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Research paper

miR-181a regulate porcine preadipocyte differentiation by targeting TGFBR1

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ABSTRACT

miRNAs have been shown to regulate a variety of biological process. It has been shown that miR-181a regulates porcine adipogenesis by targeting Tumor Necrosis Factor-α (TNF-α), but the overall functions of miR-181a in porcine preadipocyte differentiation remain unclear. This study aimed to explore the functions of miR-181a in porcine preadipocyte differentiation via the TGFβ/Smad pathway. The TargetScan program was used to predict miRNAs targeting TGFBR1, and miR-181a was selected as a candidate. To investigate the functions of miR-181a, miRNA mimics and inhibitors were used to overexpress or knockdown miR-181a, respectively. RT-qPCR and Western blotting were used to measure the expression of aP2, PPARγ, C/EBPα and TGFBR1 in porcine preadipocytes. Lipid accumulation and adipocyte apoptosis were detected using Oil Red O staining and flow cytometry, respectively. Taken together, our results indicated that miR-181a promoted porcine preadipocyte differentiation by directly targeting TGFBR1.

1. Introduction

Due to the physiological similarity between *Homo sapiens* and *Sus scrofa*, porcine adipocytes are used as a model to study human metabolic diseases, including obesity and diabetes (Bellinger DA1 and Nichols, 2006; Brambilla and Cantafora, 2004). Adipocytes are derived from mesenchymal stem cells (MSCs), which differentiate first into preadipocytes and then into mature adipocytes (Dani1 et al., 1997). Preadipocytes differentiating into rounded mature adipocytes have a wide range of transcription factors, such as C/EBPα (Cao et al., 1991), PPARγ (Willson et al., 2001), and aP2 (Furuhashi et al., 2015), and adipocyte differentiation is regulated by a number of signaling pathways, such as TGF-β (Rita Bortell et al., 1994), Wnt/β-catenin (Bennett et al., 2002), MAPK (Nishimoto and Nishida, 2006) and Notch (Bi et al., 2014).

TGFBR1 is a crucial component of the TGF-β signaling pathway (Loboda et al., 2016), and TGF-β1 can negatively regulate adipocyte differentiation (Tsurutani et al., 2011). Zhang et al. (2015) showed that knockdown of TGFBR1 facilitated adipogenic differentiation of ST2 cells. The TGF-β superfamily includes TGF-β, BMPs, GDF and activin (Zieba et al., 2016), and TGF-β signaling affects a wide range of biological processes, including cell proliferation, migration and apoptosis (Vuckovic et al., 2016). Yu et al. (2017) showed that miR-9 could repress the activation of hepatic stellate cells (HSCs) by targeting TGFBR1 (Yu et al., 2017), and Shi et al. (2014) showed that miR-199a prevented the invasion ability of the human kidney carcinoma cell line 786-O via suppressing TGFBR1 (Shi et al., 2014). However, the biological mechanisms of TGFBR1 in porcine adipocyte differentiation remain unknown.

MicroRNAs (miRNAs) are 19–25 nucleotides in length and have no coding capability but are known to play significant regulatory roles in some biological processes. miRNAs negatively regulate the levels of target genes by directly binding to their 3′ untranslated regions (3′ UTRs), and several miRNAs have been reported to play roles in adipocyte differentiation. For example, miR-125a suppresses adipocyte differentiation (Ji et al., 2014), and miR-140 facilitates adipocyte differentiation (Liu et al., 2013), which reveals that miRNAs critically function in preadipocyte differentiation. miR-181a can affect 3T3-L1 cell differentiation (Ouyang et al., 2016), and miR-181a has been shown to repress TNF-α to regulate adipogenesis in porcine preadipocytes (Li et al., 2013). While miR-181a are strongly associated with adipocyte differentiation, their potential mechanisms in regulating adipocyte differentiation by targeting TGFBR1 remain largely unknown.

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In this study, we used the TargetScan database to predict miRNAs targeting TGFBR1, and we selected miR-181a as a candidate miRNA. To confirm whether miR-181affects TGFBR1 expression, we measured the expression of TGFBR1 after transfecting miR-181a into porcine preadipocytes.

2. Materials and methods

2.1. Animals and sample collection

Briefly, 5-day-old Junmu No. 1 white pigs were obtained from the experimental farm of Jilin University. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Jilin University. The piglets were sacrificed by electric shock at 5 days old, and their adipose, heart, liver, muscle, spleen, lung and kidney tissues were collected to analyze the levels of miR-181a. After induction for 0, 2, 4, 6, 8 and 10 days, the adipocytes were used to analyze levels of miRNA at various differentiation stages.

2.2. Cell culture and adipogenic differentiation

Porcine preadipocytes were obtained from the subcutaneous adipose tissues of 5-day-old Junmu No. 1 white pigs under sterile conditions. The subcutaneous adipose tissue was digested with 0.2% collagenase I (Sigma, USA) at 37 °C for 1.5 h, and the digested cells were isolated using 80 μm and 200 μm nylon mesh. The preadipocytes were collected by centrifugation at 1450 r/min for 5 min and seeded in DMEM/F12 growth medium (Gibco, USA) containing 10% FBS (BI, Israel). When the cells reached confluence, the growth medium was supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 5 μg/mL insulin and 1 μM dexamethasone (DEX) for 48 h. After 48 h, the cell medium was replaced with maintenance medium (consisting of growth medium and 5 μg/mL insulin), and normal medium was then changed every 48 h until maturation.

