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MicroRNA-212 targets *SIRT2* to influence lipogenesis in bovine mammary epithelial cell line

Xubin Lu[1], Hailei Xia[1], Jingyi Jiang[1], Xin Xu[1], Mingxun Li[1], Zhi Chen[1], Yujia Sun[2], Huimin Zhang[1] and Zhangping Yang[1]

[1]College of Animal Science and Technology, Yangzhou University, Yangzhou, Jiangsu 225002, China and [2]Joint International Research Laboratory of Agriculture and Agri-Product Safety, Yangzhou University, Yangzhou 225009, Jiangsu Province, China

Research Article

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Author for correspondence:
Zhangping Yang, Email: yzp@yzu.edu.cn

Abstract

In this research paper we filter and verify miRNAs which may target silent information regulator homolog 2 (*SIRT2*) gene and then describe the mechanism whereby *miRNA-212* might regulate lipogenic genes in mammary epithelial cell lines *via* targeting *SIRT2*. Bioinformatics analysis revealed that the bovine *SIRT2* gene is regulated by three miRNAs: *miR-212*, *miR-375* and *miR-655*. The three miRNAs were verified and screened by qRT-PCR, western blot, and luciferase multiplex verification techniques and only *miR-212* was shown to have a targeting relationship with *SIRT2*. The results of co-transfecting *miR-212* and silencing RNA (siRNA) showed that by targeting *SIRT2*, *miR-212* can regulate the expression of fatty acid synthetase (*FASN*) and sterol regulatory element binding factor 1 (*SREBP1*) but not peroxisome proliferator-activated receptor gamma (*PPARγ*). Measurement of triglyceride (TAG) content showed that *miR-212* increased the fat content of mammary epithelial cell lines. The study indicates that *miR-212* could target and inhibit the expression of the *SIRT2* gene to promote lipogenesis in mammary epithelial cell lines.

Fat metabolism in the mammary gland is a complex process that is regulated by many factors, and the molecular mechanism is not clearly understood. *SIRT2* encodes a member of the sirtuin family of proteins, and it can deacetylate factors in physiological and biochemical reactions in many organisms (Kim *et al*., 2018). *SIRT2* has been shown to play a key role in lipogenesis (Perrini *et al*., 2020) and the expression of *SIRT2* is negatively correlated with adipogenesis in the 3T3-L1 cell line. Feeding and seasonal environmental changes will both alter the expression of *SIRT2* in mammalian tissue (Jing *et al*., 2007).

MicroRNAs are a class of single-stranded, small-molecule, noncoding RNAs that are abundant in a variety of plants, animals and microorganisms and can cause the mRNA of the target gene to be degraded or blocked during translation *via* binding to the 3′-untranslated region (3′-UTR) of the target gene (Bartel, 2009). MicroRNAs are associated with the occurrence of a variety of traits and diseases (Fernandes *et al*., 2018). Previous studies of *miR-212* have mainly concerned its positive effect on inhibiting numerous types of cancer (Wanet *et al*., 2012; Lin *et al*., 2016) as well as regulation of the nervous system (Xie *et al*., 2018; Aten *et al*., 2019). However, the functional role of *miR-212* in mammary epithelial cells is not known, and there has been little research on the regulation of lipogenesis by *miR-212*.

This study elucidated the effect of *miR-212* on the regulation of lipogenesis in a MAC-T cell line by targeting *SIRT2*; the results contribute to an understanding of the regulation of lipogenesis in bovine mammary glands.

Materials and methods

Ethical approval was not sought since the research reported here did not include any animal studies.

Bioinformatics screening of miRNAs targeting the SIRT2 gene in dairy cows

Two kinds of online software, TargetScan (http://www.targetscan.org/vert_72/) and miRBase (http://www.mirbase.org/), were used to predict miRNAs which have the targeted regulatory effect on the *SIRT2* gene, and the predicted results are shown in Fig. S1 of the online Supplementary File.

Cells used in this study: MAC-T cells and HEK 293T cells

The bovine mammary epithelial (MAC-T) cell line is a bovine mammary epithelial cell line and HEK 293T is a human embryonic kidney cell line which is a model cell line that is

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very suitable for luciferase reporter gene validation experiments because of the high efficiency of plasmid transfection (Fanunza *et al*., 2018). Both exhibit good viability and a short proliferative cycle, making them ideal for culturing in the laboratory. In this study, the MAC-T cells and HEK 293T cells were a gift of College of Veterinary Medicine, Yangzhou University. The method of cell culture is detailed in the online Supplementary File.

