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Identification of miR-106b-93 as a negative regulator of brown adipocyte differentiation

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ABSTRACT

microRNAs (miRNAs) have been reported to play an essential role in the regulation of brown adipocyte adipogenesis. In the present study, we investigated the role of the miR-106b-93 cluster in the differentiation of brown adipocytes. We found that knockdown of miR-106b and miR-93 significantly induced the expression of brown fat-specific genes and promoted the accumulation of lipid-droplet in differentiating brown adipocytes. In addition, ectopic expression of miR-106b and miR-93 suppressed the mRNA level of *Ucp1*, a selective hallmark of brown adipocytes. Furthermore, the expression levels of miR-106b and miR-93 are higher in brown adipose tissues of high fat diet-induced obese mice compared to control mice. Taken together, our results identify miR-106b and miR-93 as negative regulators of brown adipocyte differentiation and the miR-106b-93 cluster may play an important role in regulating energy homeostasis.

1. Introduction

Adipose tissues, which contain white adipose tissue (WAT) and brown adipose tissue (BAT), are an important metabolic and endocrine organ that plays a critical role in the regulation of energy homeostasis. WAT is the predominant type of fat in adult humans and mainly serves to store excess energy as lipids. BAT, on the other hand, plays a major role in thermoregulation and anti-obesity by producing heart and promoting energy expenditure, respectively [1–4]. There has been a great interest during the past few years in understanding the mechanisms controlling the formation and function of BAT because characterizing BAT function and regulation may provide attractive strategies for treating overnutrition-induced obesity and associated complications.

Recent studies have indicated that microRNAs (miRNAs) play key roles in regulating brown fat differentiation. miRNAs are a class of small non-coding RNAs of ~22 nucleotides which regulate gene expression at the transcriptional and/or translational level by specifically binding to the 3′ untranslated region (3′UTR) of target mRNAs [5]. In 2011, Sun et al. first demonstrated that the miR-193b-365 cluster markedly promoted mouse brown adipocyte adipogenesis by inhibiting *Runx1t1* expression [6]. Subsequently, miR-196a has been implicated in regulating brown adipogenesis of white fat progenitor cells [7]. The muscle-enriched miR-133 was also reported to regulate brown fat differentiation through *Prdm16* [8]. Very recently, it was demonstrated that miR-155 regulates differentiation of brown adipocytes via *C/EBP-β* [9]. However, the identities of other miRNAs that regulate brown adipogenesis remain unknown.

The miR-106b-93 cluster is located on chromosome 7 that is transcribed as a polycistronic unit. In this study, we investigated the role of miR-106b and miR-93 in brown adipocyte differentiation. We demonstrated for the first time that miR-106b-93 functions as a negative regulator of mouse brown adipogenesis.

2. Materials and methods

2.1. Cell culture and differentiation

The mouse brown preadipocyte cell line was a generous gift from Dr. Jiandie Lin, University of Michigan and has been described previously [10]. Cells were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2, and then exposed to differentiation medium containing 125 nM indomethacin (Sigma), 250 μM isobutylmethylxanthine (IBMX, Sigma), 0.5 μM dexamethasone (Sigma), 20 nM insulin (Sigma) and 1 nM triiodothyronine (T3, Sigma) for 2 days. Cells were then maintained in medium containing insulin and T3 at the same

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concentrations as described above. Medium was changed every other day and mature brown adipocytes were obtained after 7 days.

Primary mouse stromal vascular fraction (SVF) cells from BAT were fractionated and cultured according to established methods with a few modifications [11–12]. Briefly, 2-week-old C57BL/6J mice were killed. Interscapular BAT was isolated, minced, and digested with 1.5 mg/ml of collagenase (Sigma) at 37 °C in a shaking water bath for 45 min. SVF cells were collected by centrifugation, red blood cells were lysed with NH_4Cl, and the samples were then filtered through a sterile 250 μm membrane to remove undigested fragments. Primary SVF cells were incubated to fully confluence and then differentiated to functional brown adipocytes in DMEM with 10% FBS, 20 nM insulin and 1 nM T3.

