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Research paper

MicroRNA-204-5p regulates 3T3-L1 preadipocyte proliferation, apoptosis and differentiation

Jingjing Du[a,1], Peiwen Zhang[a,1], Mailin Gan[a], Xue Zhao[a], Yan Xu[a], Qiang Li[c], Yanzhi Jiang[b], Guoqing Tang[a], Mingzhou Li[a], Jinyong Wang[d], Xuewei Li[a], Shunhua Zhang[a,*], Li Zhu[a,*]

[a] College of Animal Science & Technology, Sichuan Agricultural University, Chengdu 611130, Sichuan, China
[b] College of Life and Science, Sichuan Agricultural University, Chengdu 611130, China
[c] Sichuan Province General Station of Animal Husbandry, Chengdu 611130, China
[d] Chongqing Academy of Animal Sciences, Chongqing 402460, China

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ABSTRACT

Obesity due to excessive lipid accumulation is closely associated with metabolic diseases such as type 2 diabetes, insulin resistance and inflammation. Therefore, a detailed understanding of the molecular mechanisms that underlie adipogenesis is crucial to develop treatments for diseases related to obesity. Here, we found that the microRNA-204-5p (miR-204-5p) was expressed at low levels in fat tissues from obese mice fed long-term with a high-fat diet (HFD). Overexpression or inhibition of miR-204-5p *in vitro* in 3T3-L1 preadipocytes significantly inhibited or promoted 3T3-L1 proliferation, respectively, an effect mediated by regulating cell proliferation factors. miR-204-5p also induced preadipocyte apoptosis by directly targeting the 3′ UTR region of *Bcl-2*, reducing the constitutive suppression of *Bcl-2* on p53-dependent apoptosis. Interestingly, overexpression of miR-204-5p during adipocyte differentiation significantly increased the number of oil red O+ cells, triglyceride accumulation and the expression of markers associated with adipocyte differentiation. In contrast, inhibition of miR-204-5p had the opposite effect on 3T3-L1 adipocyte differentiation. Luciferase activity assays and qRT-PCR showed that miR-204-5p regulates adipocyte differentiation by negatively regulating *KLF3*, a negative regulator of lipogenesis. Taken together, our findings showed that miR-204-5p inhibits proliferation and induces apoptosis of preadipocytes by regulating *Bcl-2*, but also promotes adipocyte differentiation by targeting KLF3.

1. Introduction

Under the World Health Organization (WHO) definition of obesity (BMI > 30 kg/m^2), 30% of Americans and 10%–20% of Europeans are classified as obese, and the prevalence of this condition is increasing in many developing countries (Vanderklaauw and Farooqi, 2015). It is predicted that up to 58% of the world's adult population will be overweight or obese by 2030 (Kelly et al., 2008). Increasing evidence suggests that obesity is closely associated with several diseases such as nonalcoholic fatty liver disease, type 2 diabetes, cardiovascular disease and even cancer (Ferrante et al., 2015; Hoy et al., 2017; World Health Organization, 2000). Therefore, reducing the incidence of obesity is important to improve human health worldwide. Many studies have demonstrated that an imbalance between energy intake and energy expenditure can lead to an excessive accumulation of adipose tissue, and that this is the main cause of obesity (Richard, 2015). Therefore, a detailed understanding of the molecular mechanisms that underlie adipogenesis would be expected to facilitate the development of novel methods to treat obesity and reduce the incidence of obesity-related diseases (Xiao et al., 2015).

