miRNA-374 Regulates Dexamethasone-induced Differentiation of Primary Cultures of Porcine Adipocytes

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Abstract
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To investigate the effect of glucocorticoid on adipocytes metabolism and miRNAs that may be involved in adipocyte differentiation, primary porcine preadipocytes were treated with 10^{-6} M dexamethasone and RU486 (a glucocorticoid receptor antagonist) for 48 h. PPAR-γ (peroxisome proliferators-activated receptor-γ), and C/EBP-β (CCTTA enhancer binding protein-β) gene and protein expression were measured. The expressions of miRNAs predicted to directly target C/EBP-β were determined, and the functions of the potential miRNAs were verified. The results showed that the triglyceride content in cultured adipocytes increased significantly after 10^{-6} M dexamethasone treatment for 48 h, whereas the cell viability did not differ among the 4 groups (Control: 10^{-6} M dexamethasone; 10^{-6} M RU486: 10^{-6} M dexamethasone + 10^{-6} M RU486) ($p > 0.05$). Cells treated with dexamethasone for 48 h significantly upregulated perilipin and PPAR-γ gene expression, and PPAR-γ protein expression was also significantly increased. However, C/EBP-β mRNA and protein expression levels were significantly decreased. Both miR-374a and miR-374b, targeting the C/EBP-β 3′-UTR (3′-untranslated region), were significantly increased. Dual luciferase activity assay results indicated that miR-374a/b was directly recognised and bound to the 3′-UTR of C/EBP-β and thereby suppressed C/EBP-β gene expression. The present study showed that 10^{-6} M dexamethasone promotes lipid accumulation in primary cultures of porcine preadipocytes. PPAR-γ and C/EBP-β protein abundance showed differences after 48 h dexamethasone treatment; miR-374a/b may be involved in regulating of C/EBP-β expression. These results provide new targets for further regulation of porcine lipid metabolism.

Introduction
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Glucocorticoids are well known as stress hormones that show rapid increases after stress, and it has been confirmed to have critical and complex functions during triglyceride (TG) metabolism. Depending on the physiological state, glucocorticoids have been proposed to be both adipogenic and lipolytic in their actions within adipose tissue [1–5]. Therefore, understanding their effects on the regulation of adipose tissue metabolism is critical in determining the correlation between stress and obesity. Although a number of studies about the influence of glucocorticoids on lipid metabolism has been reported [6–8], the specific mechanism of glucocorticoids on lipid metabolism is still largely unknown. Previous studies have shown that adipogenesis is regulated by various adipogenic transcription factors, such as C/EBPs (CCTTA enhancer binding protein), PPARs (peroxisome proliferators-activated receptor), HSL (hormone sensitive lipase), and FAS (fatty acid synthase), which are expressed as a transcriptional cascade that promotes adipocyte differentiation, ultimately leading to the mature adipocyte phenotype [9]. The PPAR and C/EBP family are considered to be the master regulators of adipogenesis, despite other transcription factors positively or negatively regulate adipogenesis [10]. The PAT protein family is the main protein family expressed on the surface of lipid droplets, and perilipin wraps the surface of lipid droplets in mature fat cells [11]. The degree of adipocyte differentiation can be determined by the expression level of perilipin. How these genes mediate glucocorticoid action on lipid metabolism, and whether miRNAs (microRNAs) can be involved during the process is unclear.

MiRNAs are an emerging class of highly conserved, endogenously expressed noncoding small RNAs (usually 19–25 nucleotides long) that are involved in the post-transcriptional regulation of gene expression by targeting mRNAs, leading to mRNA degradation or translation inhibition [12, 13]. miRNA expression is often tissue specific and developmentally regulated [14]. In recent years, accumulated evidences have shown that miRNAs are aberrantly expressed in fat tissue and play a vital role as a novel class of genes related to adipocyte differentiation and lipid metabolism [15, 16]. miR-335 levels are closely correlated with the expression levels of adipocyte differentiation markers, such as PPAR-γ, aP2 (adipocyte protein 2) and FAS in 3T3-L1 adipocytes [17]. miR-378/378* overexpression in ST2 mesenchymal precursor cells increases the size of lipid droplets due to the increased expression of PPAR-γ2. At present, 306 mature miRNAs have been identified in *Sus scrofa* (www.miR-base.org.com), yet the function of most miRNAs is still unknown. Therefore, in the present study, we treated primary culture of porcine preadipocytes with dexamethasone to investigate the influence of glucocorticoid on adipocyte maturation and identify specific roles of the miRNAs involved in this process. The study will further clarify the regulatory mechanism of glucocorticoid on adipocyte differentiation, and provide a new light for further regulation of porcine lipid metabolism.