Overexpression of and interference by miR-212, miR-375 and miR-655 in MAC-T cells

MiRNA mimics are substances that mimic endogenous miRNAs in living organisms and which are produced by chemical synthesis to enhance the function of endogenous miRNAs. An miRNA inhibitor is a chemically modified inhibitor that specifically targets specific miRNAs in cells. Based on the bovine *miR-212*, *miR-375* and *miR-655* sequences provided in the miRBase database, each miRNA as well as mimics and inhibitors for each were designed by Gene Pharma (Suzhou, China). The sequences are given in the online Supplementary File.

The medium used for transfection was Opti-MEM (Gibco, cat: 31985088) mixed with nothing and the transfection reagent was Lipofectamine™ 2000 (Invitrogen, cat: 11668019). The transfection concentrations of miRNA mimics and inhibitor were 100 and 200 nM. Four microliters of Lipofectamine™ 2000 and one milliliter Opti-MEM medium were placed in each well of a 6-well plate and the transfection process was performed according to the instruction manual of Lipofectamine™ 2000. The MAC-T cells were plated in the 6 wells for 24 h and cultivated in complete DMEM/F-12 medium mixed with 10% FBS at 37°C, 5% CO_2. When the cells occupied over 70–80% of the plate, we started the transfection step and changed the medium to Opti-MEM and after 6 h transfection, we changed the Opti-MEM medium to complete DMEM/F-12 medium mixed with 10% FBS and cultivated the cell for 48 h at 37°C, 5% CO_2.

Extraction and purification of total RNA

The total RNA in the Mac-T cells was extracted by TRIzol Reagent (Invitrogen) following with the manufacturer's protocol. Spectrophotometry (Nanodrop ND-1000, Thermo Fisher, USA) and agarose gel electrophoresis were used to ensure the quantity and quality of the RNA. The mRNA and miRNA in the total RNA were reverse-transcribed into cDNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, cat: RR047A) and a Mir-X™ miRNA First-Strand Synthesis Kit (Takara, cat: 638313).

RT-qPCR analysis

According to the *miR-212*, *miR-375* and *miR-655* sequences provided in the miRBase database, upstream primers for fluorescent quantitative PCR were designed. Details of these primers are provided in the online Supplementary File. Based on the user manual of Mir-X™ miRNA First-Strand Synthesis Kit, the RT-qPCR assays of miRNAs were performed with a 25 µl reaction system consisting of 9 µl of ddH_2O, 12.5 µl of SYBR Advantage Premix (2×), 0.5 µl of ROX Dye (50×), 0.5 µl upstream and downstream primers each and 2.0 µl of cDNA. PCR conditions for miRNAs: Denaturation at 95°C for 10 s, (95°C for 5 s and 60°C for 20 s) × 40 Cycles, then dissociation curve at 95°C for 60 s, 55°C for30 s and 95°C for 30 s.

The forward primer of *SIRT2* was 5′-GAAATACCGTCTT CCCTA-3′ and the reverse primer of *SIRT2* was 5′-GATGAA GTAGTGGCAGAT-3′. Based on the user manual of TB Green™ Premix Ex Taq™ II (Takara, cat: RR820A) the RT-qPCR assay of *SIRT2* was performed with a 20 µl reaction system consisting of 6 µl of ddH_2O, 10 µl of TB Green Premix Ex Taq II (2×), 0.4 µl of ROX Dye II (50×), 0.8 µl upstream and downstream primers (10 µM) each and 2.0 µl of cDNA. PCR conditions for *SIRT2*: Pre-denaturation at 95°C for 30 s, (95°C for 3 s and 60°C for 30 s) × 40 Cycles, then 95°C for 3 s, 60°C for 30 s in the melt stage. The expression was normalized to *GAPDH* (F: 5′-GCAAGTTCCACGGCACAG-3′, R: 5′-GGTTCACGCCCATCACAA-3′).

All the primers were synthesized by Sangon Biotech (Shanghai, China) and all real-time PCR reactions, including the controls with no templates, were carried out in a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, USA) in triplicate. The relative expression was calculated using the $2^{-\triangle\triangle Ct}$ method (Mestdagh *et al*., 2009).

