miR-378 Activates the Pyruvate-PEP Futile Cycle and Enhances Lipolysis to Ameliorate Obesity in Mice

Yong Zhang, Changyin Li, Hu Li, Yipeng Song, Yixia Zhao, Lili Zhang, Haixia Wang, Ran Zhong, Huiru Tang, Dahai Zhu

Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, PR China

Peking Union Medical College, Beijing 100005, PR China

Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, PR China

Gladdstone Institute of Cardiovascular Disease and Roodenberg Center for Stem Cell Biology and Medicine at Gladstone, San Francisco, CA 94158, USA

Abstract

Obesity has been linked to many health problems, such as diabetes. However, there is no drug that effectively treats obesity. Here, we reveal that miR-378 transgenic mice display reduced fat mass, enhanced lipolysis, and increased energy expenditure. Notably, administering AgomIR-378 prevents and ameliorates obesity in mice. We also found that the energy deficiency seen in miR-378 transgenic mice was due to impaired glucose metabolism. This impairment was caused by an activated pyruvate-PEP futile cycle via the miR-378-Akt1-FoxO1-PEPCK pathway in skeletal muscle and enhanced lipolysis in adipose tissues mediated by miR-378-SCD1. Our findings demonstrate that activating the pyruvate-PEP futile cycle in skeletal muscle is the primary cause of elevated lipolysis in adipose tissues of miR-378 transgenic mice, and it helps orchestrate the crosstalk between muscle and fat to control energy homeostasis in mice. Thus, miR-378 may serve as a promising agent for preventing and treating obesity in humans.

1. Introduction

Obesity is a major cause of debilitating diseases such as type 2 diabetes, hypertension, and cardiovascular disease (Olshansky et al., 2005), which have a devastating impact on our quality of life and lifespan (Olshansky et al., 2005). Yet, our search for drugs that prevent and treat obesity has become one of the most challenging areas in drug discovery. Glucose and lipids are macromolecules that the body uses as fuel to regulate energy fluxes and meet metabolic demands. This fuel is largely stored in skeletal muscle and adipose tissues, whose metabolic communication determines how the fuel will be selected and used under physiological and pathological conditions (Li et al., 2015; Lu et al., 2014; Shimizu et al., 2015; Viscarra and Ortiz, 2013). When this communication is dysregulated, metabolic disorders, including obesity, diabetes, and metabolic syndrome, often develop (Bliski et al., 2015; Bleau et al., 2014). While much work has focused on either glucose metabolism in muscle or lipid metabolism in adipose tissues, we do not know how communication between these processes maintains metabolic homeostasis in the body.

Metabolism is regulated by microRNAs. For example, miR-378 and miR-378*, which are embedded within the first intron of the peroxisome proliferator-activated receptor γ coactivator 1β (Ppargc1b or Pgc-1β) gene, are preferentially expressed in metabolically active tissues, skeletal muscle, and brown adipose tissue (BAT) (Carrer et al., 2012; Eichner et al., 2010). They also critically regulate fatty acid metabolism in mitochondria by targeting carnitine O-acetyltransferase (Crt) and Mediator 13 (MED13) in liver (Carrer et al., 2012). Hepatic miR-378 controls liver glucose and lipid homeostasis by modulating insulin signaling (Liu et al., 2014), and miR-378 stimulates brown-fat expansion by targeting phosphodiesterase 1b (Pde1b) (Pan et al., 2014). Furthermore, miR-378* induces a glycolytic shift by targeting the PGC-1β partners, ERRγ and CARβA, in breast cancer cells (Eichner et al., 2010), and levels of miR-378 expression is inversely correlated with the loss of adipose tissue in cancer cachexia in humans (Kulyte et al., 2014).

Here, we report that miR-378 prevents and treats obesity in mice by activating the pyruvate-phosphoenolpyruvate (pyruvate-PEP) futile cycle in the muscle and enhancing lipolysis in adipose tissues. Notably, we also show that transgenic mice globally overexpressing miR-378 provide a genetic system for investigating the metabolic crosstalk between different tissues at whole-body levels. Our significant findings support an unprecedented role of the pyruvate-PEP futile cycle in stimulating lipolysis in adipose tissues to regulate whole-body energy homeostasis.

Keywords:
- miR-378
- Futile cycle
- Lipolysis
- Energy homeostasis
- Obesity
- SCD1

Article history:
Received 13 November 2015
Received in revised form 25 January 2016
Accepted 28 January 2016
Available online 11 March 2016

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
2. Materials and Methods

2.1. Mice and Animal Care

All animal procedures were approved by the Animal Ethics Committee of Peking Union Medical College, Beijing (China). Mice were housed in the animal facility and given free access to water and a standard diet (SD) of rodent chow or a high-fat diet (HFD) according to the experimental design. miR-378 transgenic (Tg) mice were generated by the Model Animal Research Center of Nanjing University. Two transgenic lines were established from two founders identically created with the same plasmid DNA construct containing miR-378 precursor sequences and the beta-actin promoter for driving the transgene miR-378 expression (Figure S1A), and the gender- and age-matched wild-type littermates were served as control group throughout all experiments presented in the study. C57BL/6 male mice (Vital River Laboratories Company in Beijing) were used in the experiments treated with AgomiR-378.