Materials and Methods
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Primary culture of porcine preadipocyte
Meishan piglets aged 35 days were killed by exsanguination in a manner approved by the Nanjing Agricultural University Institutional Animal Care and Use Committee. Porcine preadipocytes from 5 piglets were isolated according to published protocols [18, 19] with the following modifications and pooled together. Subcutaneous adipose tissue was collected from the neck and back of the piglets and rinsed with serum-free DMEM/F-12 medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) supplemented with 15 mM NaHCO$_3$, 100 IU/ml penicillin, and 100 IU/ml streptomycin. The tissue mass was cut with scissors into fine pieces and digested with type IV collagenase (GIBCO) (Invitrogen Life Technologies, Carlsbad, CA, USA) (DMEM/F-12 + 20 g/l BSA + 1 g/l type IV collagenase) at 37 °C in a shaking water bath for approximately 1 h. Then, DMEM/F-12 medium (Invitrogen) containing 10 % fetal bovine serum (FBS) (Invitrogen) was added to stop digestion. The solution was filtered through sterile nylon meshes (150 μm pore size, 75 μm pore size, 38 μm pore size, and 23 μm pore size) to remove undigested tissue. The filtrate was centrifuged at 1 000 rpm for 10 min to separate the floating adipocyte cells from the pellet of porcine preadipocytes. The preadipocytes were then incubated with erythrocyte lysis buffer (0.154 M NH$_4$Cl, 10 mM KHCO$_3$, and 0.1 mM EDTA) at room temperature for 10 min [20], followed by centrifugation at 800 rpm for 5 min. The preadipocytes pellet was washed with DMEM/F-12, centrifuged, and resuspended in plating medium (20 % FBS, DMEM/F-12). Finally, the preadipocytes were seeded in culture plates at a density of 3×10^5 cells/cm^2 and cultured at 37 °C in a humidified atmosphere containing 5 % CO$_2$. The medium was changed every second day.

Adipogenic differentiation of preadipocytes
Cultured preadipocytes were maintained in plating medium until 85–90 % confluence. Then, to induce differentiation, the cultures were exposed to medium (without FBS) containing ITS (5 U/ml insulin, 5 μg/ml transferring, and 5 ng/ml selenium; Sigma, St. Louis, MO, USA), 400 μM oleic acid (Sigma), and BSA (Invitrogen) for 48 h, with a 6:1 ratio of oleic acid to BSA. All cells were divided into 4 groups (control; 10^{-6} M dexamethasone; 10^{-6} M RU486; 10^{-6} M dexamethasone and 10^{-6} M RU486). RU486 (Sigma) served as glucocorticoid receptor antagonist. In this experiment, a single dose of dexamethasone (10^{-6} M) and a single treatment period (48 h) were adopted which is based on our preliminary experiment. All preadipocytes were cultured in DMEM/F-12 (Invitrogen) plus l-glutamine, penicillin (100 IU/ml), streptomycin (100 IU/ml), and fungizone (4 μg/ml) at 37 °C with 5 % CO$_2$.

MTT assay
For cell viability studies, preadipocytes were seeded in 96-well culture plates at a density of 10^3/well, and then, 100 μl DMEM/F-12 medium containing 10 % FBS was added to each well. After treatment with 10^{-6} M of dexamethasone for 48 h, the old culture medium was removed, 20 μl of MTT (Keygentec, Nanjing, China) was added to each well of the 96-well assay plate containing 100 μl of fresh culture medium, and the cells were cultured for 4 h at 37 °C in a humidified, 5 % CO$_2$ atmosphere. Afterwards, the absorbance at 490 nm was recorded using a 96-well plate reader. The viable cell number is proportional to the absorbance value.

TG content determination
The intracellular TG content was measured according to the method of Oil Red-O staining extraction. Preadipocytes were seeded in 24-well culture plates until 85–90 % confluence. After treatment with 10^{-6} M of dexamethasone for 48 h, the old culture medium was removed. Cells were first washed with phosphate buffered saline (PBS) for 3 times, fixed with 10 % formalin for 5 min, and changed with fixative for 2 h. After fixation, 60 % isopropanol (1 ml/hole) was added to the plate for 30 s. After removing 60 % isopropanol, Oil Red-O working solution (Sunshinebio, Nanjing, China) was added to the plate for 1 h (1 ml/well). And then, Oil Red-O working solution was removed and 300 μl 100 % isopropanol was added to the plate to extract Oil Red-O. Finally, 100 % isopropanol was collected and the plate was observed under a microscope. A wavelength of 510 nm absorbance was used with a Microplate reader (Synergy BioTek, Vermont, USA), which can reflect the intracellular TG content [21, 22].

RNA extraction and real-time PCR
Total RNA was extracted from homogenised adipose cells using the TRIzol Total RNA Kit (Invitrogen) and subsequently purified with the RNase-Free DNase Set (Promega, Madison, WI, USA) according to the manufacturer's instructions. The total RNA concentration was then quantified by measuring the absorbance at 260 nm in an Eppendorf BioPhotometer (Gene Company Ltd., Shanghai, China). The absorption ratios (260/280 nm) for all the preparations were between 1.8 and 2.0. Two micrograms of total RNA were reverse transcribed in a final volume of 25 μl with M-MLV reverse transcriptase (Promega) and random hexamer primers (Sunshinebio, Nanjing, China). Reverse transcription was performed in a Thermal Cycler PTC0200 (Bio-Rad, Philadelphia, PA, USA).
Real-time PCR was performed in an Mx 3000P (Agilent Technologies, Stratagene, Santa Clara, CA, USA) with specific primers. All