Adipogenesis is a complex and precisely orchestrated process mediated by a network of adipogenic regulatory factors (Lefterova and Lazar, 2009). MicroRNAs (miRNAs) are a class of 17–24 base single-stranded RNA molecules that play an essential role in the post-transcriptional regulation of many important cellular pathways (Lefterova and Lazar, 2009). Recently, numerous studies have

Abbreviations: C/EBPα, CCAAT/enhancer-binding proteins; *PPAR-γ*, peroxisome proliferator activated receptor-γ coactivator; *AP2*, adipocyte fatty-acid binding protein 2; *CDK4*, cyclin dependent kinase 4; *CDK2*, cyclin dependent kinase 2; *CDK5*, cyclin dependent kinase 5; *p21*, calcium binding protein 21; *FABP4*, fatty acid binding protein 4; *KLF3*, Kruppel-like factor 3; *DVL3*, dishevelled segment polarity protein 3; *Sirt1*, sirtuin 1; *SREBP-1c*, sterol regulatory element-binding protein 1c; *SCD*, stearoyl-CoA desaturase; *FAS*, fatty acid synthase; *GSK*, glycogen synthase kinase; *DGAT*, diacylglycerol acyltransferase; *VLDL*, very-low density lipoprotein; *LPL*, lipoprotein lipase; *ACADL*, acyl-CoA dehydrogenase long chain; *ACOX2*, acyl-CoA oxidase 2; *ACSS*, acyl-CoA synthetase short-chain family member; NC, negative control; HFD, high-fat diet (HFD); NCW, normal chow

* Corresponding authors.
E-mail addresses: zhangsh1919@163.com (S. Zhang), zhuli7508@163.com (L. Zhu).
[1] These authors contributed equally to this work.

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suggested that miRNAs are closely involved in adipocyte proliferation, differentiation and apoptosis. Jin reported that miR-24 promotes 3T3-L1 preadipocyte differentiation by directly targeting *MAPK7* signaling (Jin et al., 2016). Adi showed that miR-205 knockdown induces pre-adipocyte cell proliferation and adipogenesis in the visceral adipose tissue of obese mice (Adi et al., 2011). MiR-224-5p regulates TNF-α-induced adipocyte apoptosis by controlling $NF\text{-}_{kB}$ activation (Qi et al., 2017). In this study, we found that miR-204-5p was expressed at low levels in fat tissue from obese mice fed long-term with a HFD. A detailed investigation showed that miR-204-5p inhibited 3T3-L1 preadipocyte proliferation, induced 3T3-L1 apoptosis by directly targeting *Bcl-2*, and promoted 3T3-L1 adipocyte differentiation by negatively regulating *KLF3*. The implications of these findings are discussed.

2. Materials and methods

2.1. Experimental animals

As described in our previous report (Du et al., 2016), two groups of six week-old Kunming mice were fed either a high-fat diet (HFD) or normal chow (NCW) for eighteen weeks. Mice were given free access to food and water under light and temperature-controlled conditions during the experimental trial. All experiments in the present study involving animals and tissue sample collection were performed in accordance with the guidelines of the U.K. Animals (Scientific Procedures) Act, 1986.

2.2. Serum-sample analysis

Blood samples were allowed to clot at room temperature and the serum samples were stored at −20 °C until further analysis. Serum levels of cholesterol (TC) and triglycerides (TG) were determined as described by Tan et al. (Chengdu Li Lai Biotechnology Limited Company, Chengdu, P. R. China) (Tan et al., 2017).

2.3. Glucose and insulin tolerance tests

We followed previously described methods (Kung et al., 2016). Briefly, for glucose tolerance tests (GTT), overnight-fasted mice with free access to water were given intraperitoneal injections of 2 mg glucose/g body weight. For insulin-tolerance tests (ITT), mice were fasted for 4 h and were then intraperitoneally injected with 0.5 mU insulin/g body weight. Blood was obtained from the tail vein before the injections and then 0, 15, 30, 60, and 90 min after the injections.