2.2. Metabolic-Chamber Analysis

Metabolic phenotyping of wild-type (Wt, n = 8) and miR-378 Tg (n = 8) mice on a standard diet was performed using Columbus × Oxymax/CLAMS metabolic-chamber analysis at the animal center at Peking Union Medical College.

2.3. Glucose and Insulin Tolerance Tests

Overnight-fasted mice were given intraperitoneal (i.p.) injections of glucose (2 mg/g body weight) for the glucose-tolerance test (GTT). For insulin-tolerance tests (ITT), mice fasted for 4 h and then were given 0.5 mL insulin/g body weight by i.p. injection (Novolin). Blood glucose was determined with a Lifescan One Touch glucometer.

2.4. Prevention and Treatment of HFD-induced Obesity with AgomiR-378

Cholesterol-modified AgomiR-378 and control oligos were ordered from RiboBio (Guangzhou, China). For the prevention study, C57BL/6 mice were fed an HFD, or an SD as control. During HFD feeding, AgomiR-378 was consecutively administered to the mice weekly by tail–vein injection (20 μg/g body weight) for 8 weeks. AgomiR-378 and control oligos were administered to 10 male mice, respectively. For treatment experiments, C57BL/6 mice were fed an HFD for 11 weeks to induce obesity, with mice fed an SD as control. Then, obese mice were treated weekly with AgomiR-378, or scrambled oligo as control, by tail–vein injection (20 μg/g body weight) for 4 consecutive weeks. After the last injection, mice underwent GTT and ITT and were then sacrificed for sampling.

2.5. Adenovirus-Mediated Akt1 Rescue Experiments

Constitutively active Akt1, with myristylation-signal sequences (GSKSKPKSR) in its N-terminus and a Myc-tag at its C-terminus, was overexpressed in C2C12 cells transfected with miR-378 mimic and control oligos using Lipofectamine 2000. Twenty-four h after transfection, cells were lysed and evaluated for levels of Akt1 protein by Western blotting.

2.6. C2C12-Cell Culture and Transfection

C2C12-cell culture was conducted as previously described (Wu et al., 2015a, 2015b). Briefly, C2C12 cells were cultured in growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO2 atmosphere. At 50–60% confluence, cells were switched to differentiation medium (DMEM supplemented with 2% horse serum). C2C12 cells were transfected with miR-378 mimic and control oligos using Lipofectamine 2000. Twenty-four h after transfection, cells were lysed and evaluated for levels of Akt1 protein by Western blotting.

2.7. Adipogenesis In Vitro and Transfection

3T3-L1 cells were obtained from ATCC. Maintenance and adipogenesis of 3T3-L1 cells were as described previously using methylisobutyxanthine, dexamethasone, and insulin (MDI) (Hemati et al., 1997). To induce adipogenesis, cells that were confluent for 2 days (day 0) were treated with 10% FBS, 1 μM dexamethasone, 0.5 mM methylisobutyxanthine, 1 μg/ml insulin, and 5 μM troglitazone. On day 2, cells were fed DMEM containing 1 μg/ml insulin and 10% FBS. On day 4, cells were re-fed with DMEM containing 10% FBS and were transfected with AgomiR-378 or Scd1-overexpression plasmid using Lipofectamine 2000. Lipid accumulation in adipocytes was visualized by staining with Oil Red-O.

2.8. Western-Blot Analysis

Mouse tissues or cells were lysed in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, and protease and phosphatase inhibitors. Protein lysates were resolved by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and immunoblotted with primary antibodies against total Akt1 (Cell Signaling 2938, 1:100), phosphorylated Akt1 (Santa Cruz sc7985, 1:2000), total FoxO1 (Cell Signaling 2880, 1:300), phosphorylated FoxO1 (Cell Signaling 9464s, 1:300), Myc-tag (Millipore 05–724, 1:2000), GAPDH (Millipore, 1:10,000), or β-actin (Sigma, 1:20,000). Membranes were washed in TBS washing buffer for 30 min, incubated with horseradish peroxidase (HRP)—conjugated secondary antibodies (Zsbenchangjiqiao Corporation, 1:2000) for 1 h at room temperature, and washed in TBS washing buffer for 30 min. Each membrane was then placed into Detection Solution (Thermo), incubated for 1 min at room temperature, and exposed to X-ray film.

2.9. Real-Time Quantitative PCR (RT-qPCR) Analysis

Total RNA from mouse tissues or cells was extracted with Trizol reagent (Invitrogen). Expression of miR-378 was determined with the miR-378-specific TaqMan probe (Applied Biosystems) and the iQ5 multicolor Real-Time PCR Detection System (Bio-Rad). U6 snRNA was used for normalization. mRNA expression was analyzed with SsoFast EvaGreen qPCR Master Mix (Bio-Rad) and normalized to GAPDH. All primers used for RT-qPCR are listed in Table S1.