Target	GenBank accession	PCR products (bp)	Primer sequences
Perilipin	AY973170	119	F: 5′-gcctgactttgctggatgg-3′
			R: 5′-cttggtgctggtgtaggtcttct-3′
PPAR-γ	NM138711	210	F:5′-gcccttcaccactgttgatt-3′
			R:5′-gagttggaaggctcttcgtg-3′
C/EBP-β	NM005194	158	F: 5′-gacaagcacagcgacgagta-3′
			R: 5′-agctgctccaccttcttctg-3′
GR	AY779185	108	F: 5′-ccaaactctgccttgtgtgttc-3′
			R: 5′-tgtgctgtccttccactgct-3′
FAS	EF589048	206	F: 5′-gtcctgctgaagcctaactc-3′
			R: 5′-tccttggaaccgtctgtg-3′
18S rRNA	AY265350	122	F: 5′-cccacggaatcgagaaagag-3′
			R: 5′-ttgacggaagggcacca-3′

Table 1   List of gene specific primers used for q-PCR.

microRNA (mature)	Accession	Chromosome	Primer sequence (5′–3′)
miRNA-155	MIMAT0017953	13	uccuacauguuagcauuaaca
miRNA-455	MIMAT0013880	12	ugaggggcagagagcgagacuuu
miRNA-423-5p	MIMAT0013960	1	gcaguccaugggcauauacac
miRNA-374a	MIMAT0013913		uuauaauacaaccugauaagug
miRNA-374b-5p	MIMAT0013915		auauaauacaaccugcuaagug
miRNA-191	MIMAT0013876	13	caacggaaucccaaaagcagcug
miRNA-362	MIMAT0017958		aauccuuggaaccuaggugugagug
E5 (external reference)			gtgacccacgatgtgtattcgc
Common downstream primer			tagagtgagtgtagcgagca
Reverse transcription primer			tagagtgagtgtagcgagcacagaattaatacga ctcactatagg(t)$_{16}$vn

Table 2   The primer sequences of the putative microRNAs for qRT-PCR.

qRT-PCR: quantitative reverse transcription polymerase chain reaction

primers were designed and synthesised by Takara Biotechnology (China; ○ **Table 1**). The results were expressed relative to the number of 18S transcripts used as an internal control. Mock reverse transcription and no template controls (NTC) were used to monitor possible contaminations with genomic DNA. Pooled samples, made by mixing equal cDNA quantities from each sample, were used for optimising the PCR conditions and generating the standard curves for each target gene. The quality of the PCR products was checked by 1.4% agarose gel electrophoresis. In all cases, single bands of the expected size were observed. Melting curve analyses further assessed the specificity of each PCR product.

Bioinformatics method
The miRNA targets predicted by computer-aided algorithms were obtained from miRGen (http://www.diana.pcbi.upenn. edu/miRGen.html) [23], TargetScan (http://www.targetscan. org/vert_42/) [24], PicTar (http://pictar.org/) [25], and miRanda (http://www.microrna.org/microrna/).

miRNA real-time PCR quantification
RT-PCR analysis of miRNA expression was performed in an Mx 3000P (Stratagene) with specific primers (○ **Table 2**). Briefly, total RNA was extracted from adipocytes using TRIZOL Reagent (Invitrogen) and subsequently purified with the RNase-Free DNase Set (Promega) according to the manufacturer's instructions. The treated total RNA (4 μg) was polyadenylated by poly(A) polymerase (PAP) at 37 °C for 1 h in a 20 μl reaction mixture following the manufacturer's directions for the Poly(A) Tailing Kit (AM1350, Ambion, USA) [26]. The tailing reactions contained 4 μg of RNA samples (1 μg/μl), 4 μl of 5×E-PAP buffer, 2 μl of 25 mM MnCl$_2$, 2 μl of 10 mM ATP, 0.8 μl of E-PAP, and the external controls (E1, E2, and E5) at 0.2 pmol each; this reaction solution was then brought up to a 20 μl final volume with nuclease-free

water. After phenol–chloroform extraction and ethanol precipitation, the RNAs were dissolved in DEPC-treated water and cDNAs were synthesised from tailing RNAs using a gene-specific oligo dT-adapter primer (1 μg/μl). Reverse transcriptase reactions contained 2 μg poly-A tailed RNAs and 1 μl of oligo dT-adapter (1 μg/μl). The 10 μl reactions were incubated for 5 min at 70 °C (RT1). The RT2 reactions consisted of the entire RT1 reactions, mixed with 5 μl M-MLV 5×buffer (containing 250 mM pH 8.3 Tris-HCl, 15 mM MgCl$_2$, 375 mM KCl, and 50 mM DTT), 1.25 μl 10 mM dNTP, 1 μl M-MLV RNase (200 U/μl), 0.5 μl RNase inhibitor (40 U/μl). The 25 μl reactions were incubated at 42 °C for 1 h and then at 95 °C for 5 min. The 25 μl PCR reactions included 2 μl RT product, 2 μl primers (○ **Table 2**), 8.5 μl sterile triple distilled H$_2$O, and 12.5 μl SYBR Premix Ex Taq TM (TaKaRa, Tokyo, Japan). The reactions were incubated in a 96-well optical plate at 95 °C for 5 min, followed by 28 cycles at 95 °C for 30 s and 66 °C for 30 s. The PCR reactions run on an Mx 3000P (Agilent Technologies) and analysed using the Mx 3000P System SDS software.
E5 small nuclear RNA was used as an external control to normalise RNA input. The Ct value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method, presented as the fold-expression change in dexamethasone-treated adipocytes relative to their corresponding control adipocytes after normalisation to the endogenous control. All experiments were performed in triplicate.