Western blot

After 48 h of transfection, cellular total protein was lysed in RIPA buffer (Solarbio, China) mixed with 1% Phenylmethanesulfonyl fluoride (PMSF) (Beyotime, China, cat: ST505). Then the protein was mixed with protein loading buffer and denatured at 100°C for 10 min. 20µg protein was separated by 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Millipore, USA) and probed with the primary monoclonal rabbit anti-*SIRT2* antibody (Abcam, USA, cat: ab67299) and the monoclonal mouse anti-β-*actin* antibody (Proteintech Group, 66009-1-IG, China). Polyclonal goat anti-rabbit HRP-conjugated IgG (Tiangen, China) was used as the secondary antibody. All antibodies were applied according to the manufacturers' instructions. Signals were detected using the chemiluminescent ECL western blot system (Pierce, USA).

Luciferase reporter gene validation

To generate reporter constructs for luciferase assays, the 3′ UTR of *SIRT2* gene, followed by the wild-type *miR-212*, *miR-375* and *miR-655* predicted target sites were flanked by the *SacI* and *XhoI* restriction sites that were incorporated into the amplification primers. The *SIRT2* wild-type gene fragment and the lentiviral vector GP-miRGLO were digested with *SacI* and *XhoI*. DNA T4 ligase was used to ligate the double-digested *SIRT2* wild-type gene fragment and the linearized vector. Mutagenic PCR was used to mutate the *SIRT2* target site sequences from CCAAGG to GGUUCC, AACAAA to UUGUUU, and UGUAUUA to ACAUAAU. The dual-luciferase reporter vector and miRNA mimics were cotransfected into HEK 293T cells to detect whether the miRNAs can combine with the 3′ UTR of *SIRT2*. At 48 h post-transfection the fluorescence intensity of firefly luciferase was measured with the Dual-Glo luciferase assay system according to the manufacturer's instructions (Promega, cat: N1110).

Co-transfection of the siRNA of SIRT2 and miR-212 mimics into MAC-T cell lines

In order to show that *miR-212* regulates lipogenesis by targeting only *SIRT2* and no other genes, the siRNA of *SIRT2* and

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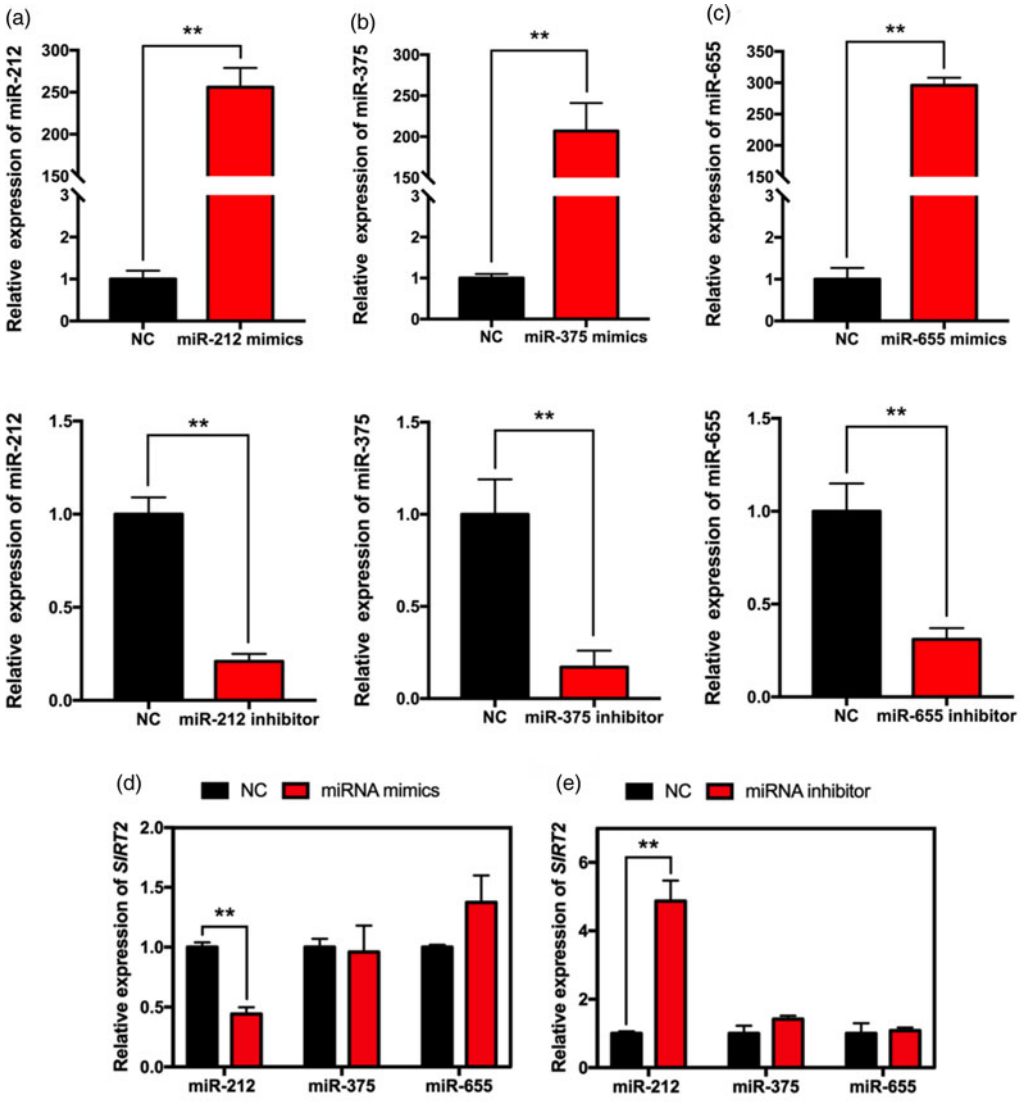

