Biochemical and Biophysical Research Communications 457 (2015) 37–42

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MiR-378a-3p enhances adipogenesis by targeting mitogen-activated protein kinase 1

Nunu Huang [a,b,1], Jiu Wang [b,1], Weidong Xie [b], Qing Lyu [a,b], Jiangbin Wu [a,b], Jie He [b], Wei Qiu [a,b], Naihan Xu [b,*], Yaou Zhang [b,*]

[a] School of Life Sciences, Tsinghua University, Beijing 100084, PR China
[b] Key Lab in Healthy Science and Technology, Division of Life Science, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, PR China

ARTICLE INFO

Article history:
Received 5 December 2014
Available online 18 December 2014

Keywords:
Adipogenesis
MiR-378a-3p
MAPK1

ABSTRACT

Previous studies showed that miR-378a plays important roles in adipogenesis and obesity; however, the precise mechanisms of action remain unknown. Here, we found that miR-378a-3p expression is up-regulated in adipose tissues of high fat diet-induced obese mice, as well as during the differentiation of 3T3-L1 preadipocytes. Mir-378a-3p induced adipogenesis by targeting mitogen-activated protein kinase 1 (MAPK1). Overexpression of miR-378a-3p or silencing MAPK1 reduced MAPK1 expression and enhanced adipogenesis, whereas blockage of endogenous miR-378a-3p had the opposite effect, suggesting that miR-378a-3p promotes the adipogenesis of 3T3-L1 cells by targeting MAPK1.

1. Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs (approximately 22nt in length) that regulate gene expression at the post-transcriptional level [1–3]. Numerous studies have demonstrated that miRNAs play crucial roles in various biological and physiological processes [4–9]. Including adipocyte differentiation and adipogenesis [10–12]. Some miRNAs enhance adipogenesis [13–16], whereas others exert negative effects by targeting essential adipogenic genes directly [17,18]. MiR-378 plays an important role in adipogenesis and lipogenesis [19–22], Both MIR378a-3p and MIR378a-5p originate from a common hairpin RNA precursor which is embedded in the first intron of PGC1β (Peroxisome proliferator-activated receptor gamma coactivator 1-beta) gene Overexpression of miR-378a-3p or miR-378a-5p in ST2 cells increases the size of lipid droplets and the accumulation of triacylglycerol, whereas knockout of these miRNAs decreases triacylglycerol accumulation [19]. Recent study report that miR-378 counterbalances the metabolic actions of PPARGC1B; hence, miR-378a gene knockout mice are resistant to high fat diet (HFD) induced obesity via counterbalancing the metabolic actions of PGC-1 β [23].

Mitogen-activated protein kinase 1 (MAPK1), which is also known as ERK2 [24], plays an important role in a wide variety of cellular processes, including cellular differentiation, proliferation, development, metabolism, and metastasis [25–28]. MAPK1 also reportedly regulates adipogenesis [29,30]; however, its roles in this process are complicated. On the one hand, MAPK1 up-regulates the expression of crucial adipogenic regulators such as peroxisome proliferator-activated receptor γ (PPARγ) and the CCAAT-enhancer-binding proteins α, β, and δ at the beginning of adipogenesis [31,32]. On the other hand, the activity of MAPK1 is down-regulated during the terminal differentiation of adipogenesis because it can phosphorylate PPARγ and then inhibit adipogenesis [33,34].

Here, we show that the expression level of miR-378a-3p was much higher in HFD-fed mice than standard diet (SD)-fed mice, and was up-regulated during adipogenic differentiation of 3T3-L1 cells. In addition, we found that miR-378a-3p could target MAPK1 directly, whose expression level is dramatically decreased at later stages of adipogenesis. Overexpression of miR-378a-3p or knockdown of endogenous MAPK1 using a specific siRNA enhanced adipogenesis whereas blockage of endogenous miR-378a-3p suppresses this process. These data uncover a potential mechanism of Mir378-3p in regulating adipogenesis, which may deepen our understanding of obesity.