Determination of protein expression
One bottle (25 cm^2) of frozen adipocytes was extracted with 1 ml lysis buffer containing 100 mM NaCl, 2 mM EDTA, 5% SDS, 0.1 mM Na$_3$VO$_4$, 50 mM NaF, 1 mM benzamidine, 100 μM AEBSF, 10 μg/ml aprotinin and 50 mM HEPES (pH 7.4). The protein content was measured with the BCA Protein Assay Kit (Pierce
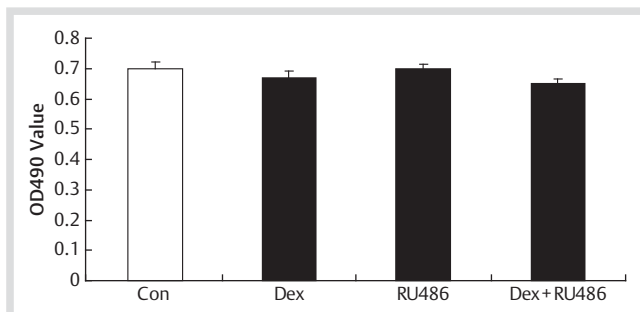
Fig. 1 Effect of 10^{-6} M dexamethasone on proliferative activity of porcine preadipocytes. Con: control group; Dex: 10^{-6} M dexamethasone group; RU486: 10^{-6} M glucocorticoid receptor antagonist group; Dex+RU486: 10^{-6} M dexamethasone and 10^{-6} M glucocorticoid receptor antagonist group. n = 6/group.

biotechnology, Rockford, IL, USA). Forty micrograms of protein extract were mixed with loading buffer and denatured by boiling for 5 min before being loaded on a 10 % SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with 3 % BSA in Tween-Tris-buffered saline for 90 min at room temperature. After repeated washing with Tween-Tris-buffered saline, the membranes were incubated with the appropriate antibodies. Western blot analysis for detecting C/EBP-β was performed with a polyclonal antibody (cs-150X, Santa Cruz Technology, CA, USA) at a dilution of 1 : 1 000. C/EBP-β was detected at 38 kDa. The PPAR-γ antibody (Bioworld Technology, MN, USA) was used at a dilution of 1 : 500. A protein band at 57 kDa was observed. An antibody against β-actin (Abcam, Cambridge, UK) was used as an internal standard at a 1 : 10 000 dilution. Goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (Bioworld Technology) were used at a dilution of 1 : 10 000. Finally, the membrane was washed, and the specific signals were detected by chemiluminescence using the LumiGlo substrate (SuperSignalWest-PicoTrial Kit, Pierce, Rockford, IL, USA). The experiments were performed in triplicate.

Plasmid construction

Genomic fragments of porcine miR-374a/b and their precursors of approximately 89 bp were synthesised by Invitrogen. There is one predicted conserved target site for miR-374a/b in the 3′-UTR of C/EBP-β (www.targetscan.org). A 389 bp fragment of the C/EBP-β 3′-UTR was amplified by PCR using the primers 5′-CCACAGTGACTCCGGGAAG-3′ and 5′-CGTAGGAACATCTTTAAG CGA-3′. The 389 bp fragment, which contains a motif for miR-374a/b that is broadly conserved in vertebrates (www.targetscan.org), was cloned downstream of the luciferase gene in the pGL3-Control luciferase reporter vector. These constructs, named pGL3-control/C/EBP-β, were transfected into HeLa cells. The PCR products were subcloned into the luciferase reporter pGL3-Control using XbaI (Invitrogen). Precursor miR-374a/b was annealed using annealing buffer (5 ×), the miRNA precursor upstream sequence (50 μM) and the downstream sequence (50 μM). The 50 μl reaction solutions were incubated in a 96-well optical plate at 95 °C for 2 min and then subjected to touchdown PCR (with decreases of 0.1 °C/8 s until 25 °C is reached); subsequently, the PCR products were subcloned into the pSilencer 3.0-H1 siRNA expression vector using BamHI and HindIII (Invitrogen).

DNA transfection

Approximately $3 \times 10^4/cm^3$ HeLa cells were seeded and cultured in 25 cm² cell culture bottles. When the cells reached 90–95 % confluence, they were co-transfected with 100 ng of pGL3-control/C/EBP-β 3′-UTR fluorescent luciferase reporter plasmid, 10 ng of pRL-TK plasmid (used to normalise for transformation efficiency), or 100 ng of pSilence 3.1 H1-neo miR-374a/b with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Negative controls were co-transfected with 100 ng of miR-SC, 100 ng of target genes 3′-UTR fluorescent luciferase reporter plasmid and 10 ng of pRL-TK plasmid. At the same time, the miRNA-374a/b inhibitor (Invitrogen) was added to the medium of the co-transfected cells. After transfection, the cells were counted, and the cell density was approximately 2×10^4 cells/cm³. The transfected HeLa cells were incubated at 5 % CO_2 and 37 °C for 24 h.

