MicroRNA-188 regulates aging-associated metabolic phenotype

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Abstract
With the increasing aging population, aging-associated diseases are becoming epidemic worldwide, including aging-associated metabolic dysfunction. However, the underlying mechanisms are poorly understood. In the present study, we aimed to investigate the role of microRNA miR-188 in the aging-associated metabolic phenotype. The results showed that the expression of miR-188 increased gradually in brown adipose tissue (BAT) and inguinal white adipose tissue (iWAT) of mice during aging. MiR-188 knockout mice were resistant to the aging-associated metabolic phenotype and had higher energy expenditure. Meanwhile, adipose tissue-specific miR-188 transgenic mice displayed the opposite phenotype. Mechanistically, we identified the thermogenic-related gene Prdm16 (encoding PR domain containing 16) as the direct target of miR-188. Notably, inhibition of miR-188 expression in BAT and iWAT of aged mice by tail vein injection of antagomiR-188 ameliorated aging-associated metabolic dysfunction significantly. Taken together, our findings suggested that miR-188 plays an important role in the regulation of the aging-associated metabolic phenotype, and targeting miR-188 could be an effective strategy to prevent aging-associated metabolic dysfunction.

Keywords
aging-associated metabolic phenotype, brown adipose tissue, energy expenditure, inguinal white adipose tissue, MicroRNA-188, PRDM16

1 INTRODUCTION
Aging is often accompanied by an irreversible decline in physiological function, especially metabolic function. The age-associated metabolic phenotype, including decreased energy expenditure, increased fat mass accumulation, and insulin sensitivity deterioration (Guillory et al., 2017), can ultimately lead to age-associated metabolic dysfunction, which correlates closely with several disease, such as type 2 diabetes mellitus.
MicroRNAs (miRNAs) belong to a class of short noncoding regulatory RNAs (22–24 nucleotides) (Li et al., 2015; Treiber, Treiber, & Meister, 2019; Wang et al., 2015; Su et al., 2019), which exert their functions by binding to the 3’ untranslated region (3’ UTR) or protein coding sequence of target mRNAs (Frankel et al., 2018; Thomou et al., 2017). MiRNAs play crucial roles in regulating many metabolic diseases (Hanin et al., 2018; Langlet et al., 2018; Pankratz et al., 2018; Yu et al., 2018; Zhang et al., 2018). However, the role of miRNAs in the regulation of the aging-associated metabolic phenotype is unknown. In our previous study, we showed that miR-188 plays crucial roles in regulating the aging-associated switch between osteoblast and adipocyte differentiation of bone marrow mesenchymal stem cells (Li et al., 2015). However, a role for miR-188 in the regulation of the aging-associated metabolic phenotype remains to be investigated.

Therefore, in the present study, we aimed to investigate the role of miR-188 in the aging-associated metabolic phenotype. The results demonstrated that the expression of miR-188 increased gradually in mouse brown adipose tissue (BAT) and inguinal white adipose tissue (iWAT) during aging. MiR-188 knockout mice were resistant to the aging-associated metabolic phenotype and had higher energy expenditure. Adipose tissue-specific miR-188 transgenic (Tg) mice had the opposite phenotype. Notably, antagonir-188-mediated inhibition of miR-188 expression in BAT and iWAT of aged mice ameliorated the aging-associated metabolic phenotype significantly. The results revealed that miR-188 regulates the aging-associated metabolic phenotype and could be a therapeutic target to treat aging-associated metabolic dysfunction.

2 | RESULTS

2.1 | The expression of miR-188 increased gradually in mouse BAT and iWAT during aging

To determine the potential role of miR-188 in regulating the aging-associated metabolic phenotype, first we examined the expression of miR-188 in the BAT and iWAT of mice at different ages using quantitative real-time PCR (qPCR) analysis. The results showed that with increasing age, miR-188 expression increased gradually in the mouse BAT and iWAT (Figure 1a,b), which suggested that miR-188 plays a role in the regulation of the aging-associated metabolic phenotype.