2.2. Animal studies

C57BL/6J WT mice were used to establish diet-induced obesity model. All animals were housed on a daily 12-h light/dark cycle with free access to water and food in a pathogen-free animal facility at the Second Xiangya Hospital of Central South University. At 6 weeks old, male mice were fed either a normal diet (ND, SLACOM) containing 4% (wt/wt) total lipids or a high-fat diet (HFD, Research Diets) containing 60% kcal fat for 16 weeks. Interscapular BAT and visceral WAT was carefully collected and stored at −80 °C for quantitative real-time PCR. All animal experiments were performed with the approval of the local ethical committee on animal research.

2.3. Transfection of miRNA inhibitors and mimics

When brown preadipocytes were grown to 50–60% confluence, miRNA mimics (100 nM, GenePharma), locked nucleic acid (LNA) miRNA inhibitors (100 nM, Exiqon) or respective negative controls were transfected into the cells with siPORT™ *NeoFX*™ (Invitrogen) according to the manufacturer's instructions. After 48 h post-transfection, cells were induced to differentiation.

2.4. Oil red O staining

After 3 days of differentiation, brown adipocytes were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 1 h at room temperature. The staining solution was prepared by dissolving 0.5 g oil red O (Sigma) in 100 ml of isopropanol; 60 ml of this solution was mixed with 40 ml of distilled water. Cells were washed twice in PBS and then stained for 2 h at room temperature with filtered staining solution. Cells were washed with distilled water to remove unbound dye, and then stored in water for visualization and photography under the inverted microscope (Olympus).

2.5. Quantitative real-time PCR assay

For quantitative real-time PCR assays, total RNAs were extracted from mouse interscapular BAT, visceral WAT or differentiating brown adipocytes using either miRNeasy Mini Kit (QIAGEN) or TRIzol reagent (Invitrogen). For miRNA analysis, complementary DNAs (cDNAs) were synthesized from 1 μg of RNA with the One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa), and subjected to real-time PCR using the SYBR® Premix Ex Taq™ II (TaKaRa) following the manufacturer's protocols. For mRNA assay, cDNAs were synthesized from 2 μg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), real-time PCR was carried out using FastStart Universal SYBR Green Master (Roche). Specific mouse primers for each gene are listed in Supplementary Table S1. U6 and β-actin were used as internal normalization controls for miRNAs and mRNAs, respectively. All the samples were analyzed in duplicates and run on a PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems).

2.6. Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.5% NP-40, 0.1% SDS, and 50 mM Tris–Cl, pH 7.5) with 1 mM PMSF and protease inhibitor cocktail (Sigma). The cell lysates were separated by 10% SDS–PAGE gels, transferred to a nitrocellulose membrane (Millipore), and probed with primary (anti-Prdm16, anti-Ucp1, and anti-β-tubulin antibodies, Abcam) and secondary antibodies. Immunoreactive proteins were visualized with the Molecular Imager® Chemi Doc™ XRS + Western Blot Detection System (BIO-RAD), and bands were quantified with Image Lab™ software.

2.7. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using the GraphPad Prism 5 (GraphPad Software). The unpaired two-tailed Student's *t*-test was used to determine statistical differences between two groups. $P < 0.05$ was considered statistically significant.