2.10. Target-Gene Prediction and Luciferase-Reporter Assays

miR-378 targets were predicted with TargetScan. The wild-type and mutant forms of 3′-UTRs in mouse Akt1 and Scd1 were amplified by PCR and cloned into the pGL3-Control vector. HEK-293 cells were co-transfected with Akt1–3′–UTR or Scd1–3′–UTR and miR-378 mimics. Empty pGL3 vector was a control. A Renilla-luciferase plasmid was co-transfected with a firefly-luciferase construct as a transfection control. The results are expressed as firefly-luciferase activity relative to Renilla-luciferase activity.

2.11. Measurement of DNA Content

DNA content was measured and used to evaluate adipocyte numbers in a given fat depot. To measure genomic DNA content, fat depots were excised, weighted, and minced. Fat samples with equal amounts were digested with 10 mM Tris buffer (pH 7.5) containing protease K.
micromole fatty acids per liter of plasma is represented as the mean ± standard deviation. Except for directly detected results, saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), monounsaturated fatty acids (MUFTAs), and polyunsaturated fatty acids (PUFTAs) were also calculated. In addition, we also calculated ratios that reflect the activities of desaturases, including D5D (C20:4n6/C20:3n6), D6D (C18:3n6/C18:2n6), SCD16 (C16:1n7/C16:0), and SCD18 (C18:1n9/C18:0).

2.18. Statistical Analysis

Values are presented as means ± s.e.m. The statistical significance of the difference between two means was calculated with a Student's t-test (two-tailed distribution, two-sample unequal variance) and displayed as p < 0.05 (one asterisk), p < 0.01 (two asterisks), or p < 0.001 (three asterisks). The tests were performed using Microsoft Excel, in which the test type is always set to two-sample equal variance.

3. Results

3.1. miR-378 Transgenic Mice Have Increased Catabolism and Reduced Fat Mass

To investigate how miR-378 controls whole-body energy homeostasis in vivo, we generated two lines (C and D lines) of transgenic mice (Tg) in which the miR-378 transgene was globally overexpressed under the control of the β-actin promoter (pCAGGS) (Figs. 1A, 1B, 1A, and 1B). miR-378 Tg mice had significantly reduced body weight compared to gender- and age-matched wild type littermates (Figs. 1C, 1C, 1C, and 1E). They showed the greatest reduction in organ mass in the muscle, inginal white adipose tissue (IWAT), and gonadal white adipose tissue (gWAT) (Fig. 1D, E, and F), but no change in the mass of other organs (Fig. 1F). Interestingly, we did not observe a change in the number of adipocytes (Fig. 1G) or the expression of adipogenesis marker genes (Fig. 1H), indicating that adipogenesis was not affected in miR-378 mice. However, these mice had smaller adipocytes in both BAT and WAT, indicating increased lipid catabolism in fat tissues (Fig. 1I and J). They also displayed increased expression of lipid metabolism genes (Fig. 1K). These findings suggest that the reduced fat mass in miR-378 Tg mice is mainly due to reduced adipocyte size caused by significantly increased lipid catabolism rather than reduced numbers of adipocytes in adipose tissues. Because our two lines of miR-378 Tg mice have a similar phenotype, we further characterized only one of our lines in detail.

3.2. miR-378 Tg Mice Display Increased Energy Expenditure

To further validate the enhanced catabolism in adipose tissues of miR-378 Tg mice, we measured energy influx and consumption in the whole bodies of both miR-378 Tg and wild-type (WT) mice with metabolic-chamber analysis. miR-378 Tg mice had a significant overall increase in body O2 consumption (Figs. 2A and 2A), CO2 production (Figs. 2B and 2B), and energy expenditure (Figs. 2C and 2C). These results were correlated with significantly increased expression of Ucp1 in BAT of miR-378 Tg mice (Fig. 2D). Interestingly, these mice consumed remarkably more food and water than controls (Fig. 2D and 2E). These data show that miR-378 Tg mice increased their energy expenditure under normal physiological conditions.

Next, we determined the metabolic mechanism underlying the increased energy expenditure in miR-378 Tg mice. First, we found that expression of lipolytic genes was increased in miR-378 Tg mice (Fig. 2E), supporting enhanced lipolysis in WAT. We also found increased expression of genes related to lipid catabolism in both BAT and skeletal muscle of miR-378 Tg mice (Figs. 2F and 2F). As consequence of the increased energy expenditure, we observed decreased levels of free fatty acids (FFA) in serum of miR-378 Tg mice compared to their WT littermates (Fig. 2G).
We then examined glucose metabolism in our miR-378 Tg mice. We found that these mice had slightly elevated levels of blood glucose when they were fed ad libitum, which significantly increased at a fasting state (Fig. 2H). Consistent with this finding, miR-378 Tg mice exhibited normal insulin sensitivity during insulin-tolerance tests (ITTs) (Fig. 2I), but impaired glucose metabolism in glucose-
tolerance tests (GTTs) (Fig. 2J). Of note, the serum concentration of insulin was similar between miR-378 Tg and Wt mice (Fig. 2K). These implicate that the hyperglycemia we observed in miR-378 Tg mice was a result of dysregulated glucose homeostasis rather than decreased insulin secretion or insulin resistance in peripheral tissues. Furthermore, these data support that the energy deficiency we observed in miR-378 Tg mice is a result of elevated lipolysis and impaired glucose metabolism.