2.2 | MiR-188 knockout mice were resistant to the aging-associated metabolic phenotype and had higher energy expenditure

To further investigate the potential role of miR-188 in regulating the aging-associated metabolic phenotype, we generated miR-188 null mice (Figure S1a). There was no significant difference in body weight between the miR-188 null mice and their wild-type (WT) littermates during the first 10 months after birth (Figure 2a). However, with increasing age, the body weights of the miR-188 null
Adipose tissue-specific miR-188 Tg mice were prone to develop the aging-associated metabolic phenotype and had lower energy expenditure

To further verify the findings reported above, we generated Tg mice that overexpressed miR-188 in an adipose tissue-specific manner (Figure S2a). The body weights of the miR-188 Tg mice showed no significant differences compared with those of their WT littermates during the first 7 months after birth (Figure 3a). However, with increasing age, the body weight of the miR-188 Tg mice gradually became higher than that of their WT littermates (Figure 3a). The increased body weight of aged miR-188 Tg mice was mainly caused by an increased fat mass component, while the lean mass component showed no significant differences between the two groups of mice (Figure 3b,c). Consistently, when the aged miR-188 Tg mice and their WT littermates were sacrificed and the adipose tissues were isolated, the gross size and mass of the eWAT and iWAT were increased in the aged miR-188 Tg mice (Figure 3d,e). In addition, histological staining of the BAT, eWAT, and iWAT in the two groups of mice revealed that the adipocytes were larger in the eWAT and iWAT of the aged miR-188 Tg mice compared with those in their WT littermates (Figure 3f). In addition, the size of the lipid droplets in the BAT of the aged miR-188 Tg mice also increased (Figure 3f). These findings suggested that the adipose tissue-specific miR-188 Tg mice were prone to developing the aging-associated metabolic phenotype.

Furthermore, we investigated the energy metabolism of aged miR-188 Tg mice and their WT littermates. The daily food intake showed no significant differences (Figure 3g); however, the O₂ consumption and energy expenditure decreased significantly in the aged miR-188 Tg mice compared with those in their WT littermate (Figure 3h–l). Although their physiological activities showed no obvious changes (Figure 3m,n), the body temperature of the aged Tg mice was significantly lower than that of their WT littermates (Figure 3o). The lower body temperature observed in the aged miR-188 Tg mice was most likely caused by decreased thermogenesis, because the mRNA levels of thermogenesis-related genes, such as Ppargc-1a (encoding peroxisome proliferator-activated receptor gamma coactivator 1-alpha), Prdm16 (encoding PR domain containing 16), Ucp1 (encoding Uncoupling protein 1), and Cidea (encoding cell death-inducing DFF-like effector A) were increased significantly in the BAT and iWAT of the aged miR-188 null mice (Figure 2p,q).

Thus, these findings suggested that the decreased body fat mass of the aged miR-188 null mice was largely caused by increased energy expenditure.
**Prdm16 mRNA**

(a) 

| 5'UTR | CDS | 3'UTR |
|-------|-----|-------|
|       | 1056 | 1525  |

miR-188 target site

position 1056-1062 of WT prdm16 3'UTR

5'...CCCAGGACUGAGUGGGAC... 3'

3'...ACGUUUGGACCAGUACACCUC... 5'

miR-188

position 1525-1531 of WT prdm16 3'UTR

5'...UAAGGUUGUGUGUGUGGAG... 3'

5'...CCCAUGGCCACUGAGUGUG... 5'

position 1056-1062 of MUT prdm16 3'UTR

5'...CCCAGGACUGAGUGGGAC... 3'

3'...ACGUUUGGACCAGUACACCUC... 5'

miR-188

position 1525-1531 of MUT prdm16 3'UTR

5'...UAAGGUUGUGUGUGGAG... 3'

(b) 

Relative luciferase activity

WT prdm16 3'UTR + + +

MUT prdm16 3'UTR − − −

miR-NC mimics − + −

miR-188 mimics − − +

(c) 

Relative Prdm16 level (fold)

AntagomiR-NC AntagomiR-188

0 1 2 3 4 5 6 7 8

(d) 

Relative Prdm16 level (fold)

AgomiR-NC AgomiR-188

0 1 2 3 4 5

(e) 

PRDM16

β-actin

(f) 

PRDM16

β-actin

(g) 

PRDM16

UCP1

β-actin

(h) 

PRDM16

UCP1

β-actin

WT miR-188 Tg
2.4 | **Prdm16 is a potential target of miR-188 in the regulation of the aging-associated metabolic phenotype**