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; HFD, high fat diet; MAPK, mitogen-activated protein kinase; MUT, mutated; NC, negative control; PPARGC1B, peroxisome proliferator-activated receptor gamma coactivator-1β; UTR, untranslated region; WT, wild-type.

* Corresponding authors at: Tsinghua Campus, University Town, Shenzhen 518055, China.

E-mail addresses: xu.naihan@sz.tsinghua.edu.cn (N. Xu), zhangyo@sz.tsinghua.edu.cn (Y. Zhang).

[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.12.055
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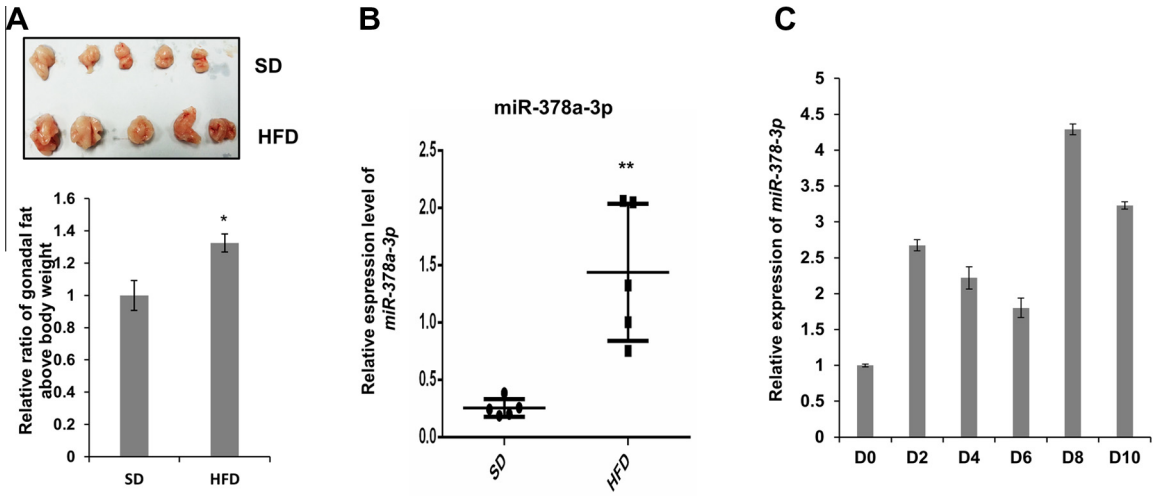

Fig. 1. MiR-378a-3p expression is up-regulated in HFD-fed mice and during adipogenic differentiation of 3T3-L1. (A) Representative images of gonadal fat of 4-week-old HFD- or SD-fed mice and the average ratios of gonadal fat to body weight of the HFD- and SD-fed mice. (B) The relative levels of miR-378a-3p in gonadal fat from HFD- or SD-fed mice ($n = 5$ per group). The expression level of RNU6 was used as control. $^{*}P < 0.05$ by a two-tailed Student's t test. (C) The expression level of miR-378a-3p during 3T3-L1 cell differentiation. Data are shown as the mean ± SD of three independent experiments. The expression level of RNU6 was used as control. $^{*}P < 0.05$ by a two-tailed Student's t test.

2. Materials and methods

2.1. Cell culture and differentiation

The 3T3-L1 preadipocytes (ATCC, CL-173) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, 8113281) supplemented with 10% calf serum (Gibco, 26170043) and 10 U/ml penicillin–streptomycin (Gibco, 15140-122), and were incubated at 37 °C in a humidified incubator containing 5% CO_2. For adipocyte differentiation, confluent 3T3-L1 cells were incubated in culture medium containing 0.5 mM methylisobutylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin for 2 days. The cells were then transferred into maintenance medium comprising DMEM supplemented with 10% FBS (PAA, A15–101) and 1 μg/ml insulin, and cultured for a further 2 days. Finally, the cells were cultured in DMEM containing 10% FBS for 4–6 days and the medium was replaced every two days.[35] HeLa cells were grown in DMEM supplemented with 10% FBS and 10 U/ml penicillin–streptomycin (Gibco/Invitrogen, 15140-122), and were incubated at 37 °C in a humidified incubator containing 5% CO_2.