3.3. miR-378 Tg Mice Are Resistant to Obesity When Fed a High-Fat Diet

Next, we tested if miR-378 protects mice against obesity induced by a high-fat diet (HFD). We found that miR-378 Tg mice had a significantly lower body weight than Wt mice after 12-weeks on a HFD (Fig. 3A), even though both lines ate the same amount of food (Fig. 3B). Additionally, miR-378 mice has significantly less fat mass than Wt controls (Fig. 3C), which was supported by a reduced number of adipocytes.
Consistent with this finding, we observed significantly improved GTT and ITT in miR-378 Tg mice on an HFD (Fig. 3G and H).

3.4. Systemically Administering miR-378 Prevents and Ameliorates Obesity in Mice

We investigated whether systemically administering miR-378 (AgomiR-378) prevents HFD-induced obesity in C57BL/6 mice (Fig. S3A). We found that simultaneously injecting mice with AgomiR-378 while they were fed an HFD (Fig. S3B) significantly reduced their body weight (Fig. S3C) and weight gain (Fig. S3A), which was not due to reduced food intake (Fig. S3D) or lean mass (Fig. S3E). Interestingly, in AgomiR-378 mice fed an HFD, BAT mass did not change compared with control mice (Fig. 4F). We also observed smaller adipocytes and less lipid deposits in both the BAT and WAT of DIO mice treated with AgomiR-378 (Fig. 4H). AgomiR-378 treatment did not alter food intake (Fig. S4C) or lean mass (Fig. S4D) in DIO mice; however, they displayed improved GTT (Fig. 4I) and ITT (Fig. 4J) compared with their control counterparts.

Next, we treated mice with diet-induced obesity (DIO) by systemically administering AgomiR-378 for four weeks (Fig. S4A). At end of treatment, DIO mice treated with AgomiR-378 had reduced body weight and weight gain compared to control mice (Fig. S4B and Fig. 4F). Notably, in AgomiR-378-treated mice, we also found that WAT mass was remarkably decreased (31%), while BAT mass did not change compared with control mice (Fig. 4G). We also observed smaller adipocytes and less lipid deposits in both the BAT and WAT of DIO mice treated with AgomiR-378 (Fig. 4H). AgomiR-378 treatment did not alter food intake (Fig. S4C) or lean mass (Fig. S4D) in DIO mice; however, they displayed improved GTT (Fig. 4I) and ITT (Fig. 4J) compared with their control counterparts.

3.5. The Pyruvate-PEP Futile Cycle in Skeletal Muscle Causes Energy Deficiency in miR-378 Tg Mice

We speculated that the impaired glucose metabolism in miR-378 Tg mice might be the primary cause of their energy deficiency. Interestingly, we found that miR-378 Tg mice displayed increased expression of key...
glycolytic genes (Fig. 5A) and enhanced activity of α-glycerophosphate dehydrogenase (α-GPDH) in their muscle tissues (Fig. 5B), indicating that they had significantly enhanced glycolytic activities. However, we did not detect differences in the levels of lactate in either their serum (Fig. S5A) or skeletal muscle (Fig. S5B). Consistent with these findings, mRNA levels of lactate dehydrogenase (Ldh) in muscle was not different between miR-378 Tg and Wt mice (Fig. S5C). Surprisingly, we detected lower levels of pyruvate in the muscle of miR-378 Tg mice than Wt mice.

