes [29], which indicated that FoxO1 possesses another pathway to inhibit adipogenesis. Adipose triglyceride lipase (ATGL) is a key gene in regulating lipid degradation [30]. Its promoter can bind FoxO1 and be enhanced by FoxO1 [31], thus the inhibition of FoxO1 decreases the expression of ATGL, resulting in the increase of lipid accumulation. A study on porcine pre-adipocytes also proved that knockdown of *FoxO1* was beneficial to the differentiation of adipocytes [32].

In conclusion, in the early stage of adipogenesis, FoxO1 inhibits the transcription of PPARγ, hindering the differentiation of pre-adipocyte. During the adipogenesis, FoxO1 promotes the expression of ATGL, which increases the degradation of triglyceride and decreases the accumulation of lipid. In the present study, we verified that *FoxO1* is the target gene of miR-15a/b, thus miR-15a/b may promote the differentiation of porcine pre-adipocytes via repressing the expression of FoxO1.

Supplementary Data

Supplementary data are available at *ABBS* online.

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