2.2. Transfection of miRNAs and siRNAs

All miRNA mimics, miRNA inhibitors, and siRNAs were purchased from Shanghai GenePharma Co. Ltd. Sequences of the specific miRNA and siRNA were as follows: miR-378a-3p mimic: sense, 5′ ACUGGACUUGGAGUCAGAAGG 3′; and antisense: 5′ CCUUCUGACUCCAAGUCCAGU 3′; siMAPK1: sense, 5′ GAUCUGAAUUGUAUAAUAATT 3′; and antisense, 5′ UUAUUAUACAAUUCAGAUCTT 3′. A small RNA with a random sequence was used as a NC. The 3T3-L1 preadipocytes were seeded into 24-well plates at a density of 3×10^4 cells/well, and suspended transfection was performed at the same time using Lipofectamine 2000 (Invitrogen, 11668-019), according to the manufacturer's instruction.

2.3. Quantitative RT-PCR

Total RNA was extracted from cells using RNAiso Plus (Takara, #9108), according to the manufacturer's instructions. RT-PCR was performed using SYBR Green Realtime PCR Master Mix (TOYOBO, QPK201) and a quantitative PCR detection system (ABI, 7300). Gene expression was normalized to that of β-actin, and miRNA expression was normalized to that of *Rnu6*.

2.4. Oil-Red O staining

On the eighth day of adipocyte differentiation, 3T3-L1 cell monolayers were fixed with 10% methanal at room temperature for 30 min. The fixed samples were then stained with 0.35% Oil-Red O/isopropanol alcohol solution for 1 h at room temperature. The stained adipocytes were photographed using a phase contrast microscope. To quantify the lipids, the stained cells were eluted with isopropanol and the OD510 values were determined using a spectrophotometer.

2.5. Western blot

The cells were lysed in ice-cold cell lysis buffer and the protein concentration was measured and equalized. Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBST and then incubated with a primary anti-MAPK1 (CST, #9108) or anti-β-actin (Proteintech, #60008) antibody, followed by horseradish peroxidase-coupled specific secondary antibodies. Protein bands were detected with ECL blotting detection reagents (KPL, 547100).

2.6. Luciferase activity assay

The 3′UTR of MAPK1 containing WT or MUT miR-378a-3p binding sites were inserted at the end of the polyA region downstream of the luciferase gene in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, 9PIE133). For luciferase activity assays, HeLa cells were co-transfected with the WT or MUT constructs and NC or miR-378a-3p mimics for 24 h using Lipofectamine 2000. Luciferase activities were measured using the Luciferase Reporter assay system (Promega, E1980). The firefly luciferase activity was normalized to that of *Renilla* luciferase. Each experiment was repeated independently in triplicate.

2.7. Animal experiments

Four-week old male NIH mice were fed a SD or HFD for 4 weeks, and then anaesthetized for the collection of gonadal fat. Water and