Fig. 4. Systematic administration of miR-378 prevents and ameliorates obesity in mice. (A–E) C57BL/6 mice fed a high-fat diet (HFD) or standard diet (SD) as control for 10 weeks. During HFD feeding, cholesterol modified AgomiR-378 (HFD-378) was administered (20 μg/g body weight) weekly for 8 consecutive weeks, which significantly prevented HFD-induced obesity (DIO) compared to control oligos (HFD-Con). n = 10 male mice in each group. (A) Reduced body-weight gain. (B) Reduced white-fat mass. (C) Reduced size of adipocytes and lipid droplets by Hematoxylin and Eosin staining (H&E). Scale bar, 20 μm. (D) Improved glucose-tolerance test (GTT). (E) Corrected insulin-tolerance test (ITT). (F–J) C57BL/6 mice were fed an HFD for 11 weeks to induce obesity (DIO). DIO mice were treated weekly with AgomiR-378 (HFD-378) or control oligos (HFD-Con) for 4 consecutive weeks. AgomiR-378 treatment (20 μg/g body weight) significantly ameliorates HFD-induced obesity compared to controls (HFD-Con). n = 10 male mice in each group. (F) Reduced body-weight gain. (G) Reduced white-fat mass. (H) Reduced size of adipocytes and lipid droplets from Hematoxylin and Eosin (H&E) staining. Scale bar, 20 μm. (I) Improved GTT. (J) Corrected ITT. Data are presented as means ± s.e.m. The statistical significance of the difference between two means was calculated using Student’s unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001. NS, statistically non-significant. See also Figs. S3 and S4.
controls (Fig. 5C), which was not caused by an enhanced activity of pyruvate dehydrogenase (PDH) (Fig. 5D), which converts pyruvate to acetyl-CoA. Biochemically, a futile cycle occurs when two metabolic pathways run simultaneously in opposite directions, thereby not causing an effect other than to dissipate energy in the form of heat (Schwender et al., 2004). Because increased glycolytic activity did not elevate pyruvate in the muscle tissues of miR-378 Tg mice, we reasoned that pyruvate was converted back to phosphoenolpyruvate (PEP) by a reverse reaction in the pyruvate-PEP futile cycle (Boiteux and Hess, 1981), which is catalyzed by pyruvate carboxlase (PC) and phosphoenolpyruvate carboxykinase (PEPCK). Indeed, we observed significantly increased levels of mRNA (Fig. 5E) and enzymatic activity (Fig. 5F) of PC and PEPCK in the muscle of miR-378 Tg mice compared with Wt controls. We also found that these tissues displayed remarkably decreased levels of acetyl-CoA (Fig. 5G) and ATP (Fig. 5H). Notably, feeding these mice an HFD restored Pepck expression to levels similar to that of Wt mice fed a standard diet (SD) (Fig. 5I), indicating that the pyruvate-PEP futile cycle was attenuated when miR-378 Tg mice were provided sufficient energy with an HFD (Fig. 5I). These data implicate that miR-378 activates the pyruvate-PEP futile cycle in skeletal muscle to at least partially cause the energy deficiency seen in miR-378 Tg mice under normal physiological conditions. This implication may explain why miR-378 Tg mice resist HFD-induced obesity (Fig. 3).

3.6. miR-378 Activates the Pyruvate-PEP Futile Cycle via the Akt1-FoxO1-PEPCK Pathway

Next, we investigated the molecular mechanism behind how miR-378 activates the pyruvate-PEP futile cycle in the muscle of miR-378 Tg mice. To identify its target, we first assessed the expression of reported miR-378 targets (Carrer et al., 2012; Eichner et al., 2010; Pan et al., 2014; Ruckerl et al., 2012) (i.e., Pde1b, Pde3b, Crt, Med13, Erry, and Akt1) (Fig. 5A6) and found that only Akt1 expression was significantly
decreased in muscle cells (Figs. S6B and S6C) and tissues of miR-378 Tg mice (Fig. 6A). To learn how miR-378 and Akt1 work together to regulate the pyruvate-PEP futile cycle in these mice, we sought an Akt1-regulated substrate. Interestingly, the transcription factor FoxO1 is a direct substrate of Akt1 (Biggs et al., 1999; Nakae et al., 2001; Rena et al., 1999), and FoxO1 transcriptionally regulates Pepck, the key rate-limiting enzyme in the pyruvate-PEP futile cycle (Puigserver et al., 2003). Therefore, we speculated that miR-378 activates the pyruvate-PEP futile cycle by targeting the Akt1-FoxO1-Pepck pathway. Indeed, we found an increase in the hypophosphorylated (active) form of FoxO1 in the muscle of miR-378 Tg mice. Total FoxO1 protein levels were not overtly different between miR-378 Tg and Wt mice (Fig. 6A).

To confirm that miR-378 activates the pyruvate-PEP futile cycle by targeting the Akt1-FoxO1-Pepck pathway, we restored the protein levels of Akt1 in miR-378 Tg mice with adenovirus containing a form of Akt without the miR-378-targeting sequence in its 3′-UTR (Ad-CaAkt) (Figs. S6D and S6E). This adenovirus restored protein expression of both total Akt and p-Akt in the muscle of miR-378 Tg mice to levels comparable to Wt mice (Fig. 6B). Moreover, the phosphorylated form of FoxO1 was expressed at similar levels in the muscle of miR-378 Tg and Wt mice (Fig. 6B). Furthermore, restoring Akt1 rescued Pepck expression in the muscle of miR-378 Tg mice to the levels comparable to Wt mice (Fig. 6C).

More significantly, in miR-378 Tg mice, restoring Akt1 attenuated lipolysis in WAT (Fig. 6D) and fatty acid use in both BAT (Fig. 6E) and muscle (Fig. 6F). It also restored FFA levels in serum of miR-378 Tg mice (Fig. 6G). Thus, activation of the pyruvate-PEP futile cycle in the muscle is the primary cause for elevated lipolysis in adipose tissues of the miR-378 Tg mice.