Dual luciferase activity assay

Twenty-four hours after transfection, firefly and renilla luciferase activities were measured using a Dual-Luciferase Assay Kit (Promega) with a plate reader (PerkinElmer, Waltham, MA, USA). The renilla and firefly luciferase signals were detected using the Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA). The firefly luciferase signal was normalised to the renilla luciferase signal. The normalised firefly luciferase activity was compared between miR-374a/b and the miRNA scrambled control (miR-SC) cells. The results were expressed as relative activity. Each target construct was tested in triplicate, and the assay was repeated to confirm the results.

Statistical analysis

All data are presented as the mean ± SEM. Statistical analyses were carried out with Statistical Program for Social Sciences (SPSS) software 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The differences were tested with a one-way ANOVA. A p-value of less than 0.05 was considered significant.
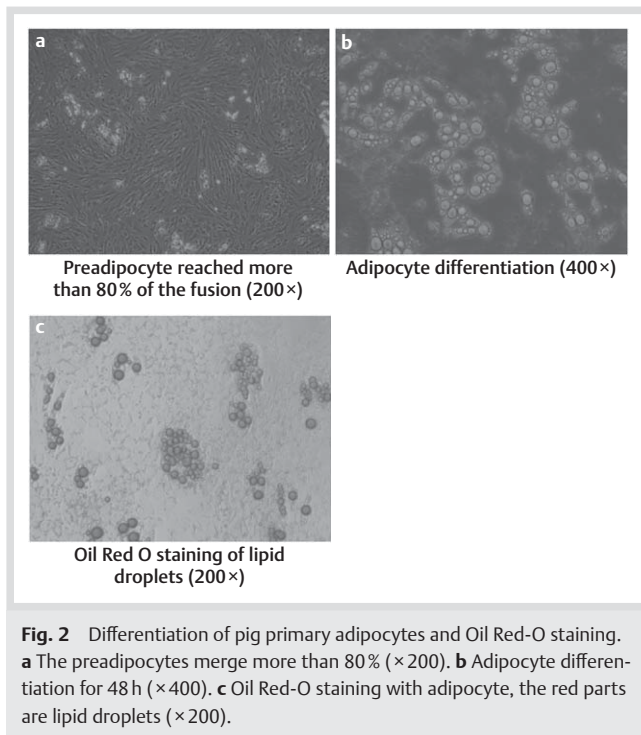
Results

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Effect of 10^{-6} M dexamethasone on the cell viability and TG deposition of porcine preadipocytes (○ Fig. 1–3)

As shown in ○ **Fig. 1**, 10^{-6} M dexamethasone had no effect on the viability of porcine preadipocytes compared with the control group. The same concentration of RU486 showed no differences when compared with other 3 groups (p > 0.05).
Treatment with 10^{-6} M dexamethasone for 48 h significantly increased the TG content when compared with the control group (p < 0.05). However, treatment with only 10^{-6} M RU486 or 10^{-6} M RU486 together with 10^{-6} M dexamethasone did not reverse the increased TG content induced by 10^{-6} M dexamethasone.

Effect of 10^{-6} M dexamethasone on the expression of target genes related to lipid metabolism

As shown in ○ **Fig. 4**, the expression of PPAR-γ and perilipin mRNA were significantly increased in the 10^{-6} M dexamethasone group compared to the control group (p < 0.05). However, C/EBP-β levels decreased significantly after 48 h treatment with 10^{-6} M dexamethasone compared with the control group. GR and FAS mRNA expression was not significantly different between the groups (p > 0.05).

Fig. 2 Differentiation of pig primary adipocytes and Oil Red-O staining. **a** The preadipocytes merge more than 80 % (×200). **b** Adipocyte differentiation for 48 h (×400). **c** Oil Red-O staining with adipocyte, the red parts are lipid droplets (×200).

Effect of 10^{-6} M dexamethasone on PPAR-γ and C/EBP-β protein expression

PPAR-γ protein level was significantly increased in the 10^{-6} M dexamethasone group compared to the control group (p<0.05) (◉ **Fig. 5a**). However, C/EBP-β protein levels were significantly decreased in the 10^{-6} M dexamethasone group compared to the control group (◉ **Fig. 5b**).

Effect of 10^{-6} M dexamethasone on the expression of miRNAs that targets the C/EBP-β 3′-UTR

As shown in ◉ **Fig. 6**, miRNA-374 (miRNA-374a and miRNA-374b) expression in the 10^{-6} M dexamethasone group was significantly increased (p<0.05) compared to the control group. However, miRNA-155, 362, 191, 455, and 423-5p expressions were not significantly different between the 2 groups.

Validation of ssc-miR-374a and ssc-miR-374b targeting C/EBP-β 3′-UTR

miRNA-374a/b had highly conserved sites (◉ **Fig. 7a**) for binding to the 3′-UTR of C/EBP-β. The miR-374a/b-targeted elements in the C/EBP-β 3′-UTR are highly conserved in many mammals, including pig, human, mouse, rat, cow, sheep, chicken, and dog (◉ **Fig. 7b**). To ascertain whether miR-374a/b are able to recognise the C/EBP-β 3′-UTR, we generated a luciferase reporter DNA construct containing the 389 bp pig C/EBP-β 3′-UTR with a putative miR-374a/b binding site and an ssc-miR-374a/b overexpression plasmid. When the pGL3-Control/C/EBP-β 3′-UTR fluorescent luciferase reporter plasmid and the ssc-miR-374a/b overexpression vector were co-transfected into HeLa cells, luciferase activity was significantly suppressed by the ectopic expression of ssc-miR-374a/b after co-transfection for 24 h. Though overexpression of ssc-miR-374a/b was able to significantly suppress luciferase activity after the addition of 50 or 100 ng of miRNA-374a/b inhibitor, adding 150 or 200 ng of miRNA-374a/b inhibitor could significantly reverse this suppression trend (◉ **Fig. 8**).