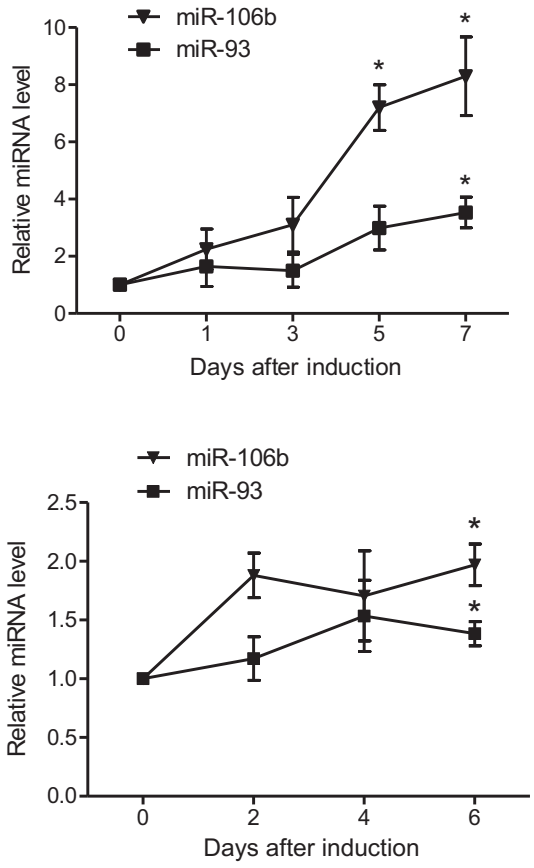

Fig. 1. Expression profiles of miR-106b and miR-93 during brown adipocyte differentiation. (A) Brown preadipocyte cell lines were stimulated to differentiate into mature brown adipocytes using differentiation medium. Cells were collected at day 0, 1, 3, 5 and 7, respectively after induction of differentiation. Real-time PCR was used to examine the expression levels of miR-106b and miR-93. $n = 4$. $^*P < 0.05$ vs. day 0. (B) SVF cells from brown fat were initiated to differentiate into classic brown adipocytes. The relative expression of miR-106b and miR-93 was measured using real-time PCR at day 0, 2, 4 and 6, respectively after induction of differentiation. $n = 3$. $^*P < 0.05$ vs. day 0.

3. Results

3.1. miR-106b and miR-93 are upregulated during mouse brown adipocyte differentiation

Several members of the miR-17 family, including miR-17-5p, miR-20a, miR-106a and miR-93, have previously been found to be expressed during early mammalian development and regulate stem cell differentiation [13]. In addition, the miR-17-92 cluster was demonstrated to accelerate adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130 [14]. Given that miR-106b and miR-93 exhibit high sequence similarity to miR-17-5p, miR-20a, and miR-106a, we asked whether these two miRNAs play a role in regulaitng brown adipocyte differentiation. By real-time PCR, we found the expression levels of miR-106b and miR-93 were gradually upregulated during brown adipocyte differentiation, which reached