2.4. Cell cultures and transfection

Mouse 3T3-L1 preadipocytes (Stem Cell Bank, Chinese Academy of Science) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco; Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco) in an incubator at 5% CO2 and 37 °C. To induce differentiation, 3T3-L1 preadipocytes were stimulated with differentiation media (DM) containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, and 5 mg/mL insulin for 2 days, at which point cells grew confluent. The medium was then replaced every other day with DMEM containing 10% FBS and 5 mg/mL insulin, and the process was continued until day 6. To explore the effects of miR-204-5p on preadipocyte proliferation and differentiation, 3T3-L1 cells were transfected with a miR-204-5p mimic (catalog number: miR10000237-1-5), an inhibitor (catalog number: miR20000237-1-5) or a negative control (catalog numbers: miR01201-1-5 and miR02201-1-5) (all purchased from RIBOBIO, Guangzhou, China) using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

2.5. Proliferation and apoptosis analysis

To evaluate the effects of miR-204-5p on preadipocyte proliferation, the Cell Counting Kit 8 (CCK-8, Beyotime; Shanghai, China) and 5-ethynyl-2′-deoxyuridine assay (EdU, RIBOBIO) were used. Briefly, 3T3-L1 cells seeded in 96-well plates were transfected with either the miR-204-5p mimic, the inhibitor or the negative control. Cell proliferation was then assessed at 0 h, 12 h, 24 h, 48 h and 96 h by means of the CCK8 kit. For EdU analysis, 3T3-L1 cells were treated with 10 μM EdU for 24 h after transfection and then incubated for an additional 14 h. EdU staining was performed according to the manufacturer's protocol. Images were captured using an Olympus IX53 microscope (Olympus; Tokyo, Japan). Additionally, to evaluate the effect of miR-204-5p on apoptosis, we used the Apoptosis and Necrosis Assay Kit (Beyotime; catalog number: C1056), following a protocol described in a previous report (Meng et al., 2012).

2.6. Quantitative PCR

Total RNA from cells and tissues was extracted using TRIzol Reagent (Invitrogen; Carlsbad, CA, USA), according to the manufacturer's instructions. Subsequently, reverse transcription of mRNA and miRNA was performed using a commercial kit (TaKaRa, China) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Premix Ex Taq kit (TaKaRa) and a CFX96 instrument (Bio-Rad, USA). Relative expression levels of mRNAs and microRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used in the qRT-PCR assays are listed in Supplemental Table 1.

2.7. Immunocytochemical analysis

Briefly, after treatments as described above, 3T3-L1 cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min. Following additional PBS washes (also performed after each step thereafter), cells were permeabilized with 0.5% Triton X-100 and blocked with 2% goat serum (diluted in PBS). After blocking, cells were incubated with an anti-adiponectin antibody and an anti-caspase 3 antibody (Bioss; Beijing, China, catalog numbers: bs-0471R and bs-0081R) for 24 h at 4 °C, followed by incubation at 37 °C for 1 h with fluorescent secondary antibodies. Images were captured using an Olympus IX53 microscope (Olympus).

2.8. Oil red O staining and triglyceride assay

Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde for 60 min, and then washed with PBS for three times. The fixed samples were stained with 0.5% Oil Red O for 1.5 h at room temperature and washed with PBS for three more times. Images were captured using an Olympus IX53 microscope (Olympus). For the triglyceride assay, the stained cells were incubated with isopropanol for 20 min, and the OD values at a wavelength of 510 nm were measured by means of a spectrophotometer.

2.9. Luciferase reporter assays

Luciferase reporter plasmids containing the wild-type 3′ UTRs of *Blc-2* and *KLF3* (WT-Blc-2, WT-KLF3), as well as mutant 3′ UTRs of *Blc-2* and *KLF3* (Mut-Blc-2, MuT-KLF3) were manufactured by TsingKe Biotech (Chengdu, China). Lipofectamine 3000 (Invitrogen) was used to co-transfect Hela cells (Stem Cell Bank, Chinese Academy of Science) with the wild-type or mutant 3′ UTR luciferase reporter plasmids and the miR-204-5p mimic or the negative control. Cells were harvested 48 h after transfection, and luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega; Madison, WI, USA), following the manufacturer's instructions. Firefly luciferase was used as

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