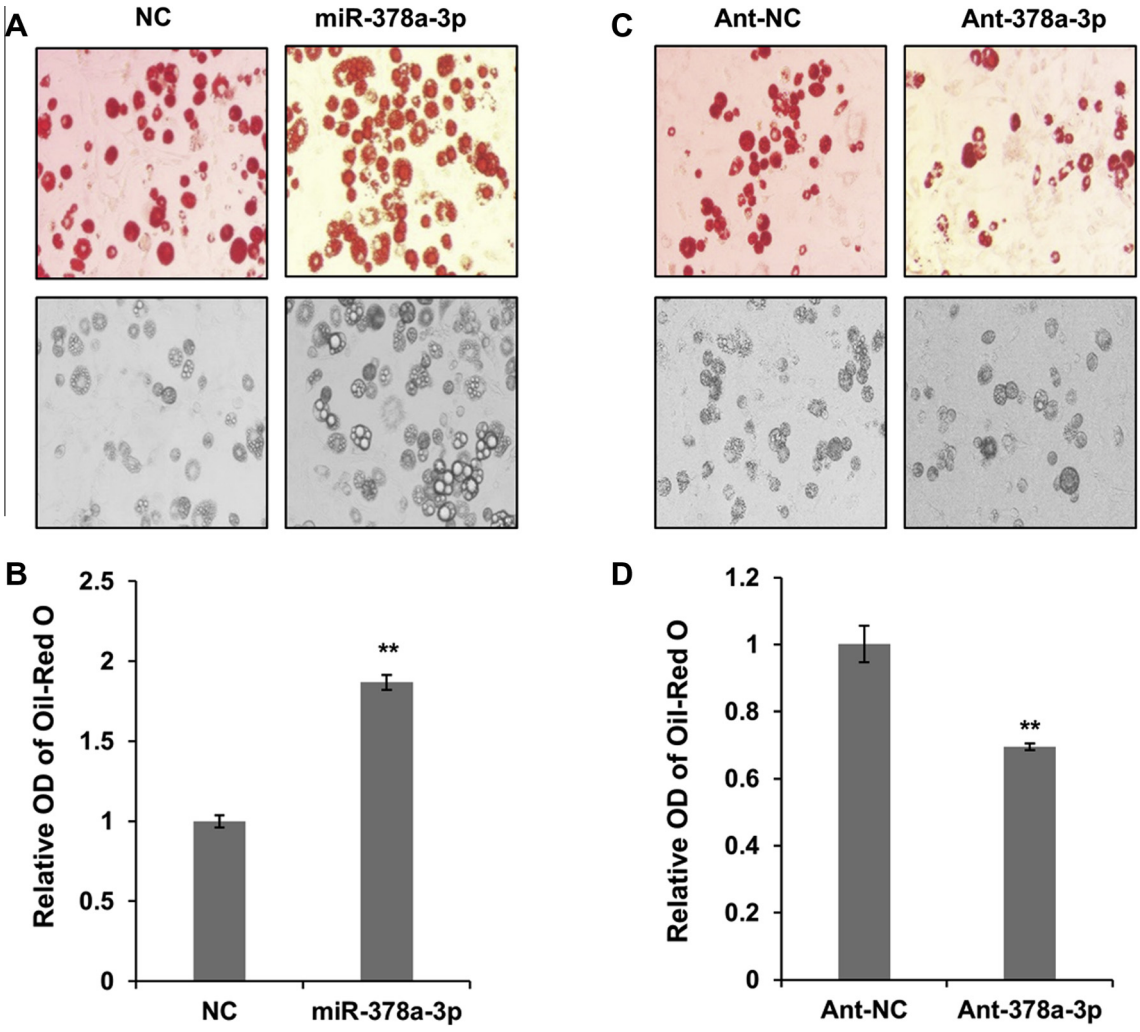

Fig. 2. Mir378a-3p enhances the adipogenesis of 3T3-L1 cells. (A) MiR-378a-3p-mediated enhancement of the adipogenesis of 3T3-L1 cells shown by white light and Oil-Red O-stained images. (B) The relative OD510 values of Oil-Red O staining of adipocytes transfected with miR-378a-3p or a negative control (NC) mimic. Data shown as the mean ± SD of three independent experiments. $^{**}P < 0.01$ by a two-tailed Student's *t* test. (C) Representative White light and Oil-Red O stained images of 3T3-L1 cells transfected with Ant-378a-3p or Ant-NC. The cells were differentiated for 8 days prior to imaging. (D) The relative OD510 values of Oil-Red O staining of were determined by spectrofluorometer. Data are shown as the mean ± SD of three independent experiments. $^{**}P < 0.01$ by a two-tailed Student's *t* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

food were available ad libitum. Total RNA extracted from the gonadal fat was used for qRT-PCR analyses. The animal experiments were carried out according to the requirements of EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