To further investigate the underlying mechanism by which miR-188 regulates the aging-associated metabolic phenotype, we used online tools, including TargetScan 6.2 (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/), to predict the potential targets of miR-188 (He, Han, et al., 2018a; Savita & Karunagaran, 2013). Among the predicted target genes, our attention was drawn to Prdm16, because sequence analysis revealed there was a conserved miR-188 binding site in its 3’ UTR (Figure 4a), meanwhile PRDM16 has been reported to function as a key transcription factor that regulates the expression of thermogenic program genes in brown and beige adipocytes (Cohen et al., 2014; Harms et al., 2014). To explore the association between Prdm16 and miR-188, we generated luciferase reporter plasmids containing the wild-type 3’ UTR of Prdm16 (WT Prdm16 3’ UTR). HEK293 cells were co-transfected with the WT Prdm16 3’ UTR luciferase reporter plasmids and miR-188 mimics. The results showed that miR-188 mimic overexpression significantly inhibited the luciferase activity (Figure 4b). However, this inhibition was largely abolished when the binding site of miR-188 in the 3’ UTR of Prdm16 was mutated (Figure 4b). In addition, in cultured primary brown adipocytes, the mRNA and protein levels of PRDM16 increased when transfected with antagoniR-188 or decreased when transfected with agomiR-188 (Figure 4c–f). Consistently, in the BAT of aged miR-188 knockout mice the protein levels of PRDM16 and UCP1 also increased, whereas in the aged miR-188 Tg mice, the protein levels of PRDM16 and UCP1 decreased (Figure 4g,h). These findings suggested that Prdm16 is a potential target of miR-188 in the regulation of the aging-associated metabolic phenotype.

2.5 | **Administration of antagoniR-188 to aged mice ameliorated aging-associated metabolic phenotype and stimulated energy expenditure**

The findings above suggested that miR-188 is a potential therapeutic target for aging-associated metabolic dysfunction. To inhibit the expression of miR-188 in the BAT and iWAT of aged mice, we injected antagoniR-188 to the tail veins of aged mice, as reported previously (Zhang et al., 2016). Six months later, we found that antagoniR-188 injection significantly inhibited the expression of miR-188 in the BAT and iWAT of the aged mice (Figure S3a). Monthly body weight measurements of the mice revealed that antagoniR-188 injection gradually decreased the body weight of the aged mice (Figure 5a). Further studies showed that the decreased body weight of the antagoniR-188-injected mice was mainly caused by a decreased fat mass component, because the lean mass component showed no obvious changes between the antagoniR-188-injected mice and the antagoniR-NC controls (Figure 5b,c). Consistently, the gross size and mass of the eWAT and iWAT in the antagoniR-188-injected mice also decreased (Figure 5d,e). In addition, histological staining showed that antagoniR-188 injection decreased the size of the adipocytes in the eWAT and iWAT of the aged mice (Figure 5f). The number of lipid droplets in the BAT of the aged antagoniR-188-injected mice also decreased (Figure 5f). Taken together, these results suggested that tail vein injection of antagoniR-188 ameliorated the aging-associated metabolic phenotype.

To evaluate the effects of antagoniR-188 injection on energy metabolism, the food intake and energy expenditure of aged antagoniR-188-injected mice and the controls were measured. The food intake was not affected by antagoniR-188 injection (Figure 5g); however, the O2 consumption and energy expenditure increased significantly increased after antagoniR-188 injection (Figure 5h–l). The increased energy expenditure observed in the aged antagoniR-188-injected mice was most likely caused by increased thermogenesis, because the two groups of mice showed no differences in their physiological activities (Figure 5m,n); however, the body temperature was significantly higher in the antagoniR-188-injected mice than in the controls (Figure 5o). Consistently, the mRNA expression of thermogenesis-related genes, Ptargc-1a, Prdm16, Ucp1, and Cidea were significantly increased in the BAT and iWAT of the antagoniR-188-injected mice (Figure 5p,q), and the protein levels of PRDM16 and UCP1 were also increased in the BAT of the antagoniR-188-injected mice compared with those in the controls (Figure S6h).

Taken together, these findings suggested that administration of antagoniR-188 ameliorated the aging-associated metabolic phenotype by increasing energy expenditure. And targeting miR-188 might be an effective way to prevent aging-associated metabolic dysfunction.

3 | **DISCUSSION**

MiRNAs have been reported to participate in the regulation of a wide variety of metabolic diseases. However, a role for microRNAs in the regulation of the aging-associated metabolic phenotype has
AntagomiR-NC AntagomiR-188

(a) (b) (c)

BAT eWAT iWAT

(d) AntagomiR-NC AntagomiR-188

(e) (f) (g)

Light Dark

7:00 11:00 15:00 19:00 23:00 3:00 7:00

VO2 consumption (ml–1 kg h–1)

Light Dark Total

VO2 consumption (ml–1 kg h–1)

Light Dark Total

RER(VCO2/VO2)

BAT iWAT

Relative expression level (fold)

Ppargc1a Prdm16 Ucp1 Cidea Dio2 Elovl6

Relative expression level (fold)

Ppargc1a Prdm16 Ucp1 Cidea Dio2 Elovl6
not been reported. In our previous study, we demonstrated that miR-188 is an important regulator of aging-associated bone mass loss (Li et al., 2015). Therefore, we questioned whether miR-188 also functions in the aging-associated metabolic phenotype.