Discussion

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Glucocorticoids are important hormones involved in body metabolism. Regarding its influence on lipid metabolism, glucocorticoids have been proposed to have both adipogenic and lipolytic actions within adipose tissue [1–5]. Previous studies have demonstrated that glucocorticoids play a direct role in the formation of cytoplasmic lipid droplets [27, 28]. The differentiation of 3T3-L1 preadipocytes can be induced by a 2-day treatment with a factor "cocktail" (DIM) containing synthetic dexamethasone, insulin, the phosphodiesterase inhibitor methylisobutylxanthine (IBMX), and fetal bovine serum [29]. In the present study, though preadipocyte cell viability was not different from the control group after treatment with 10^{-6} M dexamethasone for 48 h, yet the TG content was significantly increased [30]. The results indicated that 10^{-6} M dexamethasone contributes to porcine preadipocyte differentiation and lipid droplet synthesis. Ru486 treatment did not reverse the increase of TG content induced by dexamethasone. In our preliminary experiment, 10^{-6} M Ru486 can reverse the increase of TG content induced by 10^{-6} M dexamethasone. However, in the current experiment, the inhibitor demonstrated no effect. The reason may be due to the chemical problem of Ru486 or the pooled preadipocytes from five 35-day Meishan piglets. Therefore, in the late analysis only control and dexamethasone groups were used.

Preadipocytes that gradually filled with lipid droplets and differentiated into mature fat cells with a single chamber were regulated by a number of transcription factors, including C/EBPs and PPAR-γ. The overexpression of PPAR-γ can promote adipogenesis [31, 32]. Compared to wild-type controls, heterozygous PPAR-γ-deficient mice show decreased fat mass [17]. In the current study, PPAR-γ mRNA and protein expression was significantly upregulated after treatment with 10^{-6} M dexamethasone for 48 h. This finding is consistent with previous reports demonstrating that dexamethasone induces preadipocyte recruitment and increases PPAR-γ protein expression in porcine stromal-vascular (S-V) cells [6]. The perilipins are the most abundant proteins at the surfaces of lipid droplets in adipocytes, which play a role in regulating the packaging and storage of neutral lipids [33–35]. Further studies have shown that PPAR-γ is an important transcriptional factor for perilipin, and the upstream sequence of the perilipin promoter contains a PPRE. Previous studies have shown that treating differentiated 3T3-L1 adipocytes with a PPAR-γ agonist significantly augments perilipin mRNA expression [36, 37]. In the current study, perilipin mRNA expression was significantly increased in the 10^{-6} M dexamethasone treatment group compared to the control group.

C/EBP-β plays an important role in the induction of PPAR-γ expression and adipogenesis. Wiper-Bergeron showed that glucocorticoid-stimulated preadipocyte differentiation is mediated through the acetylation of C/EBP-β [38]. C/EBPs family includes 6 kinds of transcription factors, and C/EBP-α, C/EBP-β, C/EBP-δ, and C/EBP-ζ can expressed in adipose tissue [39]. Expression of various transcription factors in adipocyte differentiation process has a time sequence. In the current study, C/EBP-β expression significantly decreased after 48-h treatment with 10^{-6} M dexamethasone. Previous studies have shown that the expression of C/EBP-β usually occurs at a very early stage of adipocyte differentiation, which is followed by the induction of C/EBP-α and PPAR-γ, which promotes differentiation by activating adipose-specific gene expression [40, 41]. These results show that there is a time difference between PPAR-γ and C/EBP-β expression. When the adipose
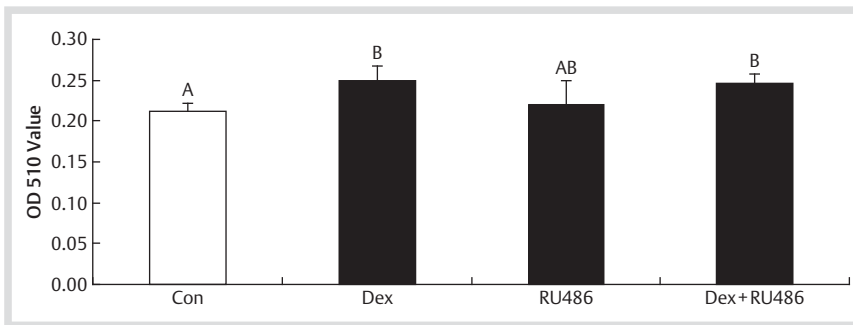
Fig. 3 Effect of 10^{-6} M dexamethasone on triglyceride deposition of primary cultured adipocyte. Con: control group; Dex: 10^{-6} M dexamethasone group; RU486: 10^{-6} M glucocorticoid receptor antagonist group; Dex+RU486: 10^{-6} M dexamethasone and 10^{-6} M glucocorticoid receptor antagonist group. Different letter means significant difference, $p < 0.05$, n=6/group.
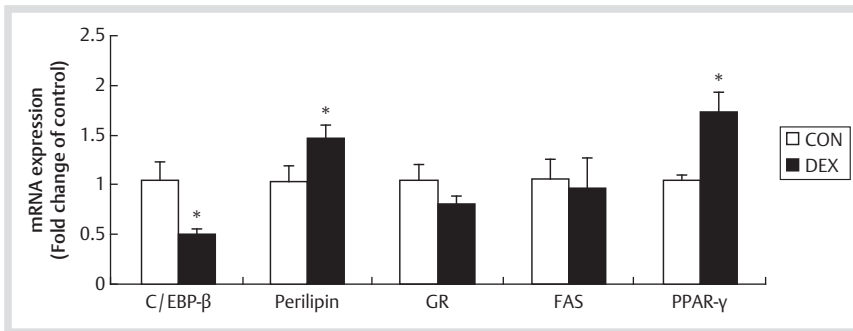