3. Results

3.1. MiR-378a-3p expression is up-regulated in HFD-fed obese mice and during adipogenic differentiation of 3T3-L1

Previous studies showed that miR-378a knockout mice are resistant to HFD-induced obesity. Therefore we generated HFD-fed obese mice and SD-fed normal mice models. HFD-fed mice had much more gonadal fat than SD-fed mice and a higher ratio of gonadal fat to body weight (Fig. 1A). In addition, the expression level of miR-378a-3p was higher in gonadal fat from HFD-fed obese mice than that from SD-fed mice (Fig. 1C). To investigate the precise molecular mechanisms of the effects of miR-378a-3p on obesity and adipogenesis, we measured the expression pattern of this miRNA during 3T3-L1 cells differentiation. These preadipocytes were induced to differentiate by culturing in induction buffer containing insulin, methylisobutylxanthine, and dexamethasone. Samples were collected every other day for qRT-PCR analyses using ABI TaqMan probes. As shown in Fig. 1C, the expression level of miR-378a-3p was upregulated during 3T3-L1 adipogenesis. These results suggests that miR-378a-3p may play a role in adipogenesis and obesity.

3.2. MiR-378a-3p promotes adipogenesis in 3T3-L1 cells

To investigate the role of miR-378a-3p in adipogenesis further, we transfected miR-378a-3p mimics into 3T3-L1 preadipocytes, which were then induced to adipogenic differentiation Enforced expression of miR-378a-3p enhanced adipogenesis significantly, as demonstrated by staining of the lipid droplets using Oil-Red O (Fig. 2A). The stained samples were eluted by isopropanol and quantified by measuring the absorbance at 510 nm (OD510) using a spectrophotometer. As shown in Fig. 2B, the lipid accumulation quantified using this method coincided with the lipid droplet staining results. To further document the physiological relevance of miR-378-3p on adipogenesis, we inhibited the expression of

