# MicroRNA-331-3p affects preadipocytes' proliferation and differentiation and fatty acid accumulation in Laiwu pigs

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The proliferation and differentiation of preadipocytes are regulated by microRNAs (miRNAs), hormones and other factors. This study aimed to investigate the effects of miR-331-3p on the proliferationand differentiation of preadipocytes in addition to fatty acid metabolism. The data indicated that miR-331-3p is a novel regulator of cellular differentiation. It was observed that miR-331-3p was capable of inhibiting cellular proliferation. Furthermore, miR-331-3p was highly expressed during cellular differentiation andappeared to promote the process. In addition, dual fluorescein analysis showed that dihydrolipoamideS-succinyltransferase (DLST) is a target gene of miR-331-3p, and over-expression of miR-331-3p could regulate the metabolism of fatty acids in the citrate pyruvate cycle by targeting DLST expression. In summary, these findings indicated that miR-331-3p exerts contrasting effects on the processes ofproliferation and differentiation of preadipocytes.

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## Abstract

The proliferation and differentiation of preadipocytes are regulated by microRNAs (miRNAs), hormones and other factors. This study aimed to investigate the effects of miR-331-3p on the proliferation and differentiation of preadipocytes in addition to fatty acid metabolism. The data indicated that miR-331-3p is a novel regulator of cellular differentiation. It was observed that miR-331-3p was capable of inhibiting cellular proliferation. Furthermore, miR-331-3p was highly expressed during cellular differentiation and appeared to promote the process. In addition, dual fluorescein analysis showed that dihydrolipoamide S-succinyltransferase (DLST) is a target gene of miR-331-3p, and over-expression of miR-331-3p could regulate the metabolism of fatty acids in the citrate pyruvate cycle by targeting DLST expression. In summary, these findings indicated that miR-331-3p exerts contrasting effects on the processes of proliferation and differentiation of preadipocytes.

Keywords: miR-331-3p, Laiwu pig, Yorkshire pig, gene expression, dual luciferase analyze

## INTRODUCTION

The Laiwu pig is a Chinese breed with many characteristics that make it favorable for the

commercial market. The meat has a high level of intramuscular fat, averaging approximately 10.32%. This is significantly higher than is observed in other commercial breeds, including Yorkshire pigs (2% intramuscular fat) as well as many others that average around 5% intramuscular fat. This high fat content results in its rich aroma. Although some studies have been carried out on the intramuscular fat deposition of Laiwu pigs (*Cui et al., 2016; Wang et al., 2017; Chen et al., 2018*), the actual mechanism remains elusive.

The amount of fat deposited in an animal depends on body's balance of synthesis and catabolism rates. Mammalian adipose tissue is mainly deposited in the subcutaneous, visceral, intermuscular and intramuscular tissues. The deposition of intramuscular fat takes place later in development than in other parts of the body. In fact, intramuscular fat is typically the last fat tissue to be deposited in livestock. The proliferation and differentiation of intramuscular precursor fat cells can affect both the number and volume of cells (*Margawati et al., 2012*), directly affecting the internal fat content, and therefore the meat quality. For example, intramuscular fat is positively correlated with meat traits such as flavor, tenderness, juiciness and marbling (*Cho et al., 2011*).

The essence of intramuscular fat formation is the process of differentiation, proliferation, and growth of fat cells in muscle tissues. Factors affecting the differentiation and growth of fat cells may also affect the formation of intramuscular fat. Precursor cells, which have the ability to proliferate and differentiate into adipocytes *in vivo*, have become an important model helping to complete the current understanding of adipose tissue formation and proliferation. Recent studies have shown that animal fat deposition is the result of not only an increase in the number of fat cells, but also an increase in the volume and accumulation of lipid droplets. It has been reported that the proliferation, differentiation and fat deposition of precursor fat cells are regulated by various factors (*Mannen et al., 2011*). In recent years, many studies have shown that microRNAs (miRNA) plays an important role in the regulation of fat formation (*Peng et al., 2014; Xie, Bing & Lodish., 2009*). With the development of high-throughput sequencing technology, an increasing number of miRNAs have been discovered to be involved in the fat metabolism

pathway, thus promoting the study of miRNAs and their target genes involved in adipose tissue function. *Xie* et al. (2011) used the Illumina sequencing technology to analyze differential expression of miRNAs in the livers of Tongcheng and Yorkshire pigs. They were able to identify 58 differentially expressed miRNAs. Furthermore, high throughput sequencing was employed to analyze subcutaneous fat of 7 and 240 day-old Rongchang pigs. A total of 93 up-regulated and 33 down-regulated miRNAs were discovered at 240 days of age (*Li et al., 2011*). Similarly, *Chen* et al. (2012) also identified 9 differentially expressed miRNAs in Meishan pig backfat, indicating that miRNAs may regulate fat deposition in pigs.

MicroRNAs mainly interact with the 3'UTR of the target gene, which often leads to degradation of the target gene mRNA or inhibition of translation. In other words, they exert post-transcriptional regulation of target genes, thereby controlling many biological processes such as cell proliferation, differentiation, apoptosis and metabolism. Currently, numerous studies have reported that miR-331-3p plays an important role in the proliferation and differentiation of cancer cells, as well as the occurrence and development of cancer (*Epis et al., 2009; Epis et al., 2011; Rui-Min et al., 2014*). Furthermore, miR-331-3p is able to directly target the cell cycle-associated gene, E2F1. Up-regulation of miR-331-3p has the ability to inhibit the growth and clonal formation of gastric cancer cells (*Guo et al., 2010*). The expression of microRNA-331-3p in prostatic tissue is low. Transfection of microRNA-331-3p can inhibit the expression of ERBB-2 gene and inhibit the downstream PI3/Akt sex hormone receptor signaling pathway. It can inhibit the expression of PSA by reducing the activity of androgen to stimulate the promoter of prostate specific antigen (PSA). It is also found that the expression of microRNA-331-3p may be specific in the nervous system, (*Epis et al., 2014; Epis et al., 2012; Zhao, Sui & Zheng, 2016*). In addition, miR-331-3p has also been used as a marker for detection of liver cancer in serum (*Chen et al., 2015*). (*Zhang*, et al.) sequencing of subcutaneous adipose tissue (by RNA-seq) of Laiwu and Yorkshire pigs showed that 17 up-regulated and 22 down-regulated miRNAs were identified in Laiwu pigs. (*Chen, et al., 2017*) Using both Laiwu and Yorkshire pigs as research subjects, high-throughput sequencing analysis of the longissimus dorsi muscle of the 2 pig breeds was

performed using the RNA-seq technology. Consequently, 19 significantly differentially expressed miRNAs were detected and selected for further screening. Among them, relative to Yorkshire pigs, 7 differentially expressed miRNAs, including miR-331-3p, were up-regulated, whereas 12 were down-regulated in Laiwu pigs. In conclusion, miR-331-3p may influence the formation of adipose tissue as well as intramuscular fat deposition in pigs. In this study, the specific functions of microRNA-331-3p during preadipocyte proliferation, differentiation and fatty acid accumulation in Laiwu pigs were analyzed.