The results of the present study showed that the expression of miR-188 gradually increased in the BAT and iWAT of mice during aging. For the WT mice, some metabolic changes gradually appeared during aging, including body weight gain and fat mass accumulation. However, the aged mice with miR-188 knockout did not develop these phenotypes, while the adipose tissue-specific miR-188 Tg mice developed these phenotypes to a greater extent. These findings revealed that miR-188 exerts an important role in the regulation of the aging-associated metabolic phenotype.

Energy homeostasis is maintained by a balance between food intake and energy expenditure (Kim et al., 2018; Xiao et al., 2017). Oxygen consumption and energy expenditure were significantly increased or decreased compared with those in the corresponding control mice, respectively. Under normal environmental conditions, energy expenditure occurs through physiological activities and thermogenesis (Cui et al., 2016; Deng et al., 2017; Wyler, Lord, Lee, Elmqquist, & Liu, 2017). The mice's physiological activities were unchanged; however, the expression of thermogenesis-related genes increased or decreased significantly in the BAT and iWAT of aged miR-188 null mice or aged adipose tissue-specific miR-188 transgenic mice, respectively. Moreover, except for serum triglyceride, there was little difference between the genetic mice and their controls in terms of glucose and lipid metabolism (Figure S3 and Figure S4). Thus, these results suggested that miR-188 regulates the aging-associated metabolic phenotype largely by affecting the thermogenesis.

Further analysis identified Prdm16 as a downstream effector of miR-188, which may participate in the regulation of aging-associated metabolic phenotype. Prdm16 is a key transcription factor that regulates the expression of a panel of thermogenic program genes in brown adipocytes and beige adipocytes (Cohen et al., 2014; Harms et al., 2014) and plays an important role in maintaining IBAT and scWAT identity (Seale et al., 2011, 2007). The results of the present study showed that miR-188 could bind directly to the 3' UTR of Prdm16 mRNA to inhibit its expression. This suggested that Prdm16 may be the downstream target of miR-188 in the regulation of the aging-associated metabolic phenotype.

To further evaluate the therapeutic potential of targeting miR-188 in the treatment of aging-associated metabolic dysfunction, we injected antagomiR-188 into the aged mice via tail vein injection. Administration of antagomiR-188 significantly inhibited the expression of miR-188 in the BAT and iWAT of the aged mice. Furthermore, antagomiR-188 injection ameliorated aging-associated metabolic phenotype significantly. Taken together, these findings revealed a role of miR-188 in the regulation of the aging-associated metabolic phenotype, suggesting that targeting miR-188 might be an effective way to prevent aging-associated metabolic dysfunction.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Animals

C57BL/6J wild-type (WT) mice were obtained from Shanghai Laboratory Animals Co. Ltd (Shanghai, China). The miR-188 null mice were generated by transcription activator-like effector nuclease (TALEN) technique as reported previously (Li et al., 2015). To generate adipose tissue-specific miR-188 transgenic (Tg) mice, first, the pre-miR-188 cDNA (synthesized by Shanghai sangon Co.) was subcloned into a plasmid containing Fabp4 (Ap2) promoter (Shi et al., 2014), resulting in Ap2-pre-miR-188 vector. Then, the Ap2-pre-miR-188 plasmid and empty plasmid were transfected into 3T3-L1 adipocytes by Lipofectamine 2000 (invitrogen), the expression of miR-188 in 3T3-L1 adipocytes was measured by qRT-PCR analysis. After the successful construction of Ap2-pre-miR-188 plasmid, the plasmids were linearized and purified, then micro-injected into C57BL/6J F2 mouse oocytes, and the injected oocytes were then surgically transferred into pseudopregnant C57BL/6J dams. Two lines with high levels of miR-188 expression in BAT and iWAT were selected from six transgenic founders and crossed with C57BL/6J strain for six generations to obtain offsprings with a defined genetic background. One line with a fivefold overexpression of miR-188 was extensively studied. The WT mice were used as controls. All mice were kept in C57BL/6J background and maintained in standard, specific pathogen-free facility of the Laboratory Animal Research Center at Central South University, with a 12-hr dark/light cycle and 4–5 mice per cage. In this study, all mice were kept on a standard normal chow diet purchased from Shanghai Laboratory Animals Co. Ltd (Shanghai, China). All animal care protocols and
experiments were reviewed and approved by the Animal Care and Use Committees of the Laboratory Animal Research Center at Xiangya Medical School of Central South University, and this study was compliant with all relevant ethical regulations regarding animal research.