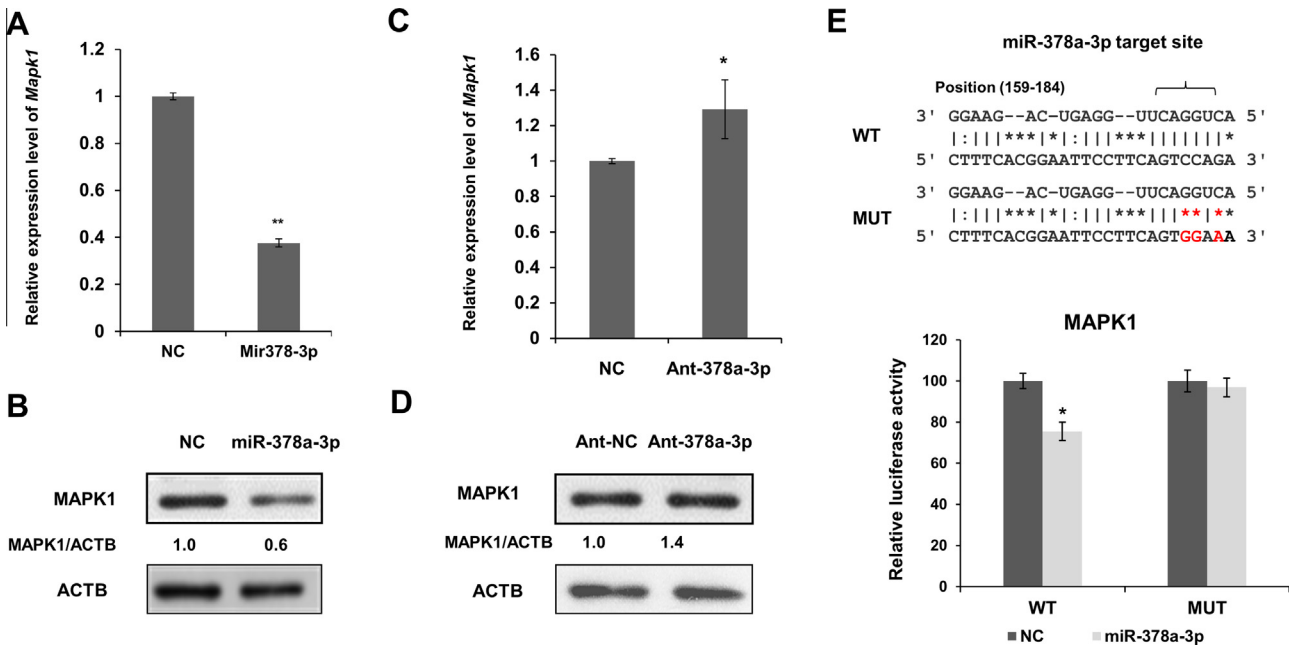

Fig. 3. MAPK1 is a direct target of miR-378a-3p. (A) Quantitative RT-PCR analyses of *Mapk1* mRNA levels in 3T3-L1 cells transfected with miR-378a-3p or NC mimics. Data are shown as the mean ± SD of three independent experiments. $^{**}P < 0.01$ by a two-tailed Student's *t* test. (B) Western blot analyses of MAPK1 in 3T3-L1 cells transfected with miR-378a-3p or NC mimics. (C, D) The relative *Mapk1* mRNA (C) and MAPK1 protein (D) levels in 3T3-L1 cells transfected with Ant-378a-3p or Ant-NC. Data are shown as the mean ± SD of three independent experiments. $^{*}P < 0.05$ by a two-tailed Student's *t* test. The expression level of ACTB was used as loading control. (E) Bioinformatics-based prediction of miR-378a-3p target sites in the 3′UTR of the *Mapk1* mRNA. Red letters indicated the mutated sites in the miR-378a-3p seed region. Relative luciferase activities 30 h after co-transfection of HeLa cells with miR-378a-3p or NC mimics and a luciferase reporter vector containing the WT or MUT 3′UTR of *Mapk1*. Data are shown as the mean ± SD of three independent experiments. $^{*}P < 0.05$ by a two-tailed Student's *t* test.

endogenous miR-378a-3p with specific antagomir (ant-378-3p). Compared with negative control (ant-NC), ant-378a-5p slightly reduced lipid droplets accumulation, as revealed by quantification of Oil-Red O staining (Fig. 2C and D). Collectively, these data suggest that miR-378a-3p promotes adipogenesis in 3T3-L1 cells.

3.3. MAPK1 is a direct target of miR-378a-3p

Bioinformatics-based predictions can be used to identify candidate miRNA targets. Using TargetScan [36] and FindTar [37], we found that MAPk1 was a putative target of Mir378-3p. MiRNAs exert their function by binding to complementary sequences in the 3′UTRs of target mRNAs, and then inducing mRNA cleavage or translation repression. To examine the effect of miR-378a-3p on MAPK1 expression, 3T3-L1 cells were transfected with miR-378a-3p, negative control (NC) mimics, ant-miR-378a-3p or ant-NC, and then total mRNAs and proteins were harvested for qRT-PCR and western blot assays. Overexpression of miR-378a-3p suppressed the expression of *Mapk1* mRNA and MAPK1 protein significantly (Fig. 3A and B), and blocking endogenous miR-378a-3p increased MAPK1 expression slightly (Fig. 3C and D). To determine whether miR-378a-3p binds directly to its predicted target sites in the *Mapk1* 3′UTR, we performed luciferase reporter assay. Luciferase reporter plasmids containing either the wide type (WT) or the mutated (MUT) miR-378a-3p binding sites in MAPK1 3′UTR region were co-transfected with miR-378a-3p or NC. Overexpression of miR-378a-3p reduced the luciferase activity of the WT but not the mutated reporter vector; indicating that miR-378-3p directly binds to the 3′UTR of MAPK1 (Fig. 3E).

3.4. MiR-378a-3p promotes adipogenesis by targeting MAPK1

To investigate its role in adipogenesis, we measured the expression level of MAPK1 during this process. Consistent with the upregulation of miR-378-3p, the level of MAPK1 protein markedly decreased at the later stage of adipogenesis (Fig. 4A). Then, we knocked down the expression of endogenous MAPK1 by use of specific siRNA (Fig. 4B). Blockage of endogenous MAPK1 enhanced adipogenesis markedly and phenocopied the effect of miR-378a-3p on adipogenesis (Fig. 4C). We quantified the values of Oil Red O staining, the results showed that miR-378a-3p can promote adipogenesis via downregulation of MAPK1 (Fig. 4D).