3.7. miR-378 Stimulates Lipolysis in WAT by Targeting Scd1

We then examined what miR-378 might target in adipose tissue to promote lipolysis. Interestingly, expression of all the known miR-378 targets, including Akt1, remained unchanged in both BAT and WAT of miR-378 Tg mice (Figs. S6F and S6G). We then searched for additional
miR-378 targets with TargetScan and miRanda. Fortunately, we found that the mRNA of stearoyl-CoA desaturase 1 (Scd1) contains a miR-378 binding site in its 3′-UTR (Fig. 7A). Scd1 catalyzes the production of monounsaturated fatty acids to regulate lipid partitioning between lipogenesis and oxidation (Ntambi, 1995, 1999). Interestingly, Scd1 knockout (Scd1<sup>−/−</sup>) mice display a similar phenotype to that of miR-378 Tg mice, including a lean body type and resistance to obesity (Cohen et al., 2002; Miyazaki et al., 2001; Ntambi et al., 2002). We first validated that Scd1 is a target of miR-378 in HEK-293 cells with luciferase-reporter assays (Fig. 7B). Then we evaluated its expression in adipocytes and found that endogenous Scd1 was reduced in adipocytes overexpressing miR-378 (Fig. 7C). Interestingly, we also found that overexpressing miR-378 upregulated lipolytic genes in adipocytes (Fig. 7D). Consistent with our <em>in vitro</em> data, we observed reduced Scd1 expression (Fig. 7E) and decreased Scd1 activity by measuring cellular fatty-acid ratios (C16:1/C16:0 and C18:1/C18:0) in both BAT and WAT of miR-378 Tg mice (Fig. 7F and G). Furthermore, we observed reduced Scd1 expression in both BAT and WAT of C57BL/6 mice treated with AgomiR-378 and fed an HFD (Fig. 7H). Together, these data support that miR-378 promotes lipolysis in adipose tissues by targeting Scd1 both <em>in vitro</em> and <em>in vivo</em>.

4. Discussion

In this report, we not only uncover the pharmacological function of miR-378 in preventing and treating obesity, but we also unravel a unique function of miR-378 in regulating metabolic communication between the muscle and adipose tissues to control energy homeostasis at whole-body levels. miR-378 activates the pyruvate-PEP futile cycle by targeting the Akt1-FoxO1-PEPCK pathway in the muscle, which then enhances lipolysis via miR-378 targeting Scd1 in adipose tissues. Importantly, we demonstrate that miR-378 activates the pyruvate-PEP futile cycle in skeletal muscle to control whole-body energy homeostasis in mice.

Glycolysis is a metabolic pathway in which glucose is converted to pyruvate to generate two molecules of ATP per molecule of glucose.

---

**Fig. 7.** miR-378 stimulates lipolysis in WAT by targeting Scd1. (A) Alignment of miR-378-target sequence in the Scd1 3′-UTR predicted with TargetScan. The nucleotide coordinate of Scd1 was based on the mouse Refseq (NM_009127). Indicated form of UTR (m-utr) was mutated from the seed matches. (B) Effect of miR-378 or a non-specific-control oligonucleotide (NC) on luciferase activity in HEK293 cells expressing the wild-type (wt-utr) or mutated 3′-UTR (m-utr) of Scd1. Empty pGL-3 vector served as negative control. Values are means ± s.e.m. of three independent measurements. (C) Expression (exp) of Scd1 decreased in 3T3-L1-induced adipocytes transfected with miR-378. Values are means ± s.e.m. of three independent experiments. (D) Expression of the lipolytic genes Hsl and Atgl in 3T3-L1-induced adipocytes described in (C). (E) Reduced expression of Scd1 in BAT tissues of miR-378 Tg (n = 5) and WT (n = 5) mice. (F,G) Decreased SCD1 enzymatic activities in BAT and WAT tissues of Tg mice evidenced by reduced ratios of (F) C16:1/C16:0 and (G) C18:1/C18:0. (H) Reduced expression of Scd1 in BAT (left) and WAT (right) tissues of AgomiR-378 treated DIO mice (n = 6). Data are presented as means ± s.e.m. The statistical significance of the difference between two means was calculated using Student’s unpaired t-test. *P < 0.05.
Glycolysis is also regulated by futile cycles (Bali and Thomas, 2001), in which two metabolic pathways run simultaneously in opposite directions without an overall effect other than to dissipate energy in the form of heat. In general, futile cycles have been regarded as energetically wasteful reactions that must be avoided in metabolic pathways under normal physiological conditions. However, researchers have speculated that futile cycles may uniquely regulate metabolism to maintain energy homeostasis, especially when energy is rapidly needed (Munakata et al., 2012; Orman et al., 2012; Tolla et al., 2015); although, they have yet to provide data that supports this speculation. In this report, we demonstrated that the miR-378-Akt-FoxO1-PEPCK pathway mediates the pyruvate-PEP futile cycle. Specifically, in miR-378 Tg mice, we found that pyruvate from glycolysis cannot enter the Krebs cycle to produce energy. Instead, it is converted back to PEP in a gluconeogenesis reaction mediated by PEPCK and PC, which costs an ATP molecule and produces an energy deficiency. As a consequence of this futile cycle, more energy from lipolysis is needed to balance energy homeostasis, which is supported by our observation that miR-378 Tg mice have significantly enhanced lipolysis in their adipose tissues. These results suggest that miR-378 activates the pyruvate-PEP futile cycle to orchestrate the glucose–lipid metabolic crosstalk between the muscle and adipose tissues and maintain energy homeostasis. One implication of this finding is that any dysregulated futile cycles of either glucose or fatty acids may be the primary cause of metabolic disorders in humans. Thus, the miR-378-mediated pyruvate-PEP futile cycle or other metabolic futile cycles may serve as promising targets for developing therapies against obesity and/or other metabolic diseases.