# MATERIALS AND METHODS

## Experimental animals and sample collection

Two breeds of pigs, Laiwu (weight=100kg, n=6) and Yorkshire (weight=100kg, n=6), served as the research subjects in this study. The pigs were raised in different groups at the Laiwu and Yorkshire farm (Laiwu city, Shandong, China), and were raised in the same environment. All groups were also provided identical diets, which met their nutrient requirements as previously described (NRC, 1998). Subsequently, all pigs were ethically sacrificed and processed for market. Tissue samples, including heart, liver, kidney, spleen, lung, muscle (longissimus dorsi), and fat (backfat) were collected and stored at -80. In addition, ear tissue samples from the Laiwu pigs were collected and stored at -20 ℃. All animal experiments were performed in accordance with the Institutional Animal Care, as well as the National (GB 13078–2001 and GB/T 17237–1998) and the Agricultural Standards (NY 5148-2002-NY 5151-2002) of the People's Republic of China.

## Isolation, culture and differentiation of Laiwu porcine primary preadipocytes

Laiwu pigs (<7 days of age) were obtained from the Laiwu and Yorkshire farm. Adipose tissue was isolated from porcine back and neck under sterile conditions. Adipose tissue was digested with 1 mg/mL collagenase 1 (Solarbio, Beijing, China) at 37 ℃ for 60 to 90 min. Then, FBS-supplemented medium was added to terminate the digestion, and all the liquids were sifted through a 100μm Cell Strainer. The culture medium was removed by centrifugation (100xg, 10

min). Subsequently, Red Blood Cell Lysis Buffer (Solarbio, Beijing, China) was added and incubated at room temperature for 10 min, and was centrifuged at 200×g for 10 min. Finally, the primary preadipocytes were resuspended in Dulbecco's modified Eagle's medium DMEM/F12 (Hyclone, Shanghai, China) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Pen-strep, Solarbio, Beijing, China). Identification of preadipocytes was conducted by an immunofluorescence assay. The cells were then divided into 2 groups, an experimental group and a negative control group. The experimental group was stained with a rabbit α-CD44 primary antibody (CD44). Labeling was detected using a secondary antibody (Alexa Fluor 488 Goat anti-rabbit). In the negative control group, the primary antibody (CD44) was replaced with 5% BSA. The nuclei of the cells were stained with DAPI, and the cells were then analyzed and photographed using fluorescent microscope.

Porcine preadipocytes were cultured in growth medium for 3 days to reach approximately 80% confluence (day 0). Then, the growth-arrested cells were cultured in differentiation medium (DMEM/F12, 10% FBS, 1% Pen-strep, 5ug/mL insulin (Solarbio, Beijing, China), 1um/mL dexamethasone (DEX, Solarbio, Beijing, China), and 0.5mM/mL 3-isobutyl-1-methylxanthine (IBMX, Solarbio, Beijing, China)) for 2 days (Day 2).The cells were then treated with maintenance medium, (DMED/F12, 10% FBS, 1% Pen-strep, and 5ug/ml insulin) an additional 6 days. During this period, the maintenance medium was replaced every 2 days.

## Bioinformatics analysis

Target sequences for miR-331-3p were predicted using TargetScan (http://www.TargetScan.org), MiRBase (http://www.mirbase.org) and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/).

## Construction of 3'-UTR luciferase reporter plasmids

Wild-type DLST and SLC25A1 3'-UTR fragments containing the binding site of miR-331-3p were amplified by PCR from total RNA extracted from the backfat of Laiwu pigs. Products of the PCR reactions were directionally sub-cloned into the pGL3 vector down-stream of the firefly luciferase open reading frame (Promega, USA). Mutation of the seed sequence (the miR-331-3p

target site) was accomplished by overlap extension PCR using the general primer and mutagenic primer, which are listed in table 1. First, the wild-type DLST and SLC25A1 3'UTR fragments were used as templates, including miR-331-3p target site. The mutant DLST 3'UTR fragment's up-stream and down-stream regions were respectively amplified with primers F1, R3 and F3, R1 (the same as above). The mutant SLC25A1 3'UTR fragments were amplified using primers F2, R4 and F4, R2. Next, the wild-type and mutant DLST and SLC25A1 3'-UTR fragments were both inserted into the pGL3 vector. Positive clones were identified through bacterial-liquid PCR and were confirmed by sequencing (Sangon Biotech, Shanghai, China). The wild-type and mutant DLST and SLC25A1 3'-UTR vectors were named as pGL3-DLST-wt and pGL3-DLST-mut, respectively. The wild-type and mutant SLC25A1 3'-UTR vectors named as pGL3-SLC25A1-wt and pGL3-SLC25A1-mut, respectively.

## Oil Red O staining and quantification of fatty acid

Preadipocytes were fixed for 1 h in 10% formalin, washed with PBS, stained for 30 min by complete immersion in a working solution of Oil red O. The cells were then washed twice in water, and data was collected by imaging the cells. In order to quantify fatty acids, excess water was evaporated by placing the stained cultures at a temperature of about 32 ℃ for 15 min. Next, 1 ml of isopropyl alcohol was added to the stained culture dish, and the extracted dye was immediately removed. The absorbance of the resulting solution was monitored spectrophotometrically at 510 nm.

## Dual luciferase reporter assay

PK-15 cells (Procell, Wuhan, China) cultured in 24-well plates were transfected using Lipofectamine™ 3000 Transfection Reagent (Thermo, USA) according to the manufacturer's instructions. The transfection mixture for each well contained 250 ng 3'UTR luciferase reporter vector, 12.5 ng of pGL4.74 (Promega, USA) for normalization, and either 500 ng pcDNA3.1(+)-pre-miR-331-3p expression plasmids, miR-331-3p mimics, or negative control mimics (GenePharmn, Shanghai, China). Each group of experiments was repeated at least 3 times. After 48 h of transfection, the luciferase activity was measured using the Dual-luciferase Reporter

Gene Assay System Kit (Promega).

## RNA Extraction and Gene expression Assay Using Quantitative Real-Time PCR (qRT-PCR).

Total RNA was extracted from the cell and tissue samples using the MicroElute Total RNA Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. The quantity of the isolated RNA was determined by measuring the UV260/280 absorbance ratio using a biophotometer (Eppendorf, Germany). For miRNA reverse transcription, Mir-XTM miRNA First-Strand Synthesis Kit (Takara Biotechnology, Dalian, China) was used. Quantitative Real-Time PCR (qRT-PCR) were performed using the SYBR Premix Ex Taq kit (Takara Biotechnology, Dalian, China) on a LightCycler® 96 (Roche). The miRNA specific primers for the qRT-PCR included the forward primer GGTATGGGCCTATCCTAGAA and universal primer used for the reverse primer. The housekeeping gene U6 was used as an internal control.