Fig. 4 Effect of 10^{-6} M dexamethasone on the expression of target genes related to lipid metabolism in primary cultured porcine adipocyte. $^*p < 0.05$ compared with the CON group, n=6/group.

differentiation process progresses, C/EBP-β expression shows a declining trend, while PPAR-γ expression gradually increases [42]. Therefore, the present results illustrate that after treatment with 10^{-6} M dexamethasone for 48 h, the differentiation of porcine preadipocytes has reached a late stage of differentiation.

It is largely unknown which factors induce the C/EBP-β expression decrease as the adipose differentiation process progresses. In recent years, it has been shown that miRNAs are involved in the regulatory network of many biological processes through the post-transcriptional regulation of transcription factors. Several miRNAs were reported to be expressed in mammalian adipocytes and seem to play a role in the regulation of adipogenesis [15]. In the present study, we identified seven candidates of C/EBP-β-targeting miRNAs by bioinformatic analyses. Among the predicated miRNAs, only miRNA-374a and miRNA-374b were found to be upregulated in the 10^{-6} M dexamethasone treatment cells. In the previous study, miRNA-374a/b was reported to serve as a prognostic marker for patient risk stratification at early stages of non-small cell lung cancer progression [43], and miR-374 has also been found to respond to primary infections of self-healing *Plasmodium chabaudi* malaria in female C57BL/6 mice [44]. miR-374a could be involved in the phospho-ΔNp63α-dependent regulation of autophagic signalling and the control cell death of squamous cell carcinoma (SCC) cells [45]. The upregulation of miR-374a is thought to participate in the carcinogenesis of the colon without lymph node metastasis [46]. miR-374b expression in seminal plasma could also provide a novel, noninvasive approach for diagnosing male infertility [47]. Referring to the role of miR-374 in the lipid metabolism, our previously reports demonstrated that microRNA-374b mediate the effect of maternal dietary protein on offspring lipid metabolism in Meishan pigs by targeting on the C/EBP-β [48]. In the present study, both microRNA-374b and microRNA-374a were shown to participate in the regulation of adipocyte differentiation. Luciferase reporter assays were performed to fully validate the predicted miRNA-mRNA interactions. In the present study, miR-374a/b overexpression significantly reduced the activity of a luciferase reporter containing the C/EBP-β 3′-UTR after co-
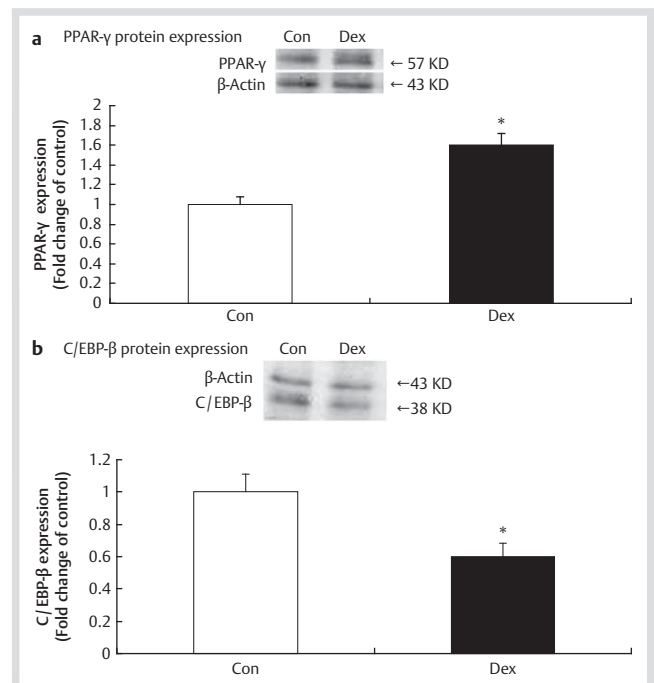

Fig. 5 Effect of dexamethasone on PPAR-γ and C/EBP-β protein expression in primary cultured porcine adipocyte. **a** PPAR-γ. **b** C/EBP-β. $^*p < 0.05$ compared with the CON group, n=6/group.

transfection for 24 h, and a miRNA-374a/b inhibitor could significantly reverse this suppression. These results indicate that miR-374a/b can directly recognise and bind to the 3′-UTR of C/EBP-β and suppress C/EBP-β expression. In contrast, miR-374a/b did not alter the activity of a luciferase reporter that has no C/EBP-β 3′-UTR (data not shown).