We also revealed that Scd1 is a functional target of miR-378 that promotes lipolysis in adipose tissues of miR-378 Tg mice. Scd1 critically regulates lipid metabolism, and dysregulation of Scd1 activity results in various metabolic disorders, including diabetes, cardiovascular disease, and obesity (Dobrzyn et al., 2015; Sampath and Ntambi, 2011, 2014; Stamatakis and Paton, 2013). Similar to our miR-378 Tg mice, Scd1-knockout mouse are also resistant to HFD-induced obesity (Cohen et al., 2002; Ntambi et al., 2002). Therefore, in miR-378 Tg mice, we propose a model in which miR-378 activates the Akt1-FoxO1-PEPCK pathway in skeletal muscle to induce the pyruvate-PEP futile cycle, which impairs glucose metabolism and causes energy deficiency. Then, at the same time in adipose tissues, miR-378 directly targets Scd1 to enhance lipolysis (Fig. S7). Because of miR-378 induced glucose futile cycle, the Tg mice utilize fatty acids to meet energy demand. As a consequence, FFA in the circulation gets low despite of elevated lipolysis in adipose tissues. To validate the primary effect of miR-378-targeted Akt-FoxO1-PEPCK, we used constitutively active Akt1 (ca-Akt1) to rescue the metabolic phenotype of miR-378 Tg mice and observed significantly attenuated lipolysis in ca-Akt1 treated Tg mice. These findings suggest that miR-378 mediates the metabolic communication between muscle and fat by targeting Akt1 and Scd1 to regulate systemic energy homeostasis.

It would be very intriguing to identify signals or molecules in mediating the metabolic communications between muscle and fat tissues in miR-378 transgenic mice. Previous publications demonstrate that some metabolites may mediate the regulation of the whole body metabolic homeostasis. For example, insufficient alanine supply mediates a muscle–liver-fat signaling by upregulating FG21 expression in liver (Shimizu et al., 2015). We observed significantly lower level of pyruvate, lactate, acetyl-CoA and FFA in Tg mice than those in wild type control. All of those metabolites are indicators for energy supply, thus the data indicating miR-378 Tg mice suffer severe energy insufficiency. Identifications of the molecules or signals which convey such energy deficiency and mediate inter-organ crosstalk will help us to understand the mechanism of miR-378 in maintaining whole body metabolic homeostasis.

Clinically, our findings support a critical role for miR-378 in human metabolic disorders. Similarly, Kulyte A et al. recently reported that miR-378 was significantly upregulated in cachectic cancer patients, and its expression was strongly and positively associated with catecholamine-stimulated lipolysis in human adipocytes (Kulyte et al., 2014). Their findings suggest that increased miR-378 expression could be an etiological factor in the disease-associated loss of adipose tissue by affecting adipocyte lipolysis and inhibiting disease-induced upregulation of miR-378. Thus, miR-378 may represent a promising target for ameliorating the severity of human diseases caused by impaired lipid metabolism.

Author contributions

Y.Z. and C.L. contributed equally to this work. Y.Z. and C.L. conceived of the study, performed experiments, and drafted the manuscript. H.L. carried out the Western–blot analysis. Y.S. performed fatty acids analysis. Y.Z. conducted tail-vein injections of the Adeno-Akt1 and AgomiR-378 on Tg mice and HFD-fed mice, respectively. L.Z. carried out the luciferase-activity assay of the Scd1 experiment. H.W. participated in discussion of the experimental design and helped to draft the manuscript. R.Z. served as technical support. H.T. participated in discussion of the experimental design. D.Z. conceived of and supervised the project and wrote the manuscript. The authors declare no competing financial interests.

Acknowledgments

This work was supported by grants from the National Basic Research Program of China (2011CB01104, 2015CB943103) and the National Natural Science Foundation of China (31271470, 91019010). The funding agencies do not involve in any research activities in the present study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.01.035.

References

Bali, M., Thomas, S.R., 2001. A modelling study of feedforward activation in human erythrocyte glycolysis. C. R. Acad. Sci. III Sci Vie 324, 185–199.

Biggs, H.H., Meienhoelder, J., Hunter, T., Cavenee, W.K., Arden, K.C., 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc. Natl. Acad. Sci. U. S. A. 96, 7421–7426.

Bleau, C., Mazer-Balty, A.I., Brzozowski, B., Magierowski, M., Jasiobs, K., Krzysiek-Maczka, G., Urbanczyk, K., Pulk-Belowska, A., Zvolenska-Wciclo, M., Mach, T., et al., 2015. Moderate exercise training attenuates the severity of experimental rodent colitis: the importance of crosstalk between adipose tissue and skeletal muscles. Mediat. Inflamm. 2015, 605071.