For miRNA reverse transcription, Mir-XTM miRNA First-Strand Synthesis Kit (Takara Biotechnology, Dalian, China) was used according to the manufacturer's instructions. qRT-PCR was performed using the SYBR Premix Ex Taq kit (Takara Biotechnology, Dalian, China) on a LightCycler® 96 (Roche). Primers used to amplify the miRNAs are listed in Table 2.

## Transfection of porcine preadipocytes

Preadipocytes were seeded in 96-, 24- or 6-well plates. Once cell densities reached 80% confluence, they were transfected with either the mimics, inhibitor or negative control for miR-331-3p (Sangon, Shanghai, China), respectively. The miR-331-3p mimics and inhibitor were transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at concentrations of 3pmol, 15pmol and 75 pmol/well for 4 h. After that, the growth medium was replaced.

## Detection of cell proliferation activity by CCK-8 assay

Preadipocytes were inoculated into 96-well plates at a density of $2 \times 10^3$. Five replicates were used for each group of cells, and 5 blank controls were used to remove background signals. After culture for 24, 48, 72 and 96 h, 10 μL of CCK-8 reagent was added to each well, and

incubated for 2h. The absorbance of each well was determined at 450 nm using a microplate reader (Labsystem). The cell proliferation curve was plotted, and the experiment was repeated in triplicate.

## Flow Cytometry

Preadipocytes were seeded in 96-well plates and incubated for 24h. By this time, the cells reached confluence and were transfected with either miR-331-3p mimics, miR-NC, or miR-331-3p inhibitor (GenePharma, Shanghai, China). Preadipocytes were harvested by digestion with 0.25% trypsin, and washed 3 times with PBS to remove cell debris. The cells were then fixed in cold 70% ethanol overnight, treated with 1 mg/mL RNaseA and propidium iodide (PI) for 30 min at 37 ℃. The cells were then filtered through a 0.75 μm membrane prior to analysis on a FACScan argon laser cytometer (BD, USA).

## Western blotting

Preadipocytes were washed with PBS, lysed using RIPA buffer (Beyotime, Shanghai, China) on ice, and centrifuged at 10,000 × g at 4 ℃. Whole protein concentration for each sample was measured using the BCA Protein Assay Kit (Beyotime, Shanghai, China). All protein samples and SDS-PAGE Sample Loading Buffer (Beyotime, Shanghai, China) were mixed at a ratio of 4 to 1, and boiled for 5 min. Next, all protein samples were diluted to the same concentration. Protein bands were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% Blocking Buffer (Beyotime, Shanghai, China) for 1h. Next, primary antibodies directed against SLC25A1 or actin (Cell Signaling Technology, CST, USA) were incubated on the membranes at 4 ℃ overnight. The membranes were then treated with HRP-conjugated secondary antibodies (Beyotime, Shanghai, China) at room temperature for 2h. Signals were detected using the enhanced chemiluminescence system Reagents (Beyotime, Shanghai, China). All experiments were repeated in triplicate.

## Statistical analysis

All data are presented as means ± standard deviation (SD). Statistical significance was assessed by Student's t-test. Differences were considered statistically significant at $P< 0.05$.

# RESULTS

## Expression analysis of miR-331-3p in different tissues of Laiwu and Yorkshire pigs.

To investigate the differences in miR-331-3p expression between Laiwu and Yorkshire pigs, RNA was extracted from various tissues, and tested the expression levels by qRT-PCR (Fig.1A, B). Significant differences were observed between the 2 breeds. In liver, muscle and backfat of Laiwu pigs, miR-331-3p expression was greater than that of Yorkshire pigs ($P<0.05$). However, the opposite was observed in spleen, lung and kidney ($P<0.05$). Furthermore, the different expression in the longissimus dorsi between the 2 breeds corroborated the results obtained by RNA-Seq. Compared with other tissues, the expression of miR-331-3p in muscle was the lowest, but was the significantly up-regulated in the liver of Laiwu pigs. The highest expression level observed was in the spleens of Yorkshire pigs. Among the results, expression tendency of miR-331-3p in muscle and backfat conformed the observations from the high throughput sequencing.

Through a biological analysis website, 2 candidate target genes of miR-331-3p were predicted (DLST and SLC25A1). Expression levels of the 2 genes were assessed in various tissues between the 2 breeds of pigs by qRT-PCR. Compared with Yorkshire pigs (Fig.1C), DLST transcription was lowest in liver, spleen, muscle and backfat. The opposite was observed in the kidneys for DLST ($P<0.05$). Expression of SLC25A1 (Fig.1D) relative to that of Yorkshire pigs, was most highly transcribed in muscle and backfat. In contrast, DLST expression in the liver and kidney were reversed ($P<0.05$).

## miR-331-3p suppressed Proliferation of Porcine Preadipocytes

Primary preadipocytes from Laiwu pigs were isolated and photographed on days 3 (Fig.2Aa) and 8 (Fig.2Ab). Pictures Fig.2A, C, and E, and Fig.2A, D, F were photographed in the same

field It was observed that the preadipocyte purity achieved was 90%, which satisfies the needs of the experiment.

Preadipocyte proliferation and differentiation form the basis of adipogenesis. To elucidate whether miR-331-3p regulates intramuscular adipogenesis, the effects of miR-331-3p on preadipocyte proliferation were first explored. This was accomplished by transiently transfecting preadipocytes with miR-331-3p mimicss, miR-NC, or miR-331-3p inhibitor. Cellular proliferation was detected by the addition of the dye, Cell Counting kit 8(CCK-8). As is presented in Fig.3A, miRNA-331-3p expression significantly suppressed cell proliferation at 24h, 48h and 72h ($P<0.05$). In contrast, the miR-331-3p inhibitor significantly suppressed cell proliferation at 48h and 72h. To verify the accuracy of this result, a series of marker genes were examined (Fig.3B), including CDK2, CDK3, CDK4 and Cyclin B. These genes were significantly down-regulated in the group receiving the miR-331-3p mimics, whereas CDK2 and CDK4 were significantly down-regulated in group receiving the miR-331-3p inhibitor. The mRNA expression of CDKN1A was significantly up-regulated after transfection of miR-331-3p mimicss.

The combined analyses of the samples by CCK-8, qRT-PCR, and flow cytometry provided insights into the function of miR-331-3p in preadipocytes proliferation.

Moreover, the cell cycle distribution in miR-331-3p transfected preadipocytes was investigated. In comparison with miR-NC group, the miR-331-3p treated group prominently increased the number of cells in G0/G1 phase while decreasing the number in S phase, both of which were significant ($P < 0.05$, Fig. 3D). Taken together, these data indicate that transfection with miR-331-3p could effectively increase the proliferation of adipocytes.