4.2 | Intravenous administration of miR-188 antagomir

The miRNA antagomir is a chemically modified, cholesterol conjugated, single-stranded RNA analog that complements the miRNAs. It efficiently and specifically silences endogenous miRNAs. AntagomiR-188 and its negative control (NC) were synthesized by RiboBio Co. For tail vein injection of antagomiR-NC or antagomiR-188, the aged mice were received antagomiR-188 once a week (10 mg/kg body weight, 0.2 ml for each injection) for six months before conducting metabolic parameters measurements. The antagomir negative control was administered at the same dose and injection intervals. The functional inhibition by the administered antagomirs in vivo was verified by qRT-PCR.

4.3 | Metabolic parameter measurements

The fat mass component and lean mass component of mice were measured by a nuclear magnetic resonance (NMR) system (Bruker, Rheinstetten, Germany). Indirect calorimetry was conducted in a comprehensive laboratory animal-monitoring system (Columbus Instruments, Columbus, OH), as described previously (Xiao et al., 2017, 2016). Rectal temperature of mice was measured at 14:00 pm by a rectal probe attached to a digital thermometer (Physitemp, NJ, USA). The measurement of daily food intake was also conducted as reported previously (Xiao et al., 2017, 2016).

4.4 | Histological analysis of tissues

Paraformaldehyde-fixed, paraffin-embedded sections of BAT, eWAT, and iWAT were stained with hematoxylin and eosin (H&E) for histology.

4.5 | Luciferase activity assays

The luciferase activity assays were conducted as reported previously (Li et al., 2015; Yang et al., 2017). Generally, the wild-type (WT) PRDM16 3’UTR firefly luciferase reporter plasmids or PRDM16 3’UTR firefly luciferase reporter plasmids with the potential miR-188 binding site mutated were co-transfected with miR-188 mimics or miR-NC mimics to the HEK293 cells, respectively. Renilla luciferase reporter plasmids were also transfected at the same time as internal control. 48 hr posttransfections, firefly and renilla luciferase activities were measured by a Dual-Glo Luciferase Assay System (Promega).

4.6 | Primary culture of brown adipocytes

The primary culture of brown adipocytes was performed as described previously (He, Tang, et al., 2018b; Hu et al., 2015). Briefly, the brown adipose tissue from three weeks old C57/BL6J mice was isolated and minced quickly. Then, the tissue pieces were digested in an isolation buffer containing 123 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 5 mM glucose, 100 mM HEPES, 4% BSA and 1.5 mg/ml collagenase B (Roche) for 45 min at 37°C. After filtered, centrifuged and washed with PBS, the preadipocytes were cultured in an adipocyte culture medium (DMEM plus GlutaMAX, penicillin and streptomycin, and 10% FBS). The preadipocytes were grown to confluence before adipocyte differentiation, which was induced by a differentiation buffer containing 5 mM dexamethasone, 0.02 mM insulin, 0.5 mM isobutylmethylxanthine, 1 nM T3, 125 mM indomethacin and 1 mM rosiglitazone. Two days after the induction, cells were incubated in fresh adipocyte culture medium containing 0.02 mM insulin, 1 nM T3 and 1 mM rosiglitazone. The culture medium was changed every other days until the appearance of multiple small lipid droplets in the cytoplasm.

4.7 | Western blot analysis

The Western Blot analysis was conducted as previously described (Li et al., 2015, 2009), primary antibodies: anti-UCP1 was purchased from Cell Signalling Technology (#14670), anti-PRDM16 was purchased from Abcam (#ab202344), anti-β-actin was purchased from Proteintech (#HRP-60008). All validation information could be found on the manufacturer’s website.

4.8 | RNA isolation and quantitative real-time PCR (qPCR)

The RNA isolation and qPCR analysis were performed as described previously (Li et al., 2015, 2009). The primer pairs used in this study are listed in Table S1.

4.9 | Quantification and statistical analysis

All the results are expressed as means ± S.E.M. Each data point derived from qRT-PCR analysis represents an average of at least three technical replicates. The statistical significance of the differences between various treatments or groups was measured by either Student’s t test or ANOVA followed by Bonferroni posttest. Data analyses were performed using GraphPad Prism 7.0. p < .05 was considered statistically significant, *p < .05; **p < .01; ***p < .001.
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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

AUTHORS’ CONTRIBUTION
YZ.X and XH.L designed the experiments and wrote the manuscript; Y.H, Y.L and YZ.X performed most of the experiments; Y.X, Q.G, FL.Z, T.L and T.S helped to collect samples. YZ.X and XH.L is the guarantor of this work and, as such, has full access to all the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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