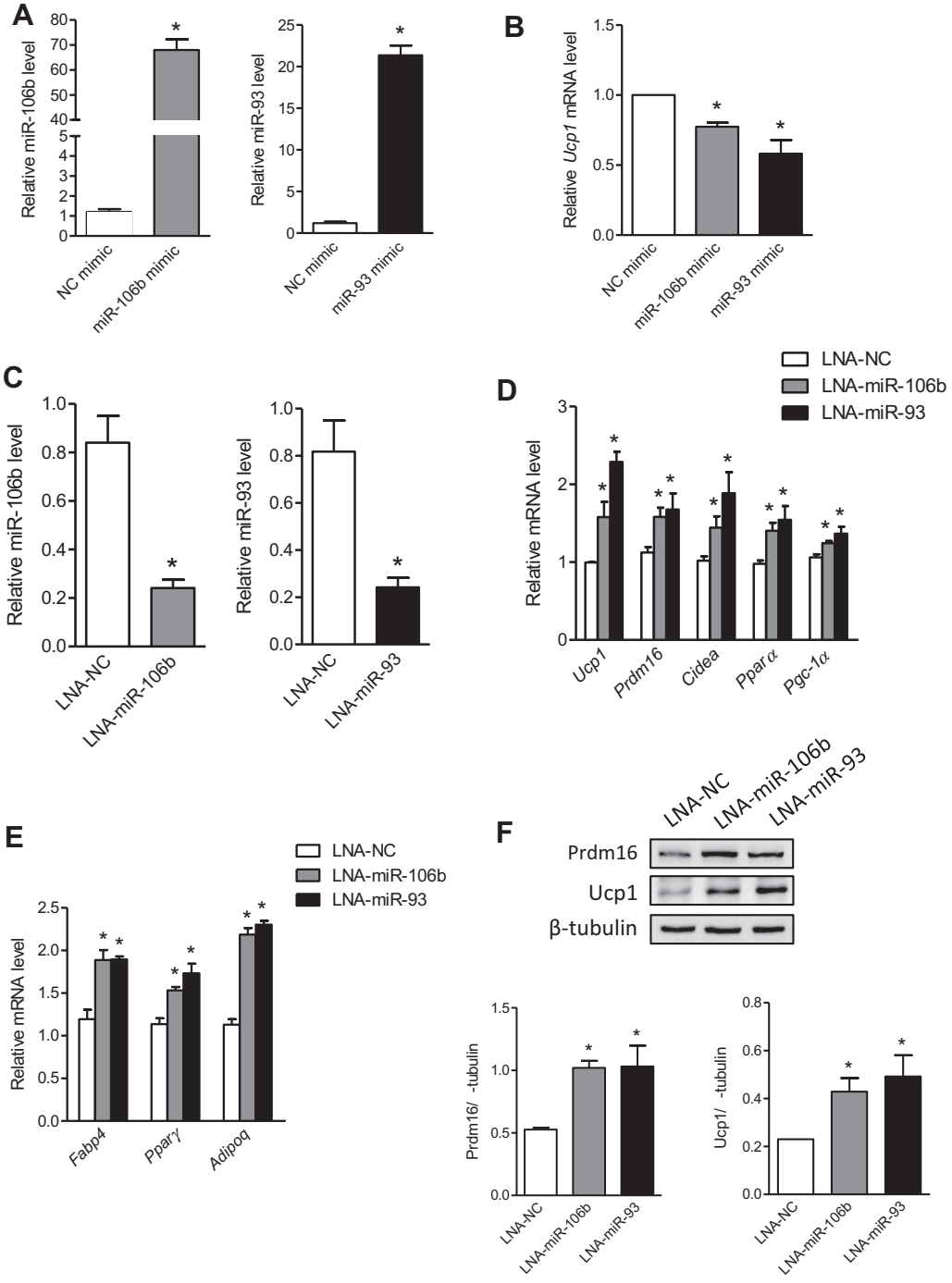

Fig. 2. miR-106b-93 modulates the expression of brown adipogenesis markers. Brown preadipocyte cell lines were transfected with miR-106b mimics (100 nM), miR-93 mimics (100 nM) or negative controls for 48 h, and then subjected to differentiation for 3 days. At day 4, cells were harvested and measured for corresponding miRNA expression (A) and *Ucp1* mRNA level (B) using real-time PCR. $n = 3$. $^*P < 0.05$ vs. negative control (NC) mimics. Cultured brown preadipocytes were transfected with LNA miR-106b inhibitors (100 nM), LNA miR-93 inhibitors (100 nM) or negative controls for 48 h, and then induced to differentiate into brown adipocytes. The expression of miRNAs (C), brown adipocyte-specific genes (D) and common adipogenesis markers (E) were analyzed 3 days after induction of differentiation by real-time PCR. $n = 4$. $^*P < 0.05$ vs. LNA-NC. Prdm16 and Ucp1 protein levels were assessed by Western blot analysis (F). $n = 3$. $^*P < 0.05$ vs. LNA-NC.