## miR-331-3p Promoted differentiation of Preadipocytes

To investigate the relationship between the expression of miR-331-3p and preadipocyte differentiation, preadipocytes were transiently transfected with miRNA-331-3p mimicss, negative control (NC), or inhibitor prior to differentiation. Two days later, complete medium was replaced with differentiation induction medium. This was considered to be day 0. Expression

levels of MiR-331-3p were measured by qRT-PCR on days 0, 2, 4, 6, and 8. As is presented in (Fig.4A), expression of miR-331-3p was conspicuously up-regulated on days 0 and 2. Expression was conspicuously down-regulated on days 4 through 8. Expression of miRNA-331-3p in the mimics treated group was greater than that of the NC group ($P< 0.05$), which was higher than the inhibitor group ($P< 0.05$). In addition, expression of a marker gene for differentiation, PPARγ, was detected (Fig.4B). The expression level of PPARγ was up-regulated in the miR-331-3p mimics group relative to the miR-NC and miR-331-3p inhibitor treated groups. Therefore, it could be reasonably hypothesized that miR-331-3p may play the same role in different stages of preadipocyte differentiation.

## The determination of target gene of miR-331-3p

To confirm target gene for miR-331-3p, 2 different bioinformatics software programs were used to predict the target genes. Interestingly, the 2 programs (Targetscan, miRWalk) produced different results. The genes were analyzed using gene ontology and Pathway analysis to confirm the likely targets of miR-331-3p. It was determined that DLST and SLC25A1, were both associated with fatty acid metabolism and are components of the citric acid pyruvate cycle. To verify direct interactions between miR-331-3p and the 3'UTR of DLST and SLC25A1, the 3'UTR regions were cloned into a luciferase reporter vector (Fig4C), and were used to transiently transfect PK-15 cells. Luciferase activity was observed after 2 days (Fig.4D). Mimicss of miR-331-3p were co-transfected with wild-type DLST 3'-UTR vector, and luciferase activity was decreased relative to the NC or mut-type DLST 3'-UTR vector groups. However, the groups co-transfected with miRNA-331-3p mimicss and wild-type SLC25A1 3'-UTR vectors, luciferase activity was increased relative to the NC or mut-type SLC25A1 3'-UTR vector groups. It can be concluded that DLST was the direct target gene of miR-331-3p, which may regulate DLST. Meanwhile, SLC25A1 is more likely to be an indirect target of miR-331-3p.

## miR-331-3p regulates DLST and SLC25A1 expression in porcine preadipocytes

To further study the mechanism by which miR-331-3p regulates DLST and SLC25A1 expression, the miR-331-3p, DLST and SLC25A1 expression levels were measured 2 days post-transfection with miR-331-3p mimicss, miR-NC and miR-331-3p inhibitor. The data are presented in Fig. 5A. The expression of miR-331-3p was significantly up-regulated ($P < 0.05$) in the experimental group and the contrary result was observed in the inhibitor group. Meanwhile, the expression of DLST was significantly down-regulated ($P < 0.05$) in the miR-331-3p mimics group and the contrary result was observed in the inhibitor group, but the expression SLC25A1 had opposite tendency with the expression of DLST, therefore the transcription level of SLC25A1 was significantly up-regulated in experimental group ($P <0.05$, Fig.5B).

To further clarify whether the miR-331-3p regulates the expression of SLC25A1, total protein was extracted for Western blot analysis. The protein level of SLC25A1 was up-regulated in the miR-331-3p mimics group compared with the miR-NC group and inhibitor groups ($P<0.05$, Fig. 6A).

## The role of miR-331-3p in adipocyte fatty acid metabolism

Both DLST and SLC25A1 are important components of citrate pyruvate cycle pathway (Fig. 6B). The DLST gene encodes the dihydrolipoamide succinyl transferase, a core enzyme of the mitochondrial α-ketoglutarate dehydrogenase complex (KGDHC). The KGDHC catalyzes α-ketoglutarate to form succinyl coenzyme A. The citrate pyruvate cycle not only results in the synthesis acetyl-CoA, but it also functions to transport it from the mitochondrial to the cell matrix. This is the only source of acetyl-CoA, which is indispensable for all fatty acid synthesis. To further study the mechanism of action, mRNA expression of all known genes involved in the citrate pyruvate cycle were analyzed, the results of which are presented in Fig.5. Citrate lyase (CS), SLC25A1 and ATP citrate lyase (ACLY) were up-regulated in the miR-331-3p mimics treated group relative to the NC and inhibitor treated groups ($P<0.05$). However, DLST, dihydrolipoamide dehydrogenase (DLD), succinate dehydrogenase complex flavoprotein subunit A (SDHA), fumarate hydratase (FH), malic enzyme 1 (ME1), pyruvate carboxylase (PC) and malate dehydrogenase 1 (MDH1) were all down-regulated in the miR-331-3p mimics treated

group ($P< 0.05$)(Fig.5C).

Preadipocytes were transfected with either miR-331-3p mimics, miR-331-3p inhibitor, or miR-NC. Then the preadipocytes were stained with oil red O (Fig.5D), and fatty acid contents of the cells were assessed (Fig.5F). It can be clearly seen that the fatty acid content of the miR-331-3p mimics treated group substantially higher than in the other treatment groups ($P< 0.05$).

## DISCUSSION

The formation of adipocytes and their aggregation to form adipose tissue are an important phase in the growth and development of mammals. MiRNAs are a class of non-coding RNAs that regulate gene expression. Studies have shown that miRNAs are involved in the regulation of many biological processes, such as cell proliferation and differentiation, biological metabolism, and adipogenesis (*Alvarezgarcia & Miska, 2005; Krützfeldt & Stoffel, 2006; O'Rourke, Swanson & Harfe, 2006*). MiRNAs have been identified to regulate the biological process of fat formation at the transcriptional stage. For example, miR-143 promotes adipogenesis by acting on the target gene ERK5 (*Esau et al., 2004; Takanabe et al., 2008*), miR-21 promotes adipogenesis by acting on the target genes TGFBR2 and STAT3 (*Kim et al., 2009; Kim et al., 2012*), and miR-519d promotes fat deposition by acting on the target gene PPARα (*Martinelli et al., 2010*). It has also been demonstrated that both miR-27 and miR-130 inhibit adipogenesis by acting on the target gene PPARγ (*Kim et al., 2010; Karbiener et al., 2009; Lee et al.,2011*). Another miRNA that inhibits adipogenesis is miR-224, which exerts the effect by acting on the EGR2 gene (*Peng et al., 2013*). However, there are still many mechanisms of miRNA action, and the role of miRNAs in adipocyte proliferation, differentiation, and lipid metabolism requires further research.