4. Discussion

Adipogenesis is a process by which preadipocytes are differentiated to lipid droplet-filled adipocytes. Obesity, which is now a severe global health problem, is characterized by increased volumes and numbers of adipocytes; to deal with this problem, we require a better understanding of the precise regulatory mechanisms of adipogenesis. MiRNAs are important regulators of adipogenesis and lipid metabolism. Some miRNAs, such as miR-143, miR-20a, miR-103, miR-375, and members of the miR-30 family, are proadipogenic factors. However, other miRNAs, including miR-130, miR-155, and miR-27, negatively regulate adipogenesis [13–16]. Mir-378 reportedly plays important roles in adipogenesis and oxidative energy metabolism [38,39]. Here, we explored the mechanism of miR-378a-3p in adipogenesis. We show that adipogenic stimuli induce miR-378a-3p upregulation in 3T3-L1 cells. MiR-378a promotes adipogenesis and lipid accumulation via directly targeting MAPK1. MiR-378 is also upregulated in HFD-induced obese mice, our data thus provide a novel link between miRNA and obesity.

MAPK intracellular signalling pathway plays a pivotal role in a number of essential physiological process, including cellular differentiation, proliferation, and development. The role of MAPK in adipogenesis is complex. Some studies suggest that MAPK inhibits the process of adipocytic differentiation of 3T3-L1 cells [33,40]. Other