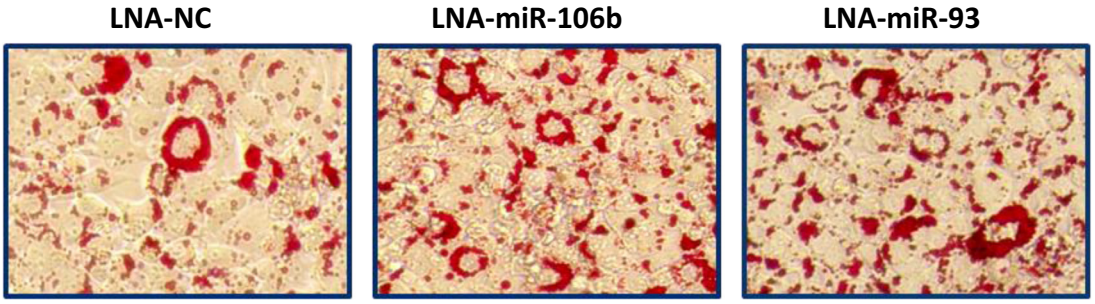

Fig. 3. miR-106b-93 inhibits triglyceride accumulation during brown adipocyte differentiation. LNA miRNA inhibitors (100 nM) were transfected into brown preadipocytes for 48 h. Cells were induced to differentiate into mature brown adipocytes for 3 days. Oil red O staining was used to determine the content of lipid-droplet in brown adipocytes at day 4. Representative images for three independent experiments were shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a peak on differentiation day 7 (Fig. 1A). To further confirm this result, we isolated and cultured primary stromal-vascular fraction (SVF) cells from mouse interscapular BAT, which contain brown preadipocytes capable of differentiating into classic brown adipocytes. Our results showed that miR-106b and miR-93 were both significantly upregulated during adipogenesis (Fig. 1B), suggesting these two miRNAs may play a role in brown fat differentiation.

3.2. Altering the cellular levels of miR-106b and miR-93 affects brown gene expression

To determine whether the miR-106b-93 cluster regulates brown adipocyte differentiation, we overexpressed miR-106b and miR-93 mimics or negative control in cultured brown preadipocytes before and during adipogenesis (Fig. 2A). As shown in Fig. 2B, the expression levels of *Ucp1* (uncoupling protein 1), an important hallmark of brown adipocytes that dissipates mitochondrial proton gradients to generate heat, were significantly decreased in cells treated with miR-106b or miR-93 mimics compared to the control-treated cells, suggesting that the miR-106b-93 cluster may function as a negative regulator of brown fat differentiation.

To provide further evidence on the negative role of the miR-106b-93 cluster in regulating brown gene expression, we treated brown preadipocytes with LNA miR-106b-93 inhibitors before induction of adipogenesis. Real-time PCR analysis showed that the expression of miR-106b and miR-93 was significantly suppressed by the miR-106b-93 antisense oligonucleotides on differentiation day 3 (Fig. 2C). Inhibition of miR-106b-93 led to significant upregulation of the brown adipogenesis markers, including *Ucp1*, *Prdm16* (PR domain containing 16), *Cidea* (cell death-inducing DNA fragmentation factor, alpha subunit-like effector A), *Pparα* (peroxisome proliferator activated receptor alpha), and *Pgc-1α* (peroxisome proliferative activated receptor, gamma, coactivator 1 alpha) (Fig. 2D). Cells treated with miRNA inhibitors also showed a significant increase in the mRNA levels of adipogenic markers common to both BAT and WAT, including *Fabp4* (fatty acid binding protein 4), *Pparγ* (peroxisome proliferative activated receptor gamma) and *adiponectin* (Fig. 2E). The enhanced expression of Prdm16 and Ucp1 was also confirmed by Western blot analysis (Fig. 2F). Together, these findings suggest that miR-106b and miR-93 are general regulators of adipogenesis; moreover, both miRNAs play an inhibitory role in the differentiation of brown adipocytes.

3.3. Lower miR-106b and miR-93 promote triglyceride accumulation in mouse differentiating brown adipocytes

To evaluate if miR-106b and miR-93 affect adipogenesis during brown adipocyte differentiation, we carried out oil red O staining experiments. Knockdown of miR-106b or miR-93 expression greatly increased triglyceride accumulation (Fig. 3), confirming that miR-106b-93 negatively regulates adipogenesis during brown adipocyte differentiation.