In the present study, miR-331-3p was identified as a novel gene that regulates proliferation, differentiation, and lipid metabolism of porcine adipocyte progenitor cells. First, the organization of miR-331-3p in Laiwu and Yorkshire pigs was analyzed. Expression of this miRNA was observed to be consistent with the previously published sequencing results, which is suggestive of the important role it plays in the regulation of fat formation. The expression of miR-331-3p in tissues was also analyzed. Compared with Yorkshire pigs, expression of miR-331- 3p was

significantly elevated in the liver, the longissimus dorsi muscle, and the backfat of Laiwu pigs. The liver is an important organ for lipid metabolism, and plays an important role in the digestion, absorption, synthesis, decomposition, and transport of lipids. The liver uses acetyl coenzyme A (acetyl-CoA) as a raw material from which it synthesizes fatty acids. The fatty acids synthesized in the liver are bound to apolipoprotein B (ApoB), which is composed of triglycerides, cholesterol, phospholipids and ApoB in the endoplasmic reticulum and Golgi. It is assembled into very low-density lipoprotein (VLDL) prior to being transported to tissues outside the liver (mainly adipose tissue, including skeletal muscle) via the blood circulation system (*Yan et al., 2015; Kamanna, ganji & kashyap, 2013*). Fat is the main tissue of lipid storage. The free fatty acids produced by the hydrolysis of triglycerides in fat can be transported to the tissues through oxidative decomposition and energy supply after being combined with plasma albumin (*Gregoire, Smas & Sul, 1998*). The longissimus dorsi muscle itself contains adipose tissue, which is referred to as intramuscular fat. The intramuscular fat is developed late and is a relatively small deposit. The intramuscular fat mainly refers to the fat deposited among the fibers in the muscle connective tissue and the muscle bundle. Intramuscular fat has certain specificities in lipid metabolism and cell differentiation, which is related to the special location of IMF (*Rajesh et a*l., *2010*). It has been well established that Yorkshire pigs are among the leanest breeds of pigs, and the liver, backfat, and longissimus dorsi muscle are important sites for the proliferation and differentiation of preadipocytes and fatty acid metabolism. Therefore, the high expression of miR-331-3p observed in Laiwu pigs may be due to the fact that miR-331-3p has the ability to orchestrate these processes.

The formation of adipose tissue is a process in which pre-adipocytes grow, proliferate, undergo terminal differentiation to form fat cells, and grow by hypertrophy. In the present study, precursor fat cells from Laiwu pigs was enriched to more than 90% purity. After over-expression of miR-331-3p, a CCK8 assay was used to detect cell growth, the rate of which was observed to have decreased. The result of flow cytometry analysis showed that the proportion of cells in G0/G1 phase increased, with a decrease in the proportion of cells in S phase served as

confirmation of the above result. In order to further elucidate the mechanism of action, qRT-PCR was used to detect several proliferation-related genes. Expression of CDK2, CDK3, CDK4, Cyclin B, and CDKN1A increased significantly after over-expression of miR-331-3p. Cyclin-dependent kinases (CDKs), such as CDK2, CDK3, and CDK4, have been recognized as key regulators of cell growth and proliferation in eukaryotic cells, and are required for G1-S phase transitions in mammalian cells (*Tsai et al., 1993; Sherr & Roberts, 1995*). Cyclin B is required for cells to enter and exit the M phase of the cell cycle (*Ito et al., 2000*). Conversely, over-expression of cellular CDKN1A may be an inhibitor of cell proliferation (*Zhang & Xu, 2018*). Therefore, miR-331-3p may inhibit the cells by affecting the expression of cell cycle-related genes. Taken together, these data indicate that miR-331-3p is likely an inhibitory regulator of the proliferation of porcine adipocyte progenitor cells.

Subsequently, over-expression of miR-331-3p was able to induce differentiation of preadipocytes. Furthermore, miR-331-3p expression was detected at 0, 2, 4, 6, and 8 days of cell differentiation. Trends in expression were increasing initially and then gradually decreasing. The expression of the PPARγ gene also showed the same trend. The PPAR family of transcription factors is important regulators of the differentiation of precursor adipocytes into mature adipocytes. The PPARs family contains 3 subtypes, alpha, beta and gamma. Among them, PPARγ is the most important marker gene in the process of precursor adipocyte differentiation (*Ntambi & Youngcheul, 1989*). The expression of the PPARγ gene was increased significantly on days 2 and 4 after over-expression of miR-331-3p. The expression trend of binding to miR-331-3p is suggestive of a role in the promotion of the differentiation process in preadipocytes.

Genes related to fatty acid metabolism (DLST and SLC25A1) were predicted bioinformatically. Next, these predicted target genes were verified experimentally by a dual luciferase reporter assay. It can be concluded that DLST was the direct target gene of miR-331-3p, which may regulate DLST. Meanwhile, SLC25A1 is more likely to be an indirect target of miR-331-3p. Preadipocytes were transfected with either miR-331-3p mimics, miR-331-3p inhibitor, or miR-331-3p NC. Compared with transfection miR-NC, mRNA expression of DLST

was significantly down-regulated in miR-331-3p mimicss group and up-regulation in miR-331-3p inhibitor group. This indicates that DLST is a target gene of miR-331-3p at the level of mRNA. However, not only the expression of SLC25A1 was significantly up-regulated, but also protein expression was significantly up-regulated in miR-331-3p mimicss group. Simultaneous, the mRNA expression of SLC25A1 was significantly down-regulated, but protein expression was not significantly changed in miR-331-3p inhibitor group. This may be due to the fact that the SLC25A1 gene is not a direct target of miR-331-3p; rather it may regulate expression indirectly by other means. The target gene DLST encodes dihydrolipoyl succinyltransferase, which is a core component of KGDHC, which is the rate-limiting enzyme for the second oxidative decarboxylation of the Krebs cycle (*Kunugi et al., 1998*). The SLC25A1 gene encodes a mitochondrial citrate transporter (CTP) that regulates citric acid transport bidirectionally between the mitochondria and cytoplasm (*Nota et al., 2013*). Both DLST and SLC25A1 are present in the citrate pyruvate cycle pathway, which transports the acetyl-COA necessary for the synthesis of fatty acids from the mitochondria into the cytoplasm. According to the quantitative fluorescence assay of the DLST, SLC25A1 genes performed here indicated that fatty acid synthesis was increased after over-expression of miR-331-3p. Oil red O staining and lipid droplet quantification showed that fatty acid synthesis indeed increased after over-expression of miR-331-3p. Transfecting the cells with an inhibitor of miR-331-3p produced a contrary result, which in turn corroborates the hypothesis that miR-331-3p can promote fatty acid synthesis through modulation of the citrate pyruvate cycle.