2.3. Transfection of miRNA mimics and inhibitors

Mimics and inhibitors of miR-181a were designed and synthesized by RiboBio Biotech (Guangzhou, China). Si-NC and si-TGFBR1 were designed and synthesized by GenePharma (Shanghai, China). Adipocytes were seeded in a 12-well plate at a density of 2×10^5 cells per well, and the riboFECT CP Transfection Kit (Guangzhou, China) was to transfect porcine preadipocytes according to the manufacturer's instructions. The final concentrations of the si-NC, si-TGFBR1, miRNA mimics and inhibitors were 100 nM.

2.4. Flow cytometry

The Annexin V Cell Apoptosis Kit (Sungene Biotech, Tianjin, China) was used to measured adipocyte apoptosis according to the manufacturer's instructions. One day after transfection, the porcine preadipocytes were digested with trypsin and centrifuged at 1000 r/min for 5 min (6×10^5 porcine preadipocytes were collected). Annexin V-FITC and propidium iodide (PI) were used to stain the dead and apoptotic cells, and flow cytometry was performed within 1 h.

2.5. Oil Red O staining

The Oil Red O Kit (Solarbio, USA) was used to stain the porcine adipocytes following the manufacturer's recommended protocol. To quantify intracellular Oil Red O staining, the stained Oil Red O cells were extracted with 100% isopropanol for 20 min, and the absorbance was measured by Microplate reader (TECAN) at 510 nm.

Table 1
Primers for reverse transcription and quantitative real-time PCR.

Primers	Sequences (from 5′ to 3′)
U6-F	GCTTCGGCAGCACATATACTAAAAT
U6-R	CGCTTCACGAATTTGCGTGTCAT
U6-RT	CGCTTCACGAATTTGCGTGTCAT
miR-181a- F	ACACTCCAGCTGGGAACATTCAACGCTGTCGG
miR-181a -R	CTCAAGTGTCGTGGAGTCGGCAA
miR-181a- RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACTCACC
TGFBR1-F	CCGTCACAGAGACCACAGAC
TGFBR1-R	TCCTGGAAAAGGACCAACAGT
GAPDH-F	ACCCAGAAGACTGTGGATGG
GAPDH-R	ACGCCTGCTTCACCACCTTC
AP2-F	TTTGCTACCAGGAAAGTGGCTGGCAT
AP2-R	GCAGTGACACCATTCATGACACATTCC
C/EBP α-F	AAGTCGGTGGACAAGAACAGCAACGAGTA
C/EBP α-R	ATTGTCACTGGTCAGCTCCAGCACCTT
PPARγ-F	CCTTAAACGAAGAGTCATCTTTTAGCG
PPARγ-R	GGCTCTTCGTGAGGTTTGTTGTACAG

2.6. RNA isolation and RT- qPCR

The miRcute mRNA Extraction and Separation Kit (Tiangen, China) was used to isolate total cellular RNA following the manufacturer's instruction, and the NanoDrop 2000 spectrophotometer (Thermo, USA) was used to evaluate the concentration and quantity of RNA. The FastQuant RT Kit (Tiangen, China) with gDNase was used to reverse transcribe RNA into cDNA according to the manufacturer's instructions. SuperReal PreMix Plus (Tiangen, China) with SYBR Green was used for real-time quantitative PCR analyses according to the manufacturer's recommended protocol. The primers sequences for the mRNAs and miRNAs are listed in Table 1.

2.7. Western blot

Two days after transfection, porcine adipocytes were washed in PBS and harvested by 0.25% trypsin digestion. Porcine adipocytes were lysed with protein lysis buffer (Beyotime, China), and total protein was separated by 12% SDS-PAGE. Next, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) for 1 h, blocked in 5% BSA for 1.5 h, incubated with primary antibodies at 4 °C overnight, and incubated with a secondary antibody for 55 min. A chemiluminescence substrate (Tanon, China) was used to visualize protein bands, and the ImageJ program was used for protein quantification.

2.8. Statistical analysis

All experimental data were presented as the mean ± SEM. One-way ANOVA was used to determine significance with SPSS 16.0.; $p < 0.05$ indicated that the difference was statistically significant.

3. Results

3.1. Prediction of miRNAs targeting TGFBR1

We used the TargetScan program, MicroCosm Targets and miRanda to predict miRNAs that could bind the 3′ UTR of mouse *TGFBR1* mRNA. By homology comparison, the porcine *TGFBR1 m*RNA sequence was shown to be 90.21% homologous with the mouse mRNA sequence. Likewise, the mature sequences of miR-181a were highly conserved across a wide variety of species. Thus, we selected miR-181a as candidate miRNAs (Fig. 1A, B).