Fig. 1. The relative expression (a) miRNA-212, (b) miRNA-375, and (c) miRNA-655 after transfecting the mimics and inhibitors and the relative expression of *SIRT2* after transfecting the (e) mimics and (f) inhibitors of miRNA-212, miRNA-375 and miRNA-655. NC, negative control. Values are presented as average ± standard deviation. $^{*}P < 0.05$ and $^{**}P < 0.01$.

miR-212 mimics were co-transfected into MAC-T cell lines. The siRNA of *SIRT2* was synthesized by GenePharma (Suzhou, China) and comprised these primers:

F: 5′-GCAUGGACUUUGACUCCAATT-3′
R: 5′-UUGGAGUCAAAGUCCAUGCTT-3′

The transfection concentrations of the *miR-212* mimics and siRNA were 100 nM respectively. Transfection reagents (4 µl) were added to each well in a 12-well plate. The transfection process was same as previously described.

Determining the expression levels of fatty acid synthesis related genes

Following co-transfection with *miR-212* mimics and siRNA for 48 h, total RNA was obtained from the MAC-T cell and reverse-transcribed into cDNA by the PrimeScript™ RT Reagent Kit with gDNA Eraser, then the RT-qPCR assays of fatty acid synthesis related genes were performed using the same method as previously described for *SIRT2*. Primer details are in the Supplementary File. Primers were synthesized by Shanghai Shenggong. Detection employed a Bio-Rad CFX96 real-time PCR detection system. The expression was normalized to *GAPDH*. All the real-time PCR reactions, including the controls with no templates, were carried out in a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, USA) in triplicate. The relative expression was calculated using the $2^{-\triangle\triangle Ct}$ method.

Cellular TAG content assay

Lipofectamine™ 2000 was transfected into the MAC-T cells with the *miR-212* mimic. After 48 h of cell incubation, the cells were harvested with a lysis buffer (50 mmol/l Tris-HCL, pH 7.4, 150 mmol/NaCl, 1% Triton X-100). A triglyceride kit (Sigma-Aldrich, USA, cat: MAK040) and an atomic absorbance spectrophotometer (Perkin Elmer, USA) were used to measure the TAG (triglyceride) content according to the manufacturer's