## CONCLUSIONS

In summary, miR-331-3p functions as a regulator of the proliferation and differentiation of adipocytes and fatty acid metabolism. Over-expression of miR-331-3p can inhibit cellular proliferation, and was observed to be highly expressed during preadipocyte differentiation. These data indicate that it has a promoting effect on differentiation of preadipocytes. Furthermore, miR-331-3p can also promote the synthesis of fatty acids by regulating the target gene DLST to promote acetyl-CoA translocation from the mitochondria into the cytoplasmic matrix through the

citrate pyruvate cycle.

# ADDITIONALINFORMATIONANDDECLARATIONS

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## Competing Interests

The authors declare there are no competing interests.

## Author Contributions

- Tao Chen performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Lixia Ma and Jinhong Geng assisted in completing the experiment.
- Yongqing Zeng and Wei Chen conceived and designed the experiments.

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## Table 1(on next page)

Table 1

1 Table 1 Primer sequences used for construction of luciferase reporter plasmids.

| Target | Primer sequence (5′–3′) |
|---|---|
| F1 | AAGGTACCGCCTTTCCTTCATTAG |
| R1 | AACTCGAGAACAATGTGGTAGGAT |
| F2 | AAGGTACCTAAGATACTACGATGT |
| R2 | AACTCGAGTTTATTCTGCCTTGGA |
| F3 | AAGCAC AGCCTGATCG ACCTGGT |
| R3 | ACCAGGTCGATCAGGCT GTGCTT |
| F4 | TCACTGCATTCTGATCGCTCCATGC |
| R4 | GCATGGAGC GATCAGAATG CAGTGA |

2

## Table 2(on next page)

Table 2

Table 2 Primer sequences used for quantitative real-time polymerase chain reaction and gene cloning.

| Gene | GenBank accession no. | Primer sequence (5′–3′) |
|---|---|---|
| DLST | NC_010449.5 | AGCCCCAAAAGCAGAACC |
| | | GGGCAGCAGTGGGTTTTA |
| SLC25A1 | NC_010456.5 | TGACCAGACCTCTTCCAA |
| | | AACGAAGAAGCGGATGGC |
| GAPDH | NC_010447.5 | AGGTCGGAGTGAACGGATTTG |
| | | ACCATGTAGTGGAGGTCAATGAAG |
| CDK2 | NC_010447.5 | AAGATGGACGGAGCTTGTTATCGC |
| | | CTGGCTTGGTCACATCCTGGAAG |
| CDK3 | NC_010454.4 | TCATCCACCGAGACCTGAAGCC |
| | | AGACATCCACAGCCGTCGAGTAG |
| CDK4 | NC_010447.5 | TGAGATGGAGGAGTCTGGAGCAC |
| | | CTCGGAAGGCAGAGATTCGCTTG |
| Cyclin B | NC_010458.4 | GACTGGCTAGTGCAGGTTCAGATG |
| | | ATGGCAGTGACACCAACCAGTTG |
| CDKN1A | NC_010449.5 | CGAGAGCGATGGAACTTCGACTTC |
| | | TCCACATGGTCCTCCTGAGACG |
| PPARγ | NC_010455.5 | AGGACTACCAAAGTGCCATCAAA |
| | | GAGGCTTTATCCCCACAGACAC |
| CS | NC_010447.5 | ACACTCAACTCAGGACGGGT |
| | | TTCCAGGAGGACATTGGGCA |
| ACLY | NC_010454.4 | CTTGATCCGCAAACCTGCCT |
| | | CCGTCACCATCAGGCACATC |
| DLD | NC_010451.4 | GGCGCAGTGCACATTGACTA |
| | | ACCATGCCATCTGTGTCAGC |
| SDHA | NC_010458.4 | CCTGGAGTTCGTGCAGTTCC |
| | | AACCTCTCACCCTGGCTGTT |
| FH | NC_010452.4 | TGGTGCCCAGACTGTGAGAT |
| | | AGCTGCACGCTTCAAGATCC |
| ME1 | NC_010443.5 | TTGCAGCCCTTCGAATCACC |
| | | AGGTGTGCAATCCCTAAGGC |
| PC | NC_010444.4 | AGAACGAGATCCCAGGAGGC |
| | | GACTCAGCCCATTCTGCACC |
| MDH1 | NC_010445.4 | CATGCCAAGAAGGGATGGCA |
| | | CCTTGGGAATGGATGGAGCC |