3.4. Impaired brown adipose tissue is accompanied by high expression of miR-106b and miR-93 in HFD-induced obese mice

It has been reported that the differentiation and function of BAT are impaired in obese individuals [3,4,15]. To explore the physiological role of miR-106b-93 *in vivo*, we examined the levels of these two miRNAs in diet-induced obese mice. Feeding mice with a high fat diet (HFD) led to a significant increase in body weight and visceral fat mass (Fig. 4A and B). The expression levels of brown fat-specific and thermogenic genes such as *Ucp1*, *Prdm16*, *Pgc-1α*, and *Acsl1* were significantly reduced in BAT isolated from HFD-fed mice as compared to control mice (Fig. 4C). Consistent with a negative role of miR-106b-93 in regulating brown gene expression *in vitro*, significantly higher levels of miR-106b and miR-93 were observed in BAT isolated from HFD-fed mice compared to control mice (Fig. 4D). However, no significant difference in miR-106b and miR-93 levels between WAT of HFD-induced obese mice and control mice was observed (Fig. 4E). Together, these results support that miR-106b-93 functions as a negative regulator of brown adipogenesis *in vivo*.

4. Discussion

The recent discovery of functional brown fat in adult humans has greatly evoked the interest in better understanding the mechanisms regulating brown fat differentiation [2,16]. In addition to various signaling molecules and transcription factors, recent studies have indicated that miRNAs, including the miR-193b-365 cluster, miR-196a, miR-133 and miR-155, also play key roles in regulating brown adipocyte differentiation [6–9].

miR-106b and miR-93 pertain to the miR-106b-25 cluster and are co-located on chromosome 7 [17]. In the current study, we have demonstrated that the expression of miR-106b-93 was significantly increased in mature brown adipocytes compared to brown preadipocytes, suggesting that miR-106b-93 may play a role in regulating brown adipocyte differentiation. Consistent with this, knockdown of miR-106b and miR-93 by antisense oligonucleotides significantly increased brown gene expression and promoted brown adipocyte differentiation. In contrast, ectopic expression of these two miRNAs repressed brown adipocyte specific and thermogenic gene expression. However, it should also be noted that suppressing miR-106b and miR-93 levels also led to an upregulation of common adipogenesis markers such as *Fabp4*, *Pparγ* and *Adiponectin*, suggesting that the miR-106b-93 cluster may function

as a general regulator of adipogenesis in addition to their roles in regulating brown gene expression. However, while the expression level of miR-106b-93 was significantly higher in BAT of diet-induced obese mice compared to control mice (Fig. 4D), no significant difference in the level of these two miRNAs was observed between WAT of obese mice and control mice. Taken together, these results support that the miR-106b-93 cluster plays a major role in regulating brown adipocyte differentiation and function.

How the miR-106b-93 cluster negatively regulates thermogenic gene expression and brown fat differentiation remains unknown. As predicted by the TargetScanv 6.2 program, approximately 930 messages are predicted to be targets of miR-106b and miR-93, including *Pparα*, a brown-fat-selective transcriptional factor that has been shown to be critical for fatty acid oxidation [18–20] and storage [21], as well as induction of the expression of peroxisomal and mitochondrial enzymes [22–24]. Interestingly, the expression of *Pparα* was induced by inhibition of miR-106b and miR-93 (Fig. 2B), suggesting that *Pparα* might be a direct target of miR-106b and miR-93. Further studies will be necessary to demonstrate whether the miR-106b-93 cluster negatively regulates brown adipocyte differentiation by directly targeting *Pparα*.

In summary, we have identified the miR-106b-93 cluster as a negative regulator of brown adipocyte differentiation. In addition, we have demonstrated a positive correlation between diet-induced obesity and miR-106b-93 expression in mouse BAT. Better understanding the functional roles and the mechanisms of action of