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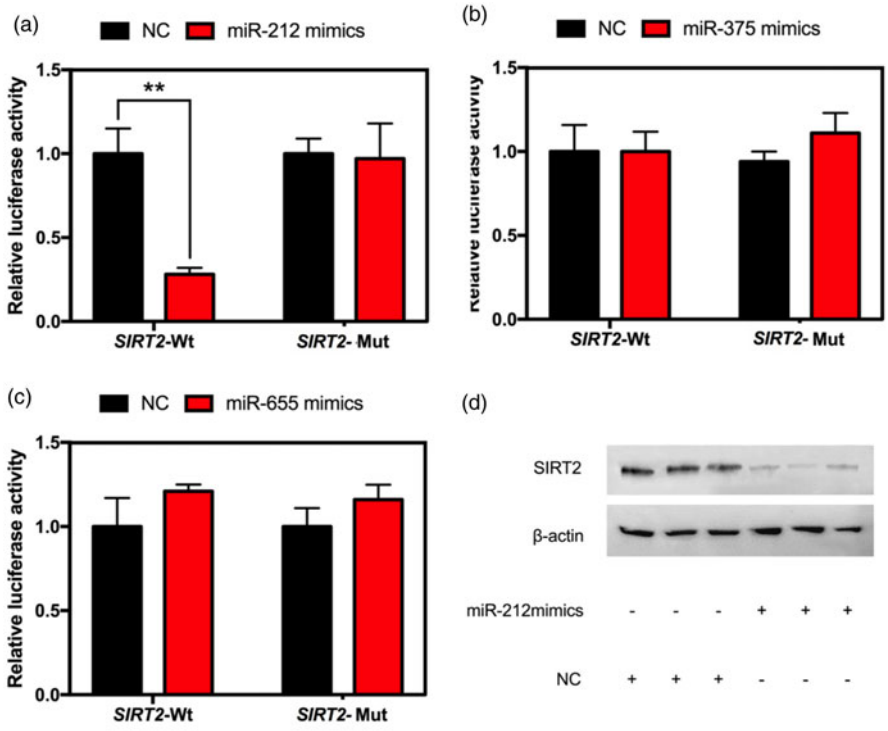

Fig. 2. Validation of (a) miRNA-212, (b) miRNA-375 and (c) miRNA-655 targeting *SIRT2*. (d)Western blot analyses of the expression of β-actin and *SIRT2* protein in cells with miR-212 mimics. NC, negative control. Values are presented as means ± standard errors; $^{*}P < 0.05$ and $^{**}P < 0.01$.

instructions. The values obtained were calibrated to the total protein content, which was measured with a BCA protein assay kit (Thermo Fisher, USA).

Statistical analysis

Statistical analyses were performed using the SPSS 18.0 statistics software package. Data are presented as the mean ± SD (standard deviation) of triplicate values. Significant differences between the groups were determined using a one-way analysis of variance (ANOVA) and two-way ANOVA with $P < 0.05$ (*) and $P < 0.01$ (**) indicating significant differences.

Data availability statement

The datasets generated during and/or analyzed in this study are available from the corresponding author on reasonable request.

Results

The transfection of mimics and inhibitors was successful

The agarose gel electrophoresis results of RNA extraction from MAC-T cells are shown in Fig. S2 of the online Supplementary File. The results of real-time PCR of miR-212, miR-375 and miR-655 after transfection are shown in Fig. 1(a–c).

There are three distinct bands in the agarose gel electrophoresis image which means that the quality of the RNA extraction is good and can be used for subsequent experiments. The expression of the miRNAs was significantly upregulated and the levels were from two hundred to three hundred times higher than the negative control groups after transfection of the mimics. In confirmation, after transfection of the respective inhibitor of the three miRNAs the expression of the miRNAs was significantly downregulated and the levels were from ten to twenty five times lower than the negative control groups.

MicroRNA-212 interferes with the expression of the SIRT2 gene

The expression of *SIRT2* after the mimics and inhibitors of miR-212, miR-375 and miR-655 were transfected into cells is shown in Fig. 1(d–e). Expression was significantly downregulated to roughly half of the negative control group after transfection with the miR-212 mimics and was significantly upregulated to approximately five times higher than the negative control group after transfection with the miR-212 inhibitor. For miR-375 and miR-655, expression of the *SIRT2* gene did not change significantly after transfection compared with negative control, which indicates that miR-212 inhibits the *SIRT2* gene and miR-375 and miR-655 have no direct effect on the *SIRT2* gene in the bovine mammary epithelial cell line.