## Figure 1(on next page)

Figure 1

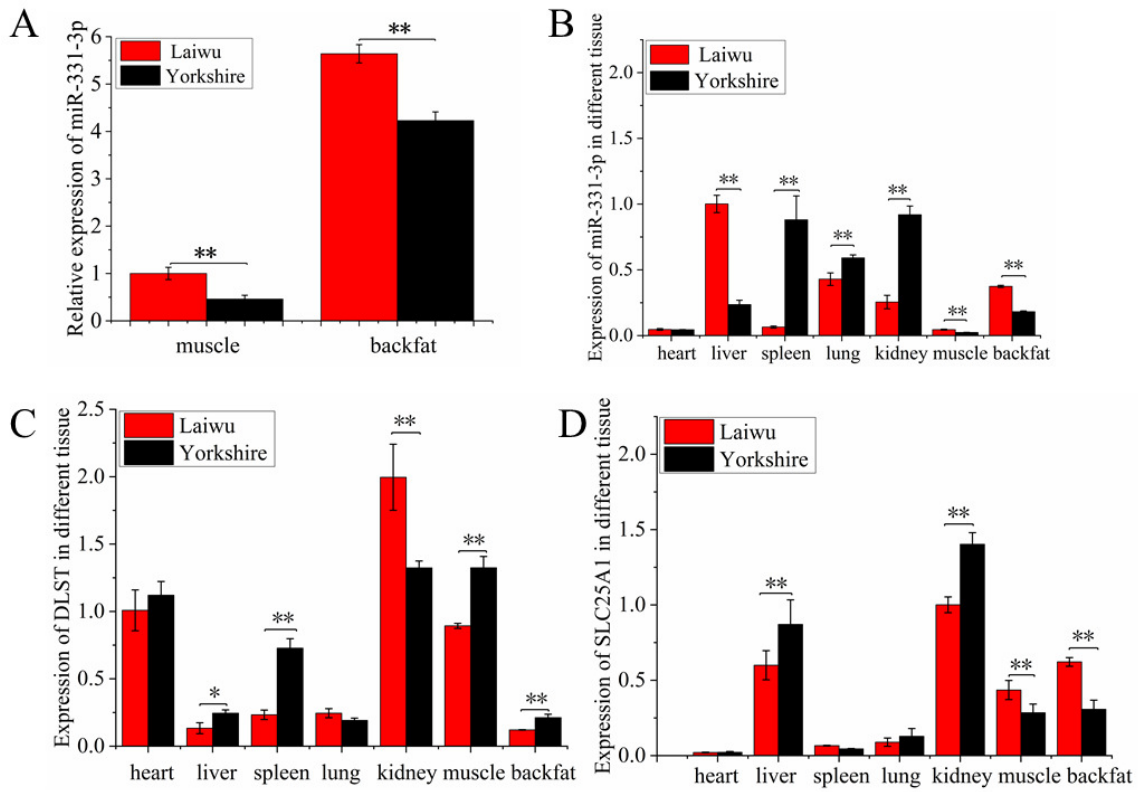


Fig 1 Expression levels of miR-331-3p, DLST and SLC25A1 in Laiwu and Yorkshire. (A) qPCR result of the expression level of miR-331-3p in muscle and backfat. And qPCR result of the expression levels of miR-331-3p (B), DLST (C) and SLC25A1 (D) in different tissues, including heart, liver, spleen, lung, kidney, muscle and backfat. U6 and GAPDH as house-keeping genes were used for the detection of miRNA and mRNA, respectively. (n=3), (*p<0.05; **p<0.01).

## Figure 2(on next page)

Figure 2

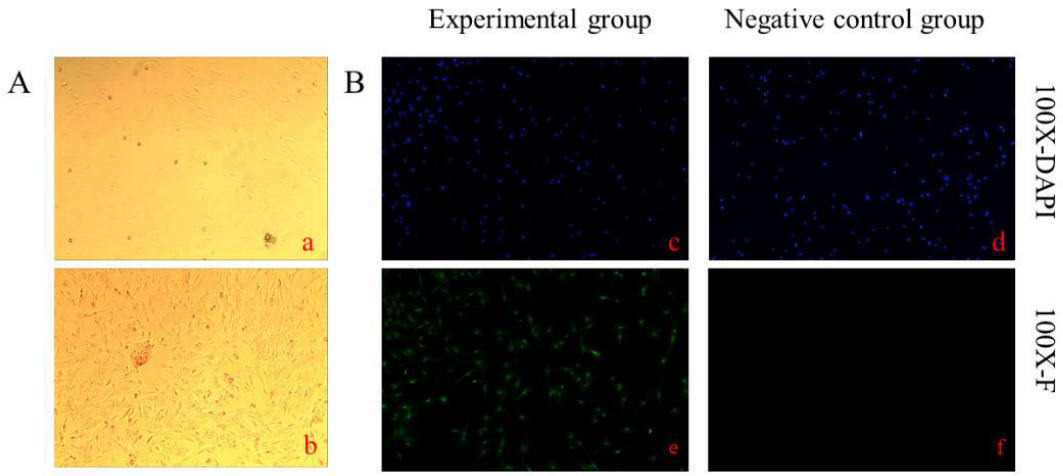


Fig.2 Isolation of primary preadipocytes from Laiwu pigs. (A) preadipocytes were observed to adhere to the dish wall and protruded outward into two or three tentacles, forming a spindle or irregular triangle on Day 3. The cells grew to approximately 90% confluency on Day 8 (40X). (B) Identification of preadipocytes by immunofluorescent imaging.

## Figure 3(on next page)

Figure 3

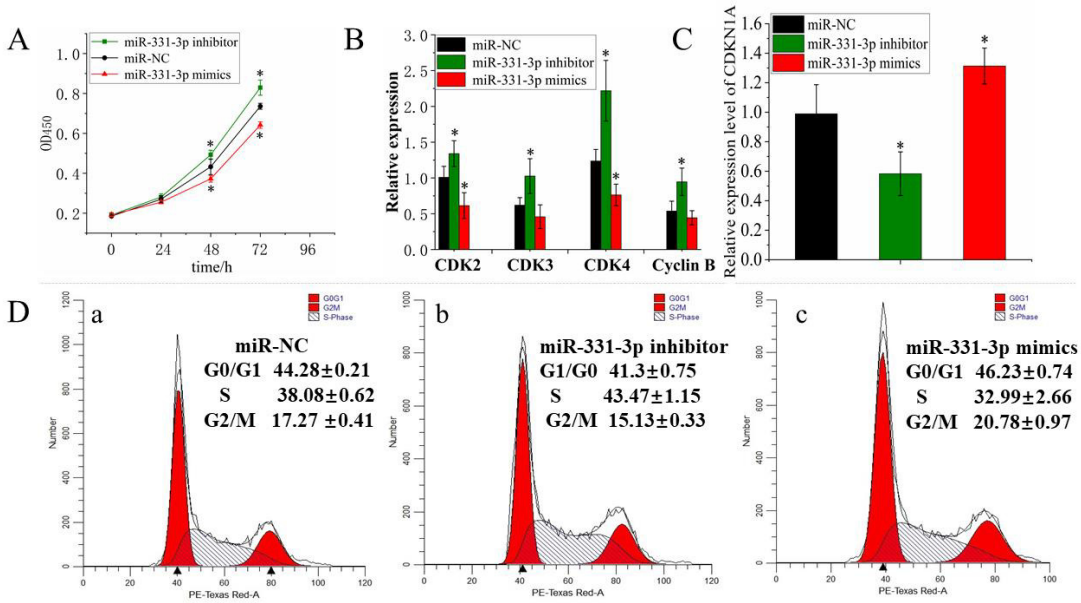


Fig.3 miR-331-3p suppressed proliferation of porcine intramuscular preadipocytes. (A) Cell Counting kit 8 (CCK-8) assays results of cell proliferation (n = 6 per treatment per time point); (B) qPCR results of the relative expression levels of cyclin-dependent kinases 2 (CDK2), Cell cycle protein B (Cyclin B), cyclin-dependent kinases 3 (CDK3), cyclin-dependent kinases 4 (CDK4), and cyclin-dependent kinase inhibitor 1A (p21) (C) when preadipocytes were transfected with mimics, inhibitors, or NC for 2 days (n = 3 per treatment); (D) Results of cell cycle analysis by flow cytometry (n = 3 per treatment). (*p<0.05).