Boiteux, A., Hess, B., 1981. Design of glycolysis. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 291, 5–22.

Carrer, M., Liu, N., Grueter, C.E., Williams, A.H., Friesld, M., Bulver, M.W., Basel-Duby, R., Olson, E.N., 2012. Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378′. Proc. Natl. Acad. Sci. U. S. A. 109, 15330–15335.

Cohen, P., Miyazaki, M., Socci, N.D., Hagge-Greenberg, A., Liedtke, W., Soukas, A.A., Sharma, R., Hudson, L.C., Ntambi, J.M., Friedman, J.M., 2002. Role for stearoyl-CoA desaturase-1 in leipotheliated weight loss. Science 297, 240–243.

Dobrzyn, P., Bednarski, T., Dobrzyn, A., 2015. Metabolic reprogramming of the heart through stearoyl-CoA desaturase. Prog. Lipid Res. 57, 1–12.

Dunn, S.E., Michel, R.N., 1997. Coordinated expression of myosin heavy chain isoforms and metabolic enzymes within overloaded rat muscle fibers. Am. J. Physiol. 293, C371–C383.

Eichner, L.J., Perry, M.C., Dufour, C.R., Bertos, N., Park, M., St-Pierre, J., Gigueure, V., 2010. miR-378(−/−) mice exhibit metabolic shift in breast cancer cells via the PGC-1beta/ERR gamma transcriptional pathway. Cell Metab. 12, 352–361.

Hemati, N., Ross, S.E., Erickson, R.L., Groblewski, G.E., MacDougald, O.A., 1997. Signaling pathways through which insulin regulates CCAAT/enhancer binding protein alpha (C/EBPalpha) phosphorylation and gene expression in 3T3-L1 adipocytes. Correlation with GLUT4 gene expression. J. Biol. Chem. 272, 29512–29519.

Kulyte, A., Lorentz-Cebrian, S., Gao, H., Meijbert, N., Agustsson, T., Arner, P., Ryden, M., Dahlman, I., 2014. MicroRNA profiling links miR-378 to enhanced adipocyte lipolysis in human cancer cachexia. Am. J. Physiol. Endocrinol. Metab. 306, E267–E274.
Lu, B., Bridges, D., Yang, Y., Fisher, K., Cheng, A., Chang, L., Meng, Z.X., Lin, J.D., Downes, M., Liu, W., Cao, H., Ye, C., Chang, C., Lu, M., Jing, Y., Zhang, D., Yao, X., Duan, Z., Xia, H., et al., Li, P., Oh da, Y., Bandyopadhyay, G., Lagakos, W.S., Talukdar, S., Osborn, O., Johnson, A., Miyazaki, M., Kim, H.J., Man, W.C., Ntambi, J.M., 2001. Oleoyl-CoA is the major de novo product of stearoyl-CoA desaturase 1 gene isosform and substrate for the biosynthesis of the Harderian gland 1-alkyl-2,3-diacylglycerol. J. Biol. Chem. 276, 39455–39461.

Munakata, K., Ookata, K., Doi, H., Baba, O., Terasima, T., Hirose, S., Kato, A., 2012. Histological demonstration of glucose transporters, fructose-1,6-bisphosphatase, and glycolgen in gas gland cells of the swimbladder: is a metabolic futile cycle operating? Biochem. Biophys. Res. Commun. 417, 564–569.

Nakae, J., Kitamura, T., Ogawa, W., Kasuga, M., Accili, D., 2001. Insulin regulation of gene expression through the forkhead transcription factor Foxo1 (Fkhrl) requires kinases distinct from Akt. Biochemistry 40, 11768–11776.

Ntambi, J.M., 1995. The regulation of stearoyl-CoA desaturase (SCD). Prog. Lipid Res. 34, 135–150.

Ntambi, J.M., 1999. Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. J. Lipid Res. 40, 1549–1558.

Ntambi, J.M., Miyazaki, M., Stoehr, J.P., Lan, H., Kendzierski, C.M., Yandell, B.S., Song, Y., Cohen, P., Friedman, J.M., Attie, A.D., 2002. Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. Proc. Natl. Acad. Sci. U. S. A. 99, 11482–11486.

Olsansky, S.J., Passaro, D.J., Hershower, R.C., Layden, J., Barnes, B.A., Brody, J., Hayflick, L., Butler, R.N., Allison, D.B., Ludwig, D.S., 2005. A potential decline in life expectancy in the United States in the 21st century. N. Engl. J. Med. 352, 1138–1145.

Orman, M.A., Androulakis, I.P., Berthiaume, F., Ierapetritou, M.G., 2012. Metabolic network analysis of perfused livers under fed and fasted states: incorporating thermodynamic and futile-cycle-associated regulatory constraints. J. Theor. Biol. 293, 101–110.

Pan, D., Mao, C., Quattrochi, B., Friedline, R.H., Zhu, L.J., Jung, D.Y., Kim, J.K., Lewis, B., Wang, Y.X., 2014. MicroRNA-378 controls classical brown fat expansion to counter obesity. Nat. Commun. 5, 4725.