The results from the validation of miR-212, miR-375, and miR-655 targeting *SIRT2* are shown in Fig. 2(a–c). After co-transfection with the wild-type vectors of the bta-miR-212 and bta-miR-212 mimics, the expression of luciferase was significantly decreased to 28.0 ± 2.9 percent of the negative control group. There was no significant difference in the expression of luciferase compared with the negative control ($P > 0.05$) after co-transfection with the *SIRT2* mutant vector plasmid and bta-miR-212. The expression of luciferase activity was not significantly different after co-transfection with either the wild-type vectors of the bta-miR-375 and bta-miR-375 mimics, the mutant vector plasmid and bta-miR-375 mimics, the wild-type vectors of the bta-miR-655 and bta-miR-655 mimics or the mutant vector plasmid and bta-miR-655 mimics.

After the transfection of miR-212 mimics into MAC-T cells, a change in the *SIRT2* protein relative to the negative control group was detected using a western blot. As shown in Fig. 2(d), the band

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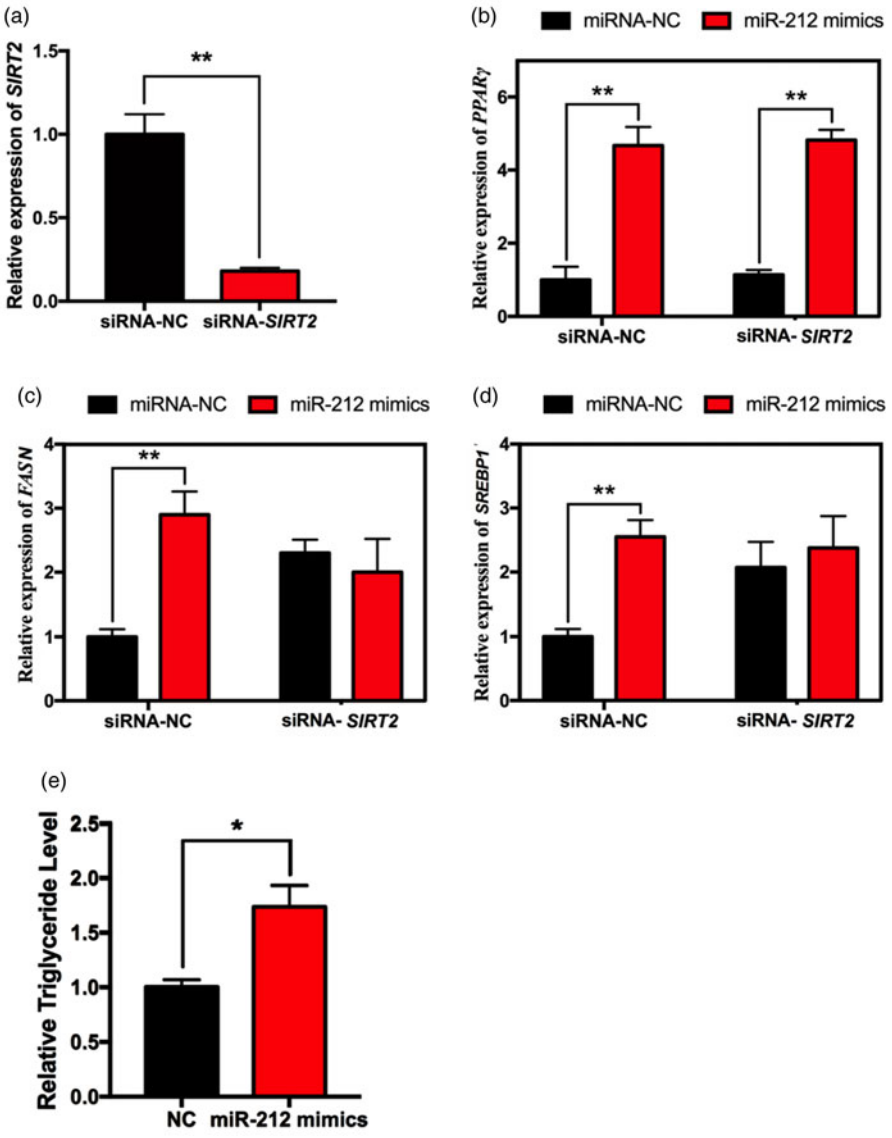

Fig. 3. The expression of *PPARγ*, *FASN* and *SREBP1* after cotransferring siRNA and miR-212 mimics and the triglyceride level in MAC-T after transfecting with miR-212 mimics. (a) The expression of *SIRT2* after transfection with siRNA; (b) The expression of *PPARγ* after co-transfection; (c) The expression of *FASN* after co-transfection; (d) The expression of *SREBP1* after co-transfection; (e) The triglyceride level in MAC-T. NC, negative control. Values are presented as means ± standard errors; $^{*}P < 0.05$ and $^{**}P < 0.01$.

corresponding to the miR-212 mimic group was significantly thinner and lighter than that of the negative control.