## Figure 4(on next page)

Figure 4

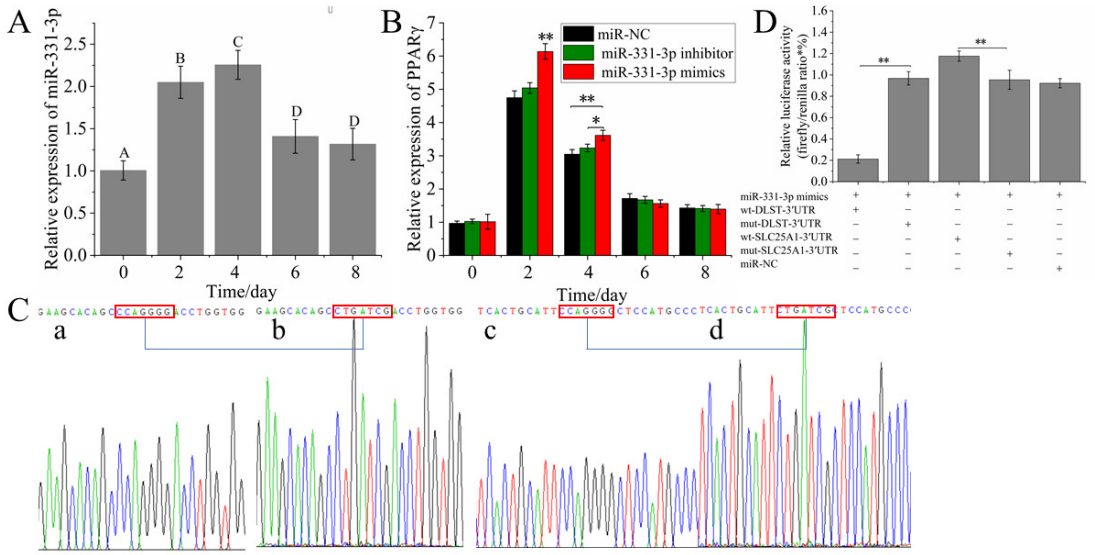


Fig.4 miR-331-3p expression during preadipocyte differentiation and confirmation of its target genes. (A) level of miR-331-3p in preadipocytes differentiation process; (B) miR-331-3p influenced PPARγ expression in preadipocytes differentiation process when preadipocytes were transfected with mimics, inhibitors, or NC; (C) The seed region of DLST and SLC25A1 CCAGGG mutated to CTGATCG using overlapping PCR. (D) Two pmirGLO vector constructs, containing either the DLST-3'UTR (or SLC25A1-3'UTR) or the DLST-3'UTR (or SLC25A1-3'UTR) with a mutation in the miR-331-3p seed region, were transfected into PK-15 cells either alone or in combination with NC or miR-331-3p mimic. Renilla luciferase activity was normalized to firefly luciferase. Data represent means ± SD. (*$p < 0.05$; **$p < 0.01$).

## Figure 5(on next page)

Figure 5

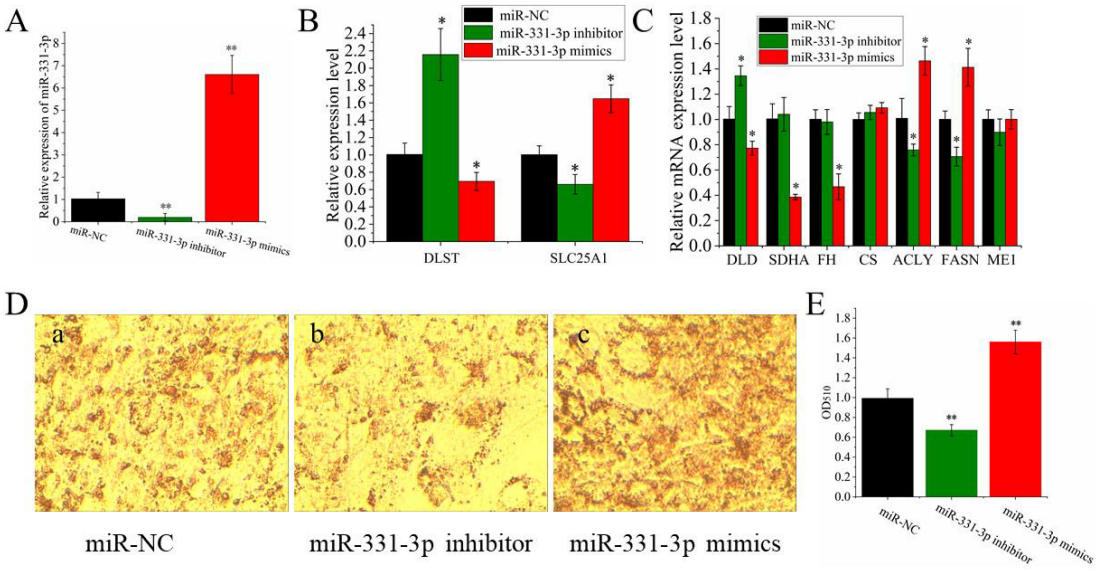


Fig 5 miR-331-3p effected fatty acid metabolism of preadipocytes. Preadipocytes were transfected with mimics, inhibitors, or NC, after differentiation. (A) Expression level of miR-331-3p measured by qRT-PCR. (B) the expression of DLST and SLC25A1 measured by qRT-PCR after transfection of miR-331-3p mimics, miR-NC and miR-331-3p inhibitor. (C) Meanwhile some genes coding enzymes of citrate pyruvate cycle was measured by qRT-PCR. (D) Imaged of cells stained with oil red O after miR-331-3p inhibitor and mimics transfection. (F) Quantification of oil red O staining. Data represent means ± SD. (*$p < 0.05$; **$p < 0.01$).

## Table 3(on next page)

Figure 6

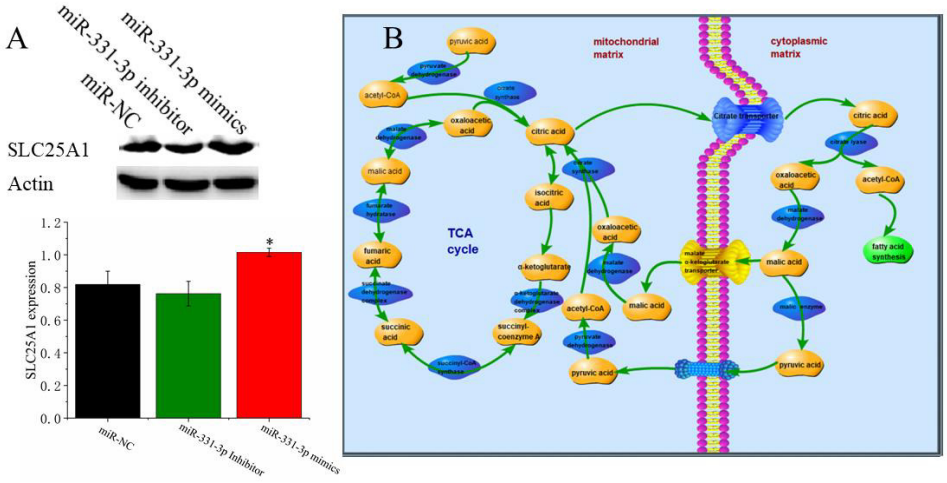


Fig.6 miR-331-3p influenced expression levels of DLST and SLC25A1. (A) SLC25A1 protein expression was influenced when preadipocytes were transfected with mimics, inhibitors, or NC; (B) DLST coding Dihydrolipoamide succinyltransferase and SLC25A1 coding mitochondrial citrate transporter played a critical role in citrate pyruvate cycle.