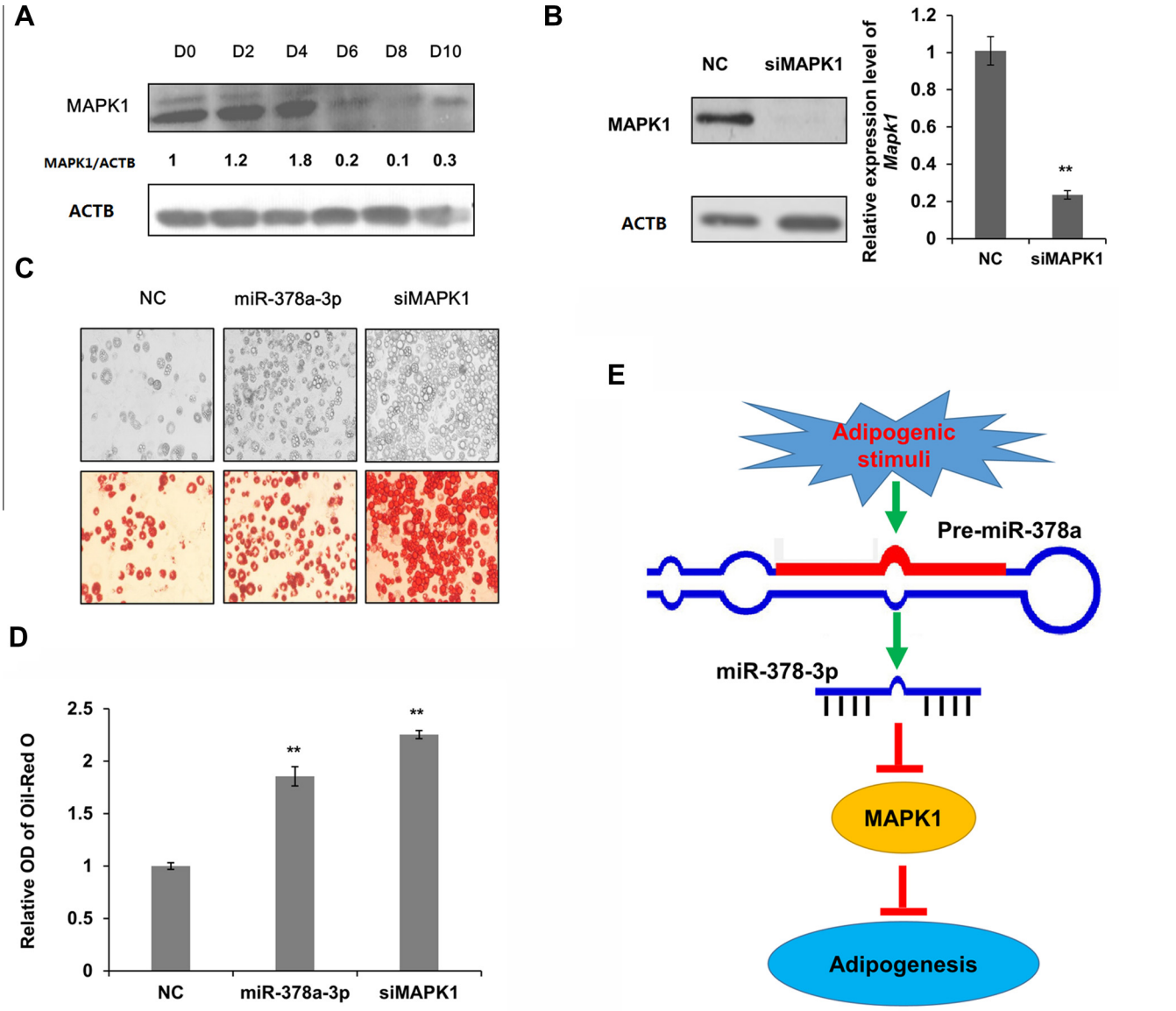

Fig. 4. MiR-378a-3p enhances adipogenesis by targeting MAPK1. (A) Western blot analysis of MAPK1 expression during 3T3-L1 differentiation. The expression level of ACTB was used as a loading control. (B) Western blot and qRT-PCR analyses of *Mapk1* mRNA and MAPK1 protein levels in 3T3-L1 cells transfected with siMAPK1 or a NC siRNA. The protein and mRNA expression levels were normalized to those of ACTB. Data are shown as the mean ± SD of three independent experiments. $^{**}P < 0.01$ by a two-tailed Student's *t* test. (C) Representative White light and Oil-Red O-stained images of 3T3-L1 cells transfected with miR-378a-3p mimics, siMAPK1 or a negative control siRNA. The cells were differentiated for 8 days prior to imaging. (D) The relative OD510 values of the Oil-Red O stained cells described in (C). Data are shown as the mean ± SD of three independent experiments. $^{**}P < 0.01$ by a two-tailed Student's *t* test. (E) Proposed model of the role of miR-378a-3p in adipogenesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

report show that MAPK is necessary to initiate the preadipocyte into differentiation process [32,41], thereafter, this signal transduction pathway needs to be shut-off to proceed with adipocyte maturation [40,42]. It is possible that both of these opinions are correct, depending on the precise context or timing. Adipogenesis is a complex process that involves several successive steps, and MAPK1 may have different roles in each step. In the initial stage of adipogenesis, 3T3-L1 cells undergo clonal expansion during the first few hours, and MAPK1 is upregulated to activate its signalling pathway [41,43]. By contrast, in the late or terminal differentiation stage of adipogenesis, MAPK1 is down-regulated because it inhibits adipogenesis by phosphorylating and inhibiting the activity of PPAR-γ [34,44].

In this study, we used an induction medium containing insulin, dexamethasone, methylisobutylxanthine, and foetal bovine serum (FBS) to promote the differentiation of 3T3-L1 preadipocytes for 2 days. Previous studies report that adipogenic inducers activate the MAPK pathway and induce MAPK1 expression from the first few hours to day 3 of differentiation, after which the expression level and activity of MAPK1 are down-regulated [41]. Here, we show that the expression level of MAPK1 was upregulated in the first few days of induction and then downregulated during the late stage of adipogenesis, and this expression pattern was inversely correlated with that of miR-38a-3p. Therefore, our results presented here demonstrate one possible regulatory mechanism of miR-378a-3p in adipogenesis. Because miRNAs usually have multiple targets, MAPK1 may not be the only target of miR-378a-3p. More research is required to identify additional targets of miR-378a-3p and their precise roles in each stage of adipocyte differentiation.

Acknowledgments

This work was supported by National Natural Science Foundation of China (31371315), International Cooperation Grant of Shenzhen (GJHZ20140416153718941) and the overseas High-caliber Personnel Foundation of Shenzhen (KQC201109050084A). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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