MicroRNA-212 targets SIRT2 to promote lipogenesis

The results of the expression of *SIRT2* after transfecting siRNA and the expression of fatty acid synthesis related genes after co-transfection with siRNA of *SIRT2* and *miR-212* mimics are shown in Fig. 3(a–d). The result of the TAG analysis is shown in Fig. 3(e). Transfection with *miR-212* significantly increased the expression of *FASN*, *SREBP1* and *PPARγ* (levels 2.90 ± 0.12, 2.55 ± 0.36 and 4.67 ± 0.51 times higher than the negative control groups, respectively). After co-transfecting with the siRNA and *miR-212* mimics and transfecting with only siRNA of *SIRT2*, there was no significant differences in the expression of either *FASN* or *SREBP1*. Likewise, the expression of *PPARγ* was not changed significantly when the siRNA of *SIRT2* was transfected alone. The triglyceride level in MAC-T cells was significantly increased after transfecting with *miR-212* mimics.

Discussion

In the present study, we identified three miRNAs (*miR-212*, *miR-375* and *miR-655)* which may have a regulatory relationship with *SIRT2* gene by two online software tools. Then we experimentally verified the relative functions of the three miRNAs and the *SIRT2* gene in MAC-T cells. The RT-qPCR, western blot and dual-luciferase reporter genes demonstrated that *SIRT2* is the target gene of *miR-212*, but not of *miR-375* or *miR-655*. The expression changes of *PPARγ*, *FASN* and *SREBP1* after co-transfection with *miR-212* mimics and siRNA as well as the TAG determination experiment showed that *miR-212* promotes lipogenesis in MAC-T by targeting *SIRT2*.

MicroRNA can down-regulate the target gene by inhibiting the translation of mRNA according to the principle of complementary base pairing. The purpose of understanding the relationship is to determine the target site (Friedman *et al.*, 2009), therefore, predicting the miRNA target gene is a pivotal step when studying the regulation of biological processes by miRNAs. The luciferase reporter assay is one of the most common approaches used to validate a direct miRNA target site (Campos-Melo *et al.*, 2014). In

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the present study, it was found that by binding to the *SIRT2* mRNA 3′-UTR, the *miR-212* mimic decreased luciferase activity. It is generally considered that the target region for miRNA is located at the 3′-UTR of the target gene. However, some studies have suggested that the 5′-UTR end of the target gene may also contain the target region (Jagtap and Shivaprasad, 2014). Therefore, if miRNA can regulate the expression of gene but there is no significant change in the result of luciferase reporter gene validation, a possibility might be that the miRNA binds to the 5′-UTR region of gene, which can be determined by redesigning vector according to the 5′-UTR region of gene and conducting the experiment of luciferase reporter gene validation again.

Recent studies suggested that miRNAs play important roles in lipogenesis and triglyceride homeostasis (Wagschal *et al*., 2015). For instance, one of the *miR-212* family members, *miR212-5p*, specifically binds to the 3′ UTR of stearoyl-CoA desaturase-1 (*SCD1*) and fatty acid synthase (*FAS*) and inhibits their activity, whilst overexpression of *miR-212-5p* decreases the protein levels of *SCD1* and *FAS* in vitro and in vivo, and silencing of *miR-212-5p* has the opposite effects in mouse primary hepatocytes (Guo *et al*., 2017). Furthermore, *miR-375* expression was increased in the serum of high fat diet (HFD)-fed mice compared to that in healthy control and the inhibition of *miR-375* can up-regulate the expression of adiponectin (Lei *et al*., 2018). *MiR-375* also promotes lipogenesis in mouse pre-adipocytes *via* regulation of *ERK1/2* signaling upstream of *PPARγ* (Deiuliis, 2016) and *MiR-146b* can promote lipogenesis by the down-regulation of *SIRT1*, which interferes with the *SIRT1-FOXO1* cascade (Ahn *et al*., 2013). In the present study, we find a new pro-adipogenic miRNA, *miR-212*, which can significantly promote lipogenesis by down-regulating *SIRT2*.