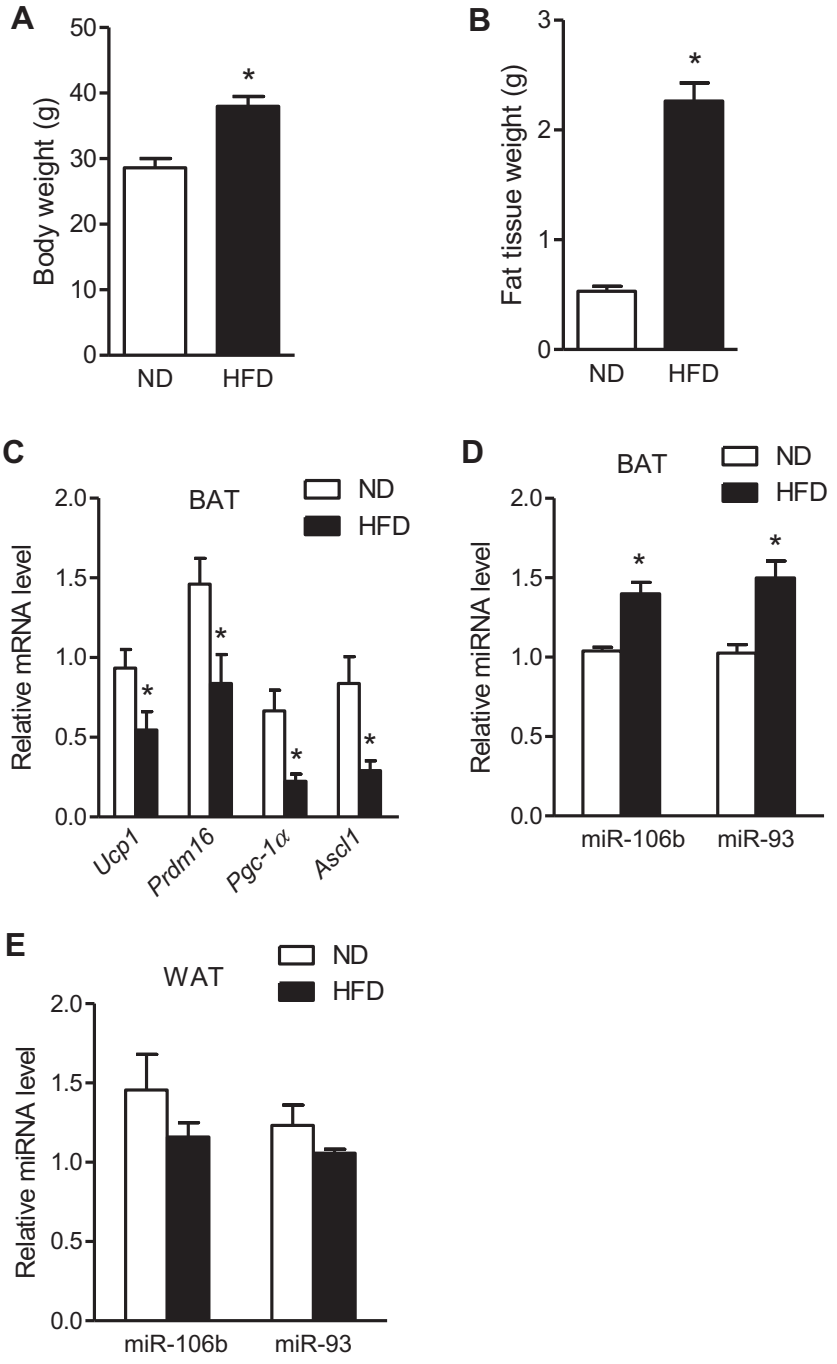

Fig. 4. Impaired BAT was associated with high expression of miR-106b-93. C57BL/6J WT mice (6 week old) were fed on a normal or high fat diet for 16 weeks after which body weight (A) and fat mass (B) were measured. $n = 5$–10 mice per group. $^{*}P < 0.05$ vs. normal diet (ND). mRNA levels of BAT-specific markers (C) and relative expression of miR-106b-93 (D, E) were determined by real-time PCR. $n = 3$-5 mice per group. $^{*}P < 0.05$ vs. ND.

these two miRNAs may lead to the development of new therapeutic strategies for the treatment of obesity and related metabolic disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.016.

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