In conclusion, the present study showed that treatment with 10^{-6} M dexamethasone promoted lipid accumulation in primary porcine preadipocytes. The expression response of PPAR-γ and C/EBP-β was different after 48-h treatment. miR-374a/b may be involved in the decrease of C/EBP-β expression. Though
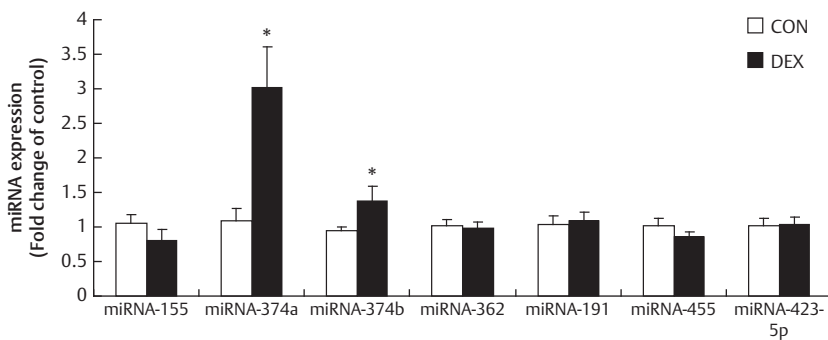
Fig. 6 Effect of dexamethasone on adipocyte miRNAs expression target C/EBP-β of primary cultured adipocyte at weaning pigs. Values are represented as means ± SEM, *p<0.05 compared with the CON group, n = 6/group.

a Conserved

Position 484-490 of C/EBP-β 3′-UTR 5′ ...GUAAUUUUAAUAUUUUAUUAUAU...

ssc-miR-374a/b 3′...   GUGAAUCGUCCAACAUAAUAUA...

b
- C/EBP-β 3′-UTR 5′   470.........480.........490.........500   3′
- Ss   UUUUUUGUAUUAUAAAAAAUAAUC
- Hs   UUUUUUGUAUUAUAAAAAU- -AA
- Mm   UUUUUUGUAUUAUAAAAAUA-GU
- Ec   UUUUUUGUAUUAUAAAAAU-AA
- Rn   UUUUUUGUAUUAUAAAAAA-GU
- Gg   UUUUUUGUAUUAUAAGAA U-AAU
- ssc-miR-374a/b seed   3′   AUAAUAU   5′

Fig. 7 The miR-374a/b target site in the 3′-UTR of C/EBP-β. **a** The predicted conserved binding site of miR-374a/b in the 3′-UTR of pig C/EBP-β. **b** Conservation of the miR-374a/b binding region in the C/EBP-β 3′-UTR among mammals. The miR-374a/b seed match is highlighted in red (TargetScan). Ss: pig; Hs: human; Mm: mouse; Ec: horse; Bt: cow; Rn: rat; Gg: chicken.
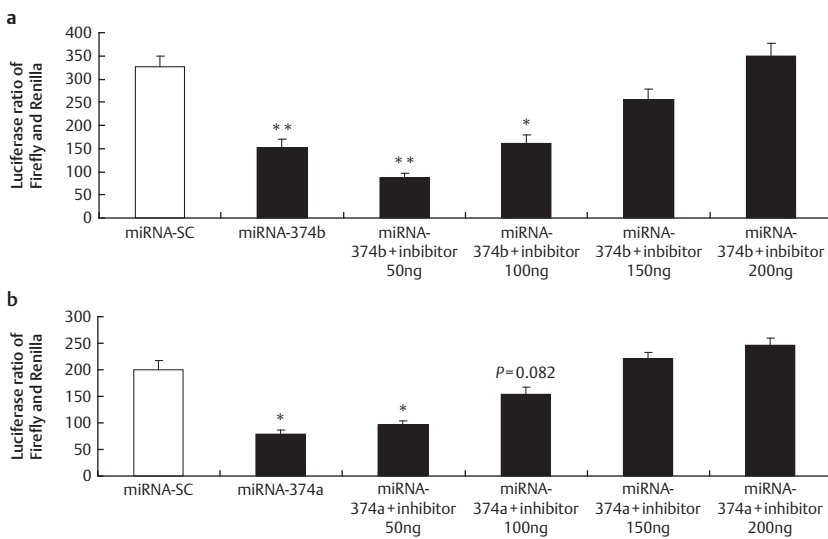

Fig. 8 Validation of ssc-miR-374a/b targeting of the C/EBP-β 3′-UTR at 24 h after transfection. **a** Validation of ssc-miR-374b targeting of the C/EBP-β 3′-UTR. **b** Validation of ssc-miR-374a targeting of the C/EBP-β 3′-UTR. *p<0.05 compared with the miRNA-SC group and **p<0.01 compared with the miRNA-SC group, n=3/group.

the reason why miR-374a/b increased during adipocyte maturation still needs further study. These results provide a possibly new target for the regulation of porcine fat deposition.

Conflict of Interest
▼

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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