We observed that *miR-212* significantly promoted lipogenesis and increased TAG accumulation in MAC-T. Furthermore, we found *miR-212* affected the mRNA expression of lipogenesis related genes, including *PPARγ*, *FASN* and *SREBP1*. It is generally accepted that *PPARγ* is a critical factor for lipogenesis and has relationships with other lipogenic genes (Farmer, 2006; Rosen and MacDougald, 2006; Siersbaek *et al*., 2012). In buffalo, *SREBP1* gene may act on *ERK1/ERK2* signaling pathway to regulate the expression of *PPARγ* gene (Xu *et al*., 2019*a*, 2019*b*) and, as a transcription regulator, *PPARγ* is able to enhance the expression of many genes related to lipogenesis including *FASN* (Lefterova *et al*., 2008; Zhang *et al*., 2016). Moreover, many other studies have indicated that these genes *(PPARγ*, *FASN*, *SREBP1*) are associated with milk fat synthesis in mammary epithelial cells (Yang *et al*., 2017). Our results revealed that *miR-212* has the ability to influence lipogenesis and TAG content by affecting the expression of lipogenesis related genes in MAC-T.

Epigenetic mechanisms including DNA methylation, histone modifications and non-coding RNA regulation are related to the regulation of lipogenesis (Ronn *et al*., 2013). Specifically, histone deacetylases and sirtuins, including SIRT1-7, have been shown to play central roles in lipid metabolism (Lomb *et al*., 2010; Feng *et al*., 2011; Hu *et al*., 2018). Previous research on Canadian Holstein cows also found that the down expression of *SIRT2* gene is associated with milk fat reduction and could affect milk fat synthesis by different pathways (Ran and Eveline, 2017). In this study, we found that reduced *SIRT2* expression promotes lipogenesis in MAC-T cells, the result is in line with previous studies in human visceral adipose stem cells, where *SIRT2* regulates lipogenesis negatively and associates with accumulation of visceral fat in human obesity (Perrini *et al*., 2020).

Many studies have confirmed that *SIRT2* is involved in lipogenesis (Perrini *et al*., 2020; Xu *et al*., 2019*a*, 2019*b*; Lantier *et al*., 2018) and the co-transfection experiment showed that by targeting *SIRT2*, *miR-212* upregulates the mRNA expression of *FASN* and *SREBP1* but not *PPARγ*. These are the first data to confirm that *SIRT2* can directly regulate *FASN* and *SREBP1* negatively, whilst the result of *PPARγ* is at odds with a recent report (Xu *et al*., 2019*a*, 2019*b*) that in bovine ovarian granular cells, *SIRT2* knockdown or treatment with inhibitors produced negative effects on *PPARγ*. It was supposed that the mechanism might be *SIRT2* mediating the transcription factors *FOXO1*. This is a sensible hypothesis because it is well-documented that *SIRT2* acetylates this transcription factor (Armoni *et al*., 2006; Wang and Tong, 2009) and in turn *FOXO1* regulates the transcription of *PPARγ* negatively in adipose tissue by binding to the promoter region to inhibit lipogenesis in cells (Kohan *et al*., 2010). However, in this study, *SIRT2* knockdown had no effect on the expression of *PPARγ*. There are two possibilities. One is that in the Mac-T cell line, *PPARγ* is regulated by *SIRT1* and not by *SIRT2*, whilst *SIRT1* was also targeted by miR-212 (in mice, *SIRT1* was identified as a target gene of miR-212: Li *et al*., 2018) to inhibit the expression of *PPARγ* (Picard *et al*., 2004). The second possibility is that the *miR-212* might have interfered with the process of *SIRT2-FOXO1-PPARγ* signaling. Regrettably, it is not possible to clarify this situation from our data, and further studies will be necessary to assess these hypothesizes.

In conclusion, we have identified *miR-212* as a new class of regulator of lipogenesis in MAC-T cells. We have shown that *miR-375* and *miR-655* cannot target *SIRT2*, which is different from the theoretical prediction. Furthermore, our subsequent results suggest that the functions of the *miR-212-SIRT2* axis could be crucial for fat synthesis in MAC-T cells by regulating lipogenesis related genes